DNA mutation frequency in vitamin C deficient mice using Big Blue mice

DNA MUTATION FREQUENCY IN VITAMIN C DEFECIENT MICE USING BIG BLUE MICE

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Abstract

Gulonolactone oxidase enzyme is important in the final stage of ascorbic acid biosynthesis. Gulonolactone oxidase is encoded by the *Gulo* gene. Most animals, such as mice, have the *Gulo* gene, through which they produce ascorbic acid from glucose, while humans, guinea pigs and primate animals carry a non functional *Gulo* gene. Ascorbic acid plays an important role in many biological processes. However, it is primarily essential as an antioxidant. Ascorbic acid protects genomic DNA from free radicals resulting from oxidative stress that might otherwise cause a variety of diseases such as cancer or heart disease. This thesis focuses on investigating the role of ascorbic acid in the elimination of oxidative stress-induced mutagenesis.

To investigate how vitamin C decreases level of the DNA mutation frequency and protects DNA from free radicals, knockout *Gulo* and Big Blue mice were used as models to determine the ability of vitamin C to minimize oxidative stress. The Big Blue mice carry the *cII* gene which is a reporter gene through which DNA mutation rate can be detected in any part of body. Therefore, we generated double transgenic mice which are Gulo deficient or a Big Blue background. Homozygote *Gulo cII* positive ($Gulo^{-/-}$ cII^{+}) were obtained by crossing heterozygote *Gulo cII* Positive and homozygote *Gulo* mice. Five $Gulo^{-/-}cII^{+}$ mice were placed under vitamin C deficient diet and another five were supplemented with vitamin C. DNA mutation frequency was analyzed in the two groups. There were no significant differences in mutation frequencies between homozygote $Gulo^{-/-}$ cII^{+} mice on vitamin C deficient diet and homozygote $Gulo^{-/-}$ cII^{+} mice fed vitamin C rich diet. One treatment mouse showed increased frequency in mutations but a second did not. Further tests can be done on other treated knockout mice to identify the mutation types generated by oxidative stress in the absence of vitamin C.

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Last, but not least, my sincere thanks go to the 2005-2008 staff of Biology Department at McMaster University for their assistance throughout the years of my study.

Above all, I thank Allah for his guidance and inspiration.

Thuraya Shaban.

Dedication

For my mother, my brother Omoura and in memory of my father.

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CHAPTER 1: BACKGROUND

1.1 Introduction

Vitamin C is an important nutrient for humans and primates (Mohan *et al.*, 2005). This nutrient plays many roles in the human body. It is necessary for humans to obtain vitamin C through their diet, as humans do not synthesize vitamin C due to the absence of the gene, *Gulo* (Maeda *et al.*, 2000;Mohan *et al.*, 2005) . This gene was present in early ancestors but was lost through selection, since more distantly related mammals contain the gene, such as mice (Li and Schellhorn, 2007). Vitamin C works as an antioxidant to protect DNA from damage that is caused by reactive oxidative species (ROS) (Gazivoda *et al.*, 2006). Ascorbate also induces immunity to minimize lung pathology during influenza infection (Li, Maeda, and Beck, 2006).

In this study, we aim to determine the effect vitamin C has on preventing DNA damage. A cross of knockout *Gulo*$^{-/-}$ mice with Big Blue mice provides a reporter gene that detects mutations resulting from a vitamin C deficient diet (Flotte and Laube, 2001;Hernandez and Heddle, 2005). This conceived to measure the effectiveness of vitamin C in minimizing the oxidation of DNA, a condition which leads to complicated diseases like cancer (Jansson, Lindqvist, and Nordstrom, 2005). Furthermore, this work will contribute to the use of adenoviral vector for gene therapy in general (Gazivoda *et al.*, 2006). There is evidence that vitamin C has an important effect in tumor angiogenesis and tumor growth (Telang et al., 2007). In addition, vitamin C acts as a nephroprotective agent, more than vitamin E (Ajith, Usha, and Nivitha, 2007).

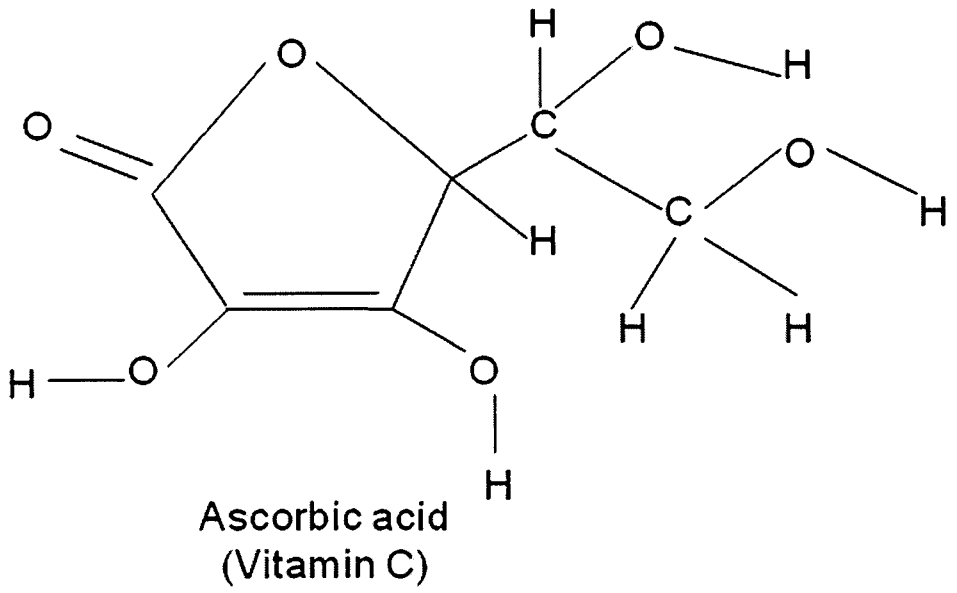

Figure 1: Chemical structure of vitamin C (Duarte and Lunec, 2005) .

Vitamin C, also known as ascorbic acid (Figure 1), is a water-soluble vitamin, an essential nutrient for cells, especially connective tissue cells (Catani et al., 2005). Ascorbic acid has a therapeutic effect in many biological molecules, such as DNA, lipid and protein (Jansson, Lindqvist, and Nordstrom, 2005). The daily recommended intake of vitamin C is 90mg/day for males 75mg/day for females (Jansson *et al.*, 2004). Ascorbic acid is not stored in the body but it is eliminated by the kidney when it accumulates (Jansson *et al.*, 2004). Ascorbic acid helps decrease the harmful effects of various diseases, such as atherosclerosis, cancer, and neurodegenerative and ocular disease (Jansson *et al.*, 2004). Vitamin C acts as an antioxidant, which maintains both intracellular components and important processes that are sensitive to free radicals, is a co-factor in many processes in the body, and plays a role in collagen synthesis, which is the main component of connective tissue in humans and animals (Jansson, Lindqvist, and Nordstrom, 2005;McNulty, Vail, and Kraus, 2005).

Scurvy is a disease that is caused by the deficiency of ascorbic acid (Hatuel *et al.*, 2006). Vitamin C is not naturally synthesized in humans, primates and guinea pigs, and

therefore, it is necessary to obtain this nutrient from their diet (Hatuel *et al.*, 2006;Margittai *et al.*, 2005). Another factor that may contribute to scurvy is inadequate delivery of vitamin C into a cell which forms collagen (Maeda *et al.*, 2000), because of the inability to synthesize vitamin C from glucose in these species (Li, Maeda, and Beck, 2006).

Vitamin C is essential in food to protect against oxidative stress (Alvarado *et al.*, 2006;Zaidi and Banu, 2004). Scurvy affects many people with starvation, like the homeless and the poor (Hatuel *et al.*, 2006). The main symptoms of scurvy are weight loss, weakness in the legs and bleeding in the gums. Other symptoms include unwillingness to move, swelling around the joints with pain and hemorrhagic tendencies (Bennett and Coninx, 2005;de Villiers, 2006;Maeda *et al.*, 2000).

Deficiency of ascorbic acid leads to impaired collagen synthesis, which results in a lack of building connective tissues (Brickley and Ives, 2006). Decrease in collagen synthesis leads to impairment and weakness in blood vessels. This can cause bleeding as a result of a rupture in blood vessel walls, thus appearing purple and causing pain, especially in the legs, hands, and skin (Hasan *et al.*, 2004). Decrease in collagen leads to reduced osteoblastic activity, the formation of a weak layer of cortex and trabecula with spaces, which results in a weakness in bone (Hasan *et al.*, 2004).

1.2 Importance of vitamin C

1.2.1Collagen synthesis

As an antiscorbutic agent, ascorbic acid helps the formation of connective tissues (Sato *et al.*, 2006). Ascorbic acid plays an important role in collagen synthesis, and it is an essential nutrient in humans, guinea pigs and primate animals that lack the enzyme for ascorbic acid production (Sato *et al.*, 2006).

L-ascorbic acid is required for collagen biosynthesis in the connective tissues of cells

(Munday, Fulford, and Bates, 2005;Parsons *et al.*, 2006). L-ascorbic acid acts as a co-factor for the enzyme prolyl hydroxylase and catalyzes the hydroxylation of peptidyl proline in post-translational processes (Hata and Senoo, 1989;Munday, Fulford, and Bates, 2005). Ascorbic acid reacts with the oxidized iron bound to prolyl hydroxylase, reduces the iron and helps to produce prolyl hydroxylase enzyme, which is important for the synthesis of hydroxyproline (Parsons *et al.*, 2006). Hydroxyproline is an amino acid which is a component of type II collagen, the main structural protein in connective tissues, such as tendons, bone, blood vessels, ligaments, teeth and skin (McNulty, Vail, and Kraus, 2005;Parsons *et al.*, 2006). The protein that constitutes collagen has three polypeptide chains, forming triple helices. An inadequate amount of ascorbic acid in the cell leads to a breakdown of the triple helices of collagen, which results in scurvy (Parsons *et al.*, 2006).

Ascorbic acid also increases the rate and the transcription of pro-collagen genes, and it increases the stability of pro-collagen mRNA to modulate the growth ability of the cells (Hata and Senoo, 1989). Therefore, deficiency of ascorbic acid in these species leads to disturbed collagen synthesis in all types of connective tissue, including the gums, teeth, and bone, which may become fatal to the organism if left untreated (Hata and Senoo, 1989). In addition, proliferation of human skin fibroblasts is stimulated significantly by the presence of L-ascorbic acid 2-phosphate (Hata and Senoo, 1989). Thus, the importance of ascorbic acid in keeping connective tissue healthy well established (Munday, Fulford, and Bates, 2005).

1.2.2 Antioxidant

Water-soluble vitamins, such as vitamin C, play an essential role as an antioxidant and a co-factor in many hormonal processes (Sanchez-Moreno et al., 2003). Ascorbic acid is primarily a component used in minimizing oxidative stress, which normally is a

by-product of normal biochemical processes in the presence of excess glutathione (Montecinos *et al.*, 2007). Reactive oxidative species (ROS) are formed by normal processes in the body, such as cell proliferation and cell signaling. Free radicals are produced, which leads to a disturbance of the bimolecular components of the cell, especially DNA, proteins and lipids. In particular, ROS leads to DNA damage, which may cause a variety of diseases such as cancer and neurodegenerative diseases (Burk *et al.*, 2006;Duarte and Lunec, 2005;Evans and Halliwell, 1999). To minimize this disturbance in cell molecules, some enzymes work defensively and protect against DNA damage (Burk *et al.*, 2006;Duarte and Lunec, 2005;Evans and Halliwell, 1999).

Ascorbic acid acts as a therapy to protect the cell membrane and other intracellular components and processes that are sensitive to oxidative species (Mohan *et al.*, 2005). Many cell studies have shown beneficial antioxidant effects of ascorbic acid when using ascorbic acid as a supplement in cell biology and vascular disease (Kraus, Roth, and Kirchgessner, 1997). These studies illustrate how vitamin C plays a role in minimizing DNA damage when the cell is under oxidative stress (Kraus, Roth, and Kirchgessner, 1997). The most common oxidized product from cells and tissues is 8-oxo-7, 8-dihydro-2-deoxyguanosine. Ascorbic acid can decrease the oxidized product 8-oxo-7, 8-dihydro-2-deoxyguanosine, which is generated during DNA oxidation (Lutsenko, Carcamo, and Golde, 2002). Ascorbic acid is able to reduce apoptosis induced by oxidative stress, even in the presence of an excess of glutathione (Witenberg et al., 1999).

1.2.3 Immunity

Vitamin C is a micronutrient in food which has the beneficial effect of enhancing the immune system in the human body (De la Fuente *et al.*, 1998). Intracellular vitamin C provides protection for neutrophils from apoptosis during any inflammation (Vissers and Wilkie, 2007). In addition, there is a lot of evidence that vitamin C has the ability to

inhibit NO production that is necessary for macrophage proliferation and activity during phagocytosis (Vandana et al., 2006). Therefore, adequate amounts of this antioxidant improves the function and restoration of leukocytes (Alvarado *et al.*, 2006).

1.3 Transport and synthesis of vitamin C

After ingesting food that contains vitamin C, vitamin C is absorbed from the lumen of the intestine and renal tubules. Subsequently, vitamin C enters enterocytes and renal epithelial cells (Wilson, 2005). Vitamin C enters the blood and gets distributed into all cells of the body by crossing the plasma membrane (Wilson, 2005). Several physiological mechanisms may contribute to elevated ascorbic acid concentration inside the cell, such as high levels of vitamin C in the liver and spleen (Kuo et al., 2005;Yokogoshi et al., 1999). Ascorbic acid enters the cell by two main modes of transport: facilitated by diffusion and active transport (Wilson, 2005).

A- Facilitated diffusion is one route by which ascorbic acid is transported into the cell. The oxidized form of ascorbic acid, which is dehydroascorbic acid (DHA), is transported into the cell by cell diffusion via a glucose transporter(Rumsey *et al.*, 1997;Welch *et al.*, 1995). The oxidized form is then reduced back to ascorbic acid, which accumulates within the cell (Figure 2) (Rumsey *et al.*, 1997;Welch *et al.*, 1995). The reduced form of ascorbic acid is taken up more slowly than the oxidized form or dehydroascorbic acid (Figure 2) (Rumsey *et al.*, 1997;Vera *et al.*, 1993;Welch *et al.*, 1995). The recycling of ascorbic acid is thought to protect the cell from ROS that causes DNA damage (McFadden et al., 2005).

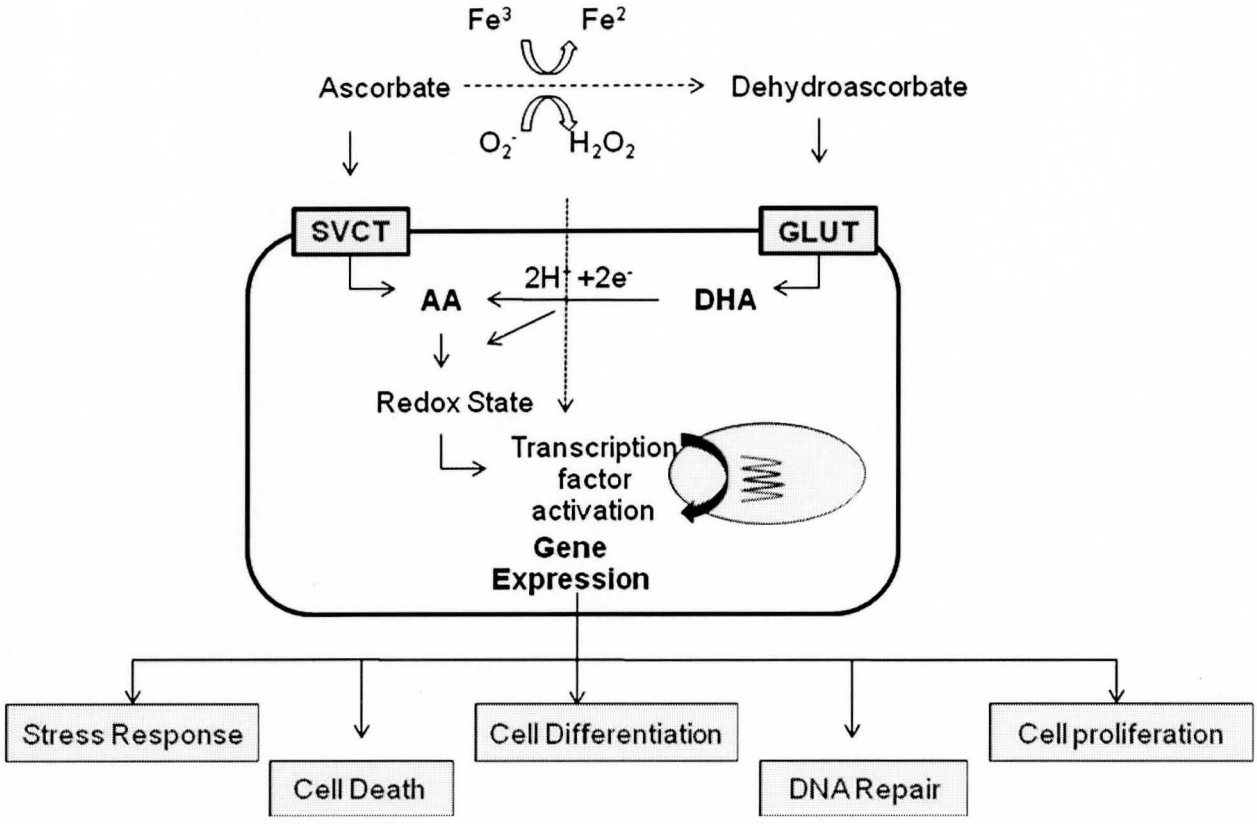

Figure 2: Transport of reduced and oxidized form of ascorbic acid into the cell (Duarte and Lunec, 2005) .

B- Active transport is the transport of the reduced form of ascorbic acid via sodium-dependent SVCT1 and CVCT2 proteins. These proteins are encoded by the genes Slc23al and Slc23a2 (Jin et al., 2005;Wilson, 2005). Therefore, the plasma levels of ascorbic acid are related to and dependent on dietary ascorbic acid intake (Nakata and Maeda, 2002).

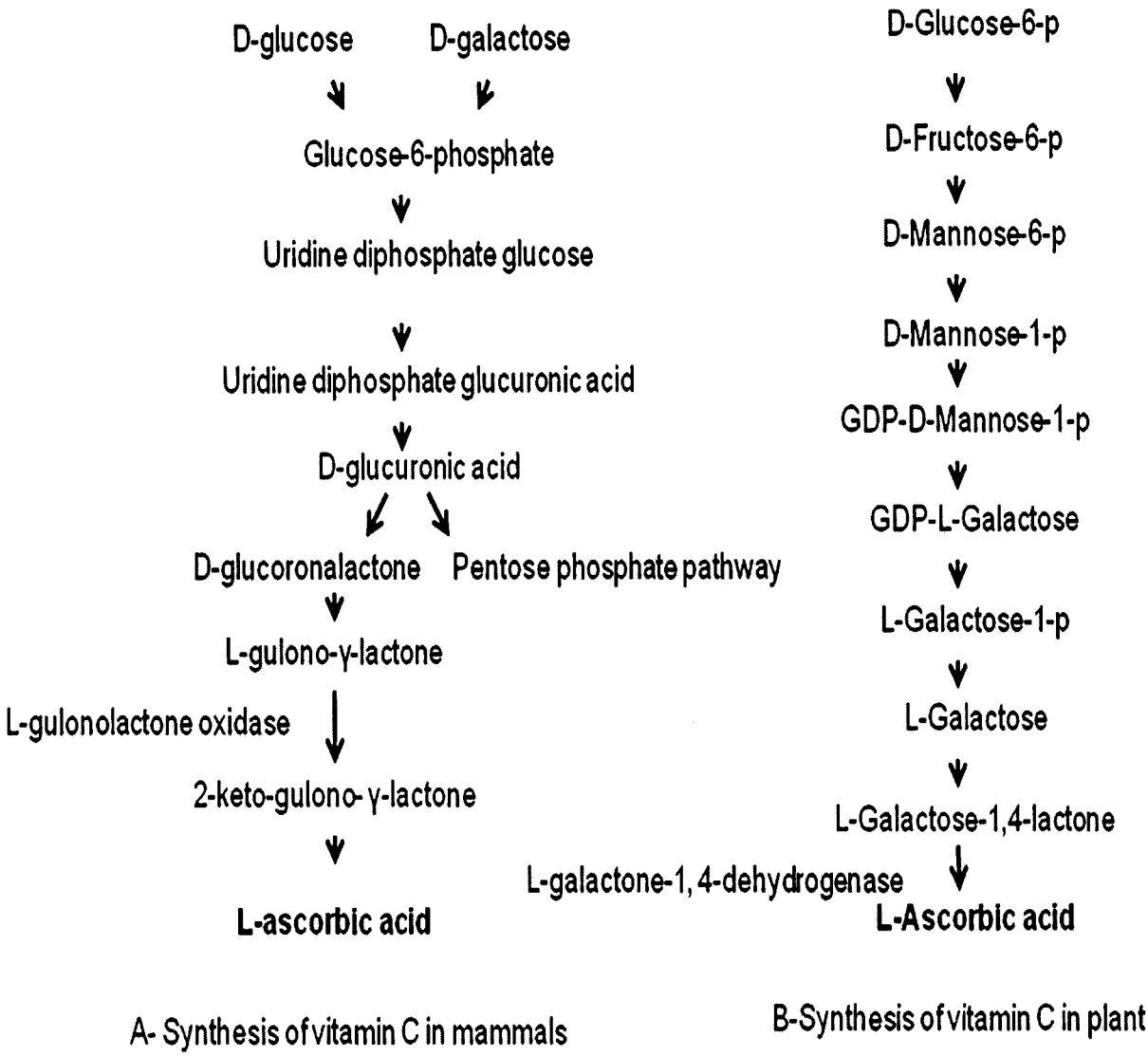

Figure 3: Biosynthesis of vitamin C in mammals and plant (Wheeler, Jones, and Smirnoff, 1998;Naidu, 2003). Ascorbic acid synthesis pathway needs L-gulonolactone oxidase to convert L-gulono-γ-lactone to ascorbic acid while in plant, mitochondrial L-galactone-1,4-dehydrogenase enzyme is responsible to convert L-galactose-1, 4-lactone to ascorbic acid.

Gulonolactone oxidase (Figure 3 A) is required for vitamin C synthesis by catalyzing the conversion of L-gulonolactone to 1-keto-gulono-γ-lactone in order to produce L-ascorbic acid (Hasan *et al.*, 2004). Humans, primates and guinea pigs lack this enzyme (Krasnov *et al.*, 1998;Mohan *et al.*, 2005) because they do not have the *Gulo* gene, which encodes the gulonolactone oxidase enzyme required for ascorbic acid synthesis (Krasnov *et al.*, 1998;Mohan *et al.*, 2005). But in plant the sources of ascorbic acid are D-mannose and L-galactose in which, L-galactose dehydrogenase, is responsible to catalysis oxidation of L-galactose to L-galactone 1, 4-lactone (Wheeler, Jones, and Smirnoff, 1998) (Figure 3 B). In plants, the last stage of ascorbic acid synthesis needs L-galactone-1,4-dehydrogenase enzyme which is responsible for the conversion of L-galactose-1, 4-lactone to ascorbic acid (Wheeler, Jones, and Smirnoff, 1998). GULO is a membrane-bound flavoenzyme found in microsomal fraction of liver cell homogenates (Hasan *et al.*, 2004). FAD molecule, a part of GULO, binds to the apoprotein via an 8α-riboflavin linkage (Hasan *et al.*, 2004). Most animals have the ability to synthesize vitamin C from D-glucose by hexuronic acid in the liver (Kim *et al.*, 2006).

Both L-galactone-γ-lactone and aldono-1,4-lactones are precursors of vitamin C biosynthesis (Kim *et al.*, 2006). Gulonolactone expression leads to an increased intracellular level of ascorbic acid, which is important for endothelial cells in producing collagen (Kim *et al.*, 2006).

1.4 Pro-oxidant effect of vitamin C

The pro-oxidant activity of ascorbic acid via redox-cycle involves metal ions, which can generate ROS (Duarte and Lunec, 2005;Jansson, Lindqvist, and Nordstrom, 2005). ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl (OH) (Figure 4). Superoxide (O_2^-) anion is formed from a reaction between molecular O_2 with an electron (Nedeljkovic, Gokce, and Loscalzo, 2003). Superoxide is changed to hydrogen peroxide

(H_2O_2) by superoxide dismutase. Hydrogen peroxide (H_2O_2) is converted to highly reactive hydroxyl radicals (Figure 4) (Nedeljkovic, Gokce, and Loscalzo, 2003). The hydroxyl radicals produced from hydrogen peroxide and Fe(II) are harmful to cells (Jansson, Lindqvist, and Nordstrom, 2005).

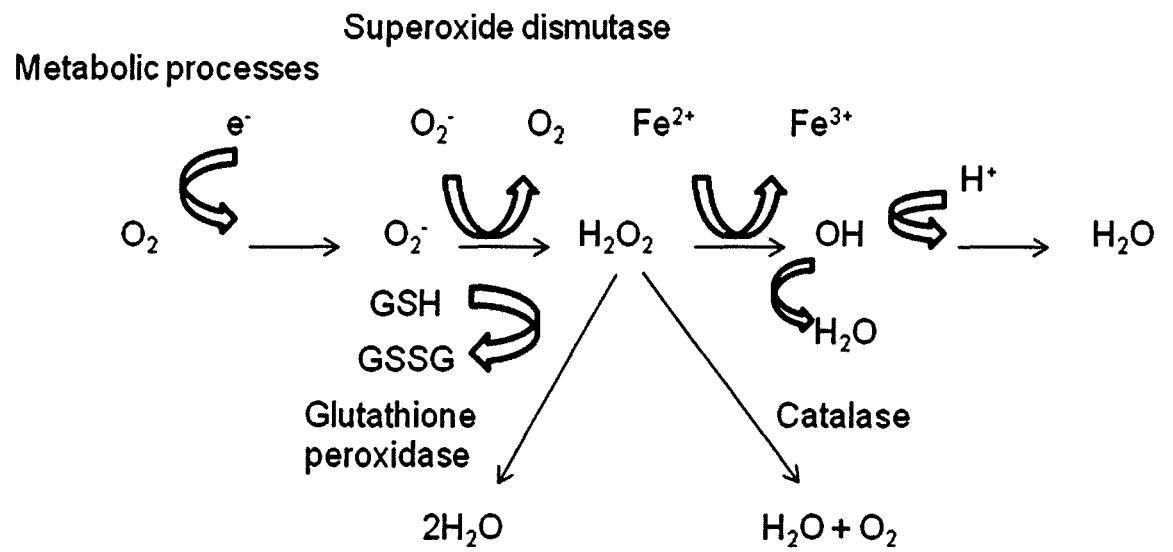

Figure 4: Generation of reactive oxygen species (Nedeljkovic, Gokce, and Loscalzo, 2003)

Vitamin C operates as a pro-oxidant *in vitro*. Vitamin C, mixed with copper or iron, leads to the modification of these oxidation effects on DNA, protein and lipid (Jansson *et al.*, 2004). The ascorbic acid reduces ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions and Cu^{2+} to Cu^{+} in the presence of high levels of iron or copper (Jansson *et al.*, 2004;Jansson, Lindqvist, and Nordstrom, 2005). The reduction of H_2O_2 to hydroxyl is carried by vitamin C-mediated Fenton reactions controlled in the human body (Jansson, Lindqvist, and Nordstrom, 2005). This reaction produces free radicals in the cell which distribute quickly to the cell membrane and cause oxidative damage (Jansson, Lindqvist, and Nordstrom, 2005). People with high iron levels are not recommended to take vitamin C as a supplement, especially those who have iron accumulation in the conditions such as

thalassaemia or haemochromatosis (Chen *et al.*, 2003).

1.5 Experimental models of vitamin C

1.5.1 Guinea pigs

Guinea pigs, similar to humans, have naturally lost the *Gulo* gene and, therefore lost the ability to synthesize gulonolactone oxidase enzyme, an essential enzyme for the production of ascorbic acid (Fiala *et al.*, 2005). Guinea pigs are used to identify the main function of ascorbic acid *in vivo* and to test whether ascorbic acid can be used as a therapy to prevent or to eliminate free radicals resulting from proliferate processes (Mody, Jr. et al., 2005). The guinea pig is essential for vitamin C studies since it lacks the *Gulo* gene (Hasan *et al.*, 2004;Lykkesfeldt and Moos, 2005). Guinea pigs have been used to study the level of ascorbic acid in cells and body fluid (Fiala *et al.*, 2005;Hasan *et al.*, 2004). In fact, the role of ascorbic acid has been studied intensively using guinea pigs for such health problems as lung cancer and oxidative stress (Fiala *et al.*, 2005).

1.5.2 Knockout mice

Small laboratory animal models have been used to study some human diseases (Maeda *et al.*, 2000). Many endogenous and exogenous factors have been shown to cause genetic diseases, through the use of mutant mice (Maeda *et al.*, 2000). Targeted modification of the mouse *Gulo* gene was done by using the neomycin resistance gene as a target vector inserted into the Gulo gene and introduced in embryonic stem cells, which can be verified by Southern blot analysis (Maeda *et al.*, 2000). This modification inactivated the *Gulo* gene, and therefore, halts the synthesis of vitamin C (Maeda *et al.*, 2000). Embryonic stem cells are used to generate knockout mice. These knockout mice like humans, require 330mg/L of vitamin C as a supplement in water to survive (Maeda

et al., 2000). These knockout mice have a nonfunctional *Gulo* gene which encodes the gulonolactone oxidase enzyme required for the final stage of ascorbic acid synthesis (Kondo et al., 2006). The symptoms shown in knockout mice when fed with a vitamin C deficient diet are anemia, a decrease in weight and death in five weeks (Maeda *et al.*, 2000). Knockout *Gulo* mice have been used to investigate the effect of vitamin C on reducing atherosclerosis and oxidative stress (Nakata and Maeda, 2002). Other studies have used knockout *Gulo* mice to investigate the important role of ascorbic acid in the synthesis of collagen and spermatogenesis (Parsons *et al.*, 2006;Yazama *et al.*, 2006).

1.5.3 Osteogenic Disorder Shionogi

Osteogenic Disorder Shionogi **(ODS)** is a rat strain that lacks L-gulonolactone oxidase due to a hereditary mutation (Sakamoto and Takano, 2005). Furthermore, there is a sub-strain of the rat strain called Wistar Shionogi rats (od/od) (Chan and Reade, 1996). They need ascorbic acid in their diet, much like humans and guinea pigs, due to a low plasma level of ascorbic acid in a vitamin C deficient diet (Sakamoto and Takano, 2005). Vitamin C is taken from the mother's milk during infancy but older animals require 3mg/day of ascorbic acid to avoid symptoms of scurvy (Sakamoto and Takano, 2005;Ueta *et al.*, 2003). Vitamin C supplement is important for fracture healing, which is supported by its treatment effect on callus in elderly rats (Alcantara-Martos *et al.*, 2007). Since vitamin C prevent scurvy, ODS rats are susceptible to this disease. The main symptoms of scurvy are spinal curvature, stunted extremities accompanied by walking difficulties, skeletal abnormalities, especially in the tibia, and weight loss (Sakamoto and Takano, 2005). In addition to ascorbic acid deficiency, ODS rats without vitamin C supplement show a reduction in bone formation and in the biomechanics of the femur, not including macro-architectural changes (Togari *et al.*, 1995). Also, ODS rats have an abnormality of odontoblast structure in their teeth (Sakamoto and Takano, 2005).

Therefore, Osteogenic Disorder Shionogi rats are a good model to be used to investigate the efficiency of vitamin C on bone healing during fractures (Alcantara-Martos *et al.*, 2007)

1.5.4 Big Blue mice

Big Blue transgenic mutation models (mouse or rat), carrying *cII* or *lacI* as reporter genes, can be use to a analyze mutations in DNA (Hill *et al.*, 1999;Mei *et al.*, 2005). Lambda *lacI* or *cII* is inserted in the chromosome of Big Blue mice and can be packaged into a lambda vector. It then is infected into *Escherichia coli* to analyze the frequency of mutations (Mei *et al.*, 2005) . The *lacI* phage particles, when infected in *E.coli* appear as blue plaques on plates in the presence of a chromogenic substrate X-gal (5-bromo-4chloro-3-indoyl-β-D-galactopyranoside)(Jakubczak *et al.*, 1996;Kohler *et al.*, 1991). Mutation frequency can be measured and the DNA sequence can be analyzed. For the *cII* gene, the Big Blue model DNA is collected, packaged into the lambda *cII* vector and infect into *E. coli* (G1250 *hfl-*) (Jakubczak *et al.*, 1996). The phage infected *E.coli* (carrying *cII*) is grown in two different conditions: (1) incubation at 30°C for count the number of phage and (2) incubation at 24 °C to enumerate the packaged lambda *cII*. Mutation frequency will be measured (Jakubczak *et al.*, 1996) (Figure 4). This transgenic mouse mutation assay is able to identify spontaneous and somatic mutations, as well as determine the mutation frequency. DNA sequence can be analyzed and determine the type of mutation (Buettner *et al.*, 1997;Hill *et al.*, 1999). The significance of this recent Big Blue mouse strain is the ability to determine the mutation frequency in any part in the body (Mei *et al.*, 2004;Mei *et al.*, 2005). Since, mutations represent a main cause of most types of cancer (Mei *et al.*, 2004).

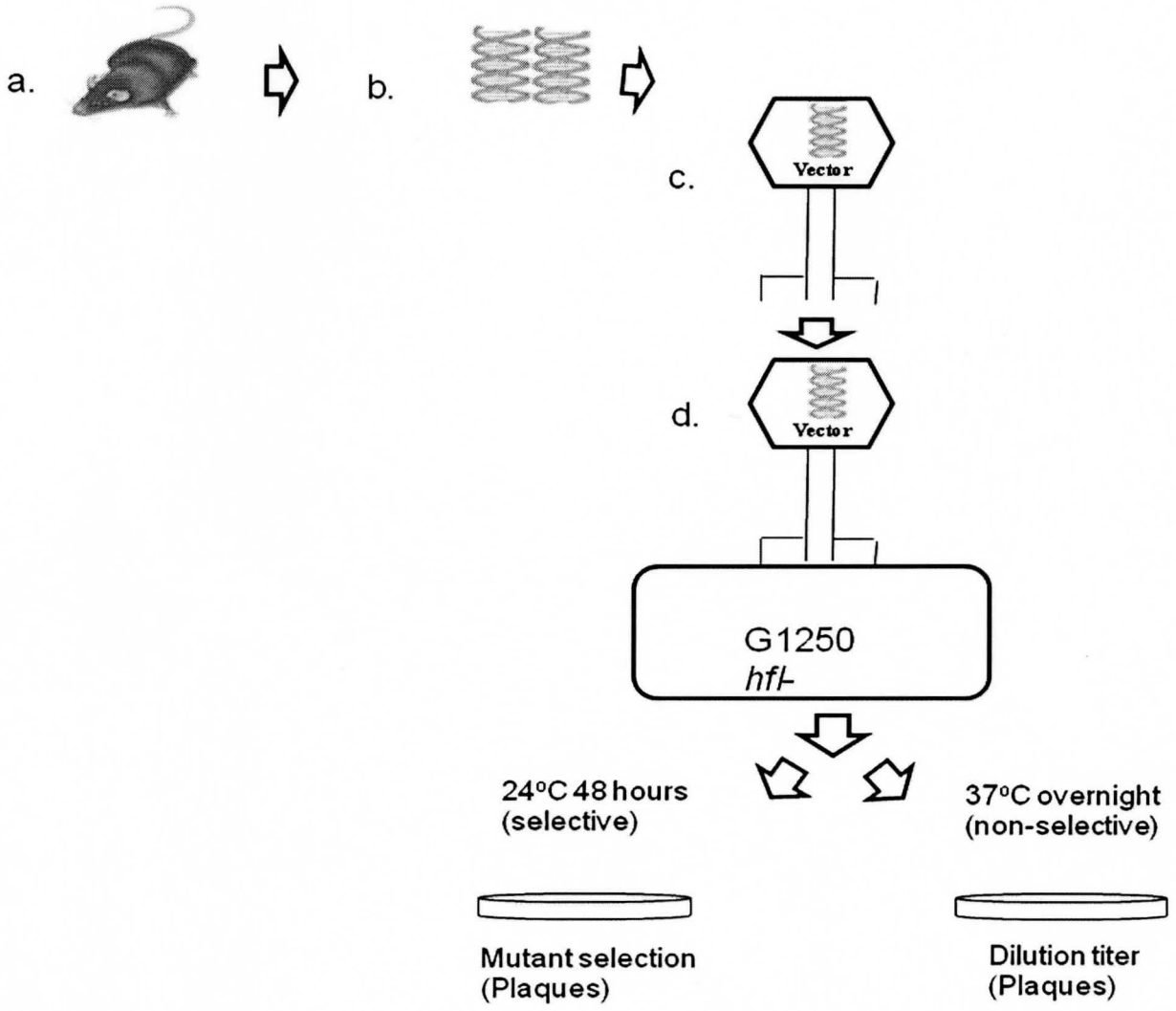

Figure 5: A scheme for the *cII* gene mutation assay of Big Blue mouse.

a. Treat Big Blue mice (cII^+).

b. Isolate DNA sample (e.g liver, lung, heart, brain, muscle, and kidney).

c. Package genomic DNA into lambda vector.

d. Infect *E.coli* (G1250 *hfl*-) host and grow under selective (24°C) and non-selective (37°C) conditions.

1.6 Effect of vitamin C on diseases

1.6.1 Cardiac vascular diseases

Arterial dysfunction or abnormalities in vascular endothelium are the main causes of atherosclerosis disease, which leads to cardiovascular disease (Cangemi *et al.*, 2007). Abnormalities in arterial dilation lead to ischemia-induced flow-mediated dilation (FMD), which causes an increased level of 8-hydroxy-2-deoxyguanosine (8-OHdG) (Cangemi *et al.*, 2007). In that way, the 8-OHdG comes from oxidative stress. Vitamin C, as an antioxidant supplement in food can help to minimize the levels of 8-OHdG (Tarng, Liu, and Huang, 2004). In addition, there is an increased risk of mortality in patients suffering from cardiac vascular diseases when the level of ascorbic acid in the serum is low (Simon, Hudes, and Tice, 2001). There is a lot of research recommending the consumption of fruits and vegetables, which are rich in antioxidant vitamins that may help decrease the risk of coronary heart disease (Knekt *et al.*, 2004). As a result, vitamin C supplied as an antioxidant reduces the occurrence of major heart diseases (Knekt *et al.*, 2004). As well, low plasma vitamin C level leads to increased mortality in hemodialysis patients (Deicher *et al.*, 2005). Therefore, the lack of vitamin C is one cause of the development of atherosclerosis. Thus, it might be possible to eliminate the risk of atherosclerosis by increasing vitamin C intake (Simon et al., 2004).

Coronary artery disease is a heart problem associated with abnormal accumulation of serum lipids (hypercholesterolemia). In addition, the coronary arteries are abnormal, leading to endothelial vasodilator dysfunction (Anderson *et al.*, 1995). Oxidative degradation of the endothelium elevates nitric oxide, a cause of oxygen-derived free radicals. Vitamin C may help to diminish free radicals that are generated from endothelium dysfunction (Ting et al., 1997). As well, cholesterol-lowering and antioxidant therapy facilitate the endothelium vasomotion by reducing the activity of myocardial ischemia and, as a result, coronary events (Anderson *et al.*, 1995).

1.6.2 Cancer

An increase in vitamin C in the diet decreases the risk of chronic diseases, such as cancer neurodegenerative and cardiovascular diseases through its antioxidant actions (Carr and Frei, 1999). One hundred and twenty milligrams of vitamin C per day is the recommended allowance for adults to protect from chronic diseases (Carr and Frei, 1999). Cancer is uncontrolled cell proliferation or cell growth. Vitamin C may help to increase the lifespan of cancer patients if treated intravenous or oral administration of high doses of vitamin C (Padayatty et al., 2006). Also, intravenous vitamin C injection at a dose of 10-100 g/day leads to a decrease in tumor size with no side effect on the kidneys (Riordan et al., 2005). Oral administration of vitamin C has a smaller effect on tumor activity than intravenous administration since only intravenous administration provides high plasma concentration of vitamin C (Padayatty *et al.*, 2004). The beneficial effect of vitamin C is as an antioxidant, and vitamin C leads to a block of carcinogenic N-nitroso compound production in the stomach (Jenab et al., 2006). In fact, You and colleagues have shown that low vitamin C in the diets of cigarette smoking people, is likely to enhance the development of gastric cancer in a high percentage of the population (You et al., 2000). More recent in vitro work shown that a high concentration of ascorbic acid is toxic to cancer cells (Padayatty *et al.*, 2004).

1.6.3 Influenza or common cold

The common cold is an upper respiratory infection caused by respiratory viruses, such as *rhino, corona*, *adeno*, *Para-influenza* and *influenza* (Pauling, 1971). In general, the symptoms of the common cold are nasal discharge, cough, lethargy and sore throat. The relation between the common cold and vitamin C has been widely studied, starting in the early 1970 (Pauling, 1971). Some trial experiments indicated that vitamin C supplementation considerably decreased infection by the common cold with no effect on

the duration of the common cold (Sasazuki et al., 2006). Vitamin C supplementation decreases the common cold during winter time (Van Straten and Josling, 2002). Therefore, the consumption of dietary vitamin C (200-280 mg) is recommended because of the beneficial effect of ascorbic acid on health (Lee et al., 2003). During common cold infection, plasma concentration of ascorbic acid in leukocytes was measured after oral administration of ascorbic acid and was found to be high. Therefore, activation of leukocytes generates an inflammatory mediator, increase consumption of intracellular and extracellular vitamin C to use for cell protection (Turow, 1997;Wilson, Greene, and Loh, 1976).

1.7 Mutation and effect of vitamin C on mutation

1.7.1 Type of mutation

Mutation is a change in the base pair sequence of genetic material (DNA or RNA). It can be induced by chemical mutagen, ultraviolet, stress and free radicals(Kim, Pfeifer, and Besaratinia, 2007;Watson, Cunningham, and Tindall, 1998).

Point mutation is caused by chemicals or malfunction of DNA replication. There are two types of point mutation, which are transitions and transversions. A transition mutation is the exchange of a purine for a purine (A→G) or a pyrimidine for a pyrimidine, (C →T). Transition mutation can be caused by nitrous acid, base mispairing, or mutagenic base analogs. A transversion mutation is the exchange of a purine for a pyrimidine or pyrimidine for a purine.

Insertion mutation is the insertion of one or more extra nucleotides into a DNA sequence. Insert mutations occur from transposable elements or errors during replication of repeated elements. Insertion, leading to a shift in the reading frame may produce a change in the mRNA. These changes alter protein production.

Deletion mutation is deletion of one or more nucleotides from DNA. This mutation can also alter the reading frame of a gene resulting in a dysfunctional protein product.

Frameshifts are insertions or deletions of more than two bases which lead to DNA misreading.

Amplifications are gene duplications which lead to multiple copies of gene.

1.7.2 Causes of mutation

Causes of mutation may come from environmental, chemical, viral, or hereditary source, as well as ionizing radiation (Jawad et al., 2007). Free radicals formed from oxidative stress leads to DNA mutation (Sakamoto et al., 2007). An example of a hereditary mutation is hemorrhagic, telangiectasia which is a defect in the ALK-1 gene that is involved in vascular remodeling and angiogenesis (Lenato and Guanti, 2006). From chemical source, vinyl carbamat is present in fermented foods and alcoholic beverages. It has the ability to cause mutations (Hernandez and Forkert, 2007). From the environment the genotoxicity of sunlight ultraviolet A (UVA) cause promutation oxidative DNA damage (Kim, Pfeifer, and Besaratinia, 2007). Tamoxifen and acrylamide are chemical compounds that have been implicated in the etiology of transversion mutation (Davies et al., 1999;Manjanatha et al., 2006). Any damage of germ cells DNA can cause mutation, and may consequently, lead to birth defect or cancer (Fraga *et al.*, 1991).

1.7.3 Vitamin C and mutation

Oxidative damage causes DNA mutation, which may result in developmental defects in humans (Cheng *et al.*, 1992). Many studies have investigated the ability of antioxidants to decrease mutation frequency in the human body (Liu and Lee, 1998). 8-hydroxyguanine is the major product of DNA damage (Cheng *et al.*, 1992), and with the

addition of vitamin C level of 8-oxodG decreases (Loft and Poulsen, 1996). Therefore, dietary ascorbic acid may play an important role to diminish damage to germ cell DNA (Fraga *et al.*, 1991). The basic focus of this study is to investigate the role of vitamin C in minimizing DNA mutation.

CHAPTER 2: MATERIAL AND METHODS

2.1 Animal used in this study

2.1.1 Gulo mice

Gulo knockout mice have a deletion of Gulo gene exons. The gulonolactone oxidase enzyme is responsible for the synthesis of ascorbic acid (Hasan *et al.*, 2004). We have obtained knockout *Gulo*$^{-/-}$ mice on a C57BL/6 background from university of California at Davis. Knockout *Gulo*$^{-/-}$ mice were held in the Canadian animal facility (CAF) in a pathogen-free room (level B), with 3-5 animals per plastic cage. Mice were kept in a 12/12 h light/dark schedule. Homozygous *Gulo*$^{-/-}$ mice were given water supplemented with vitamin C (330 mg ascorbate /liter with 10μm EDTA), which was changed once a week. Isolated DNA from tail tissues samples were used to genotype for the Gulo gene. For PCR, we used the following primers to amplify for the presence of the *Gulo* gene: P2 (5`-CGCGCCTTAGCATCCCAGTGACTAAGGAT-3`), P3 (5`GTCGGTGACAGAATGTCTTGC-3`), and P4 (5`-GCATCCCAGTGACTAAGGAT-3`). Heterozygous *Gulo*$^{+/-}$ mice identified by the presence of 230bp and 330 bp PCR fragments. Homozygous *Gulo*$^{-/-}$ mice were identified by a 230bp PCR fragment, and the wild type mice were identified by a 330 bp PCR fragment.

2.1.2 Big Blue mice

Two founder (Big Blue males) mice used in the current study were obtained from Stratagene, carrying the reporter *cII* gene. Animal were supplied water and food according to CAF protocol. They were kept in a 12/12 hrs light/dark schedule. To genotype Big Blue *cII*$^{+}$ mice, the following *cII* primers were used: *cII* I (5`-

AATTAAACCACACCTATGGTG 3`) and *cII* II (5`-CCTCTGCCGAAGTTGAGTATTT 3`). The Big Blue mice were identified by presence of a 381bp PCR fragment.

2.2 Crossing the mice

We crossed Big Blue mice ($Gulo^{+/+}$ cII^{+}) with homozygous $Gulo^{-/-}$ mice ($Gulo^{-/-}$ cII^{-}). We had previously confirmed the $Gulo^{-/-}$ mice by genotyping. From this cross, the newborn mouse genotypes were carrying $Gulo^{+/-}$ cII^{+}. The first cross is represented as the first generation of our matting plane. For a second generation, we crossed the first generation ($Gulo^{+/-}cII^{+}$) of the first cross with the parental Gulo knockout mouse ($Gulo^{-/-}$ cII^{-}). From this cross, newborn mouse genotypes were $Gulo^{+/-}$ cII^{+} $Gulo^{-/-}$ cII^{+}, $Gulo^{+/-}cII^{-}$ and $Gulo^{-/-}$ cII^{-}. The second cross, we refer to as the second generation. We further verified the $Gulo^{-/-}$ cII^{+} mice by PCR as described earlier.

2.2.1 First generation

Once the first generation mice were weaned, we used them for further breeding. DNA samples were collected from Gulo mice and Big Blue mice, by using Gulo primers and *cII* primers for genotype. Males and females were weaned, and then we kept them for four weeks to reach sexual maturity. Two males Big Blue mice were bred with knockout females $Gulo^{-/-}$. One of the male Big Blue mice was crossed with two female of knockout $Gulo^{-/-}$ mice. The other male was crossed with two females $Gulo^{-/-}$ mice. Both *Gulo* mice and Big Blue mice were housed in level B room under vitamin C supplement. To identify the breeding time and birthday, we used the breeding tag for females, in addition to special tags for the males. The duration of pregnancy for the mice was 14-20 days. After giving birth, pups were kept with the mother for three weeks, and then were tailed and genotyped by PCR. We obtained all heterozygote $Gulo^{-/+}$ mice, with some cII^{+}. These mice are the first generation of first cross (F1).

2.2.2 Second generation

Heterozygous *Gulo*$^{-/+}$ *cII*$^{+}$ mice were obtained from the first cross, and we used them for the second cross. Male heterozygous *Gulo*$^{-/+}$*cII*$^{+}$ mice were mated with homozygous *Gulo*$^{-/-}$ *cII*$^{-}$ females. We crossed one male *Gulo*$^{-/+}$ *cII*$^{+}$ mouse with two female *Gulo*$^{-/-}$ mice. As well, one female *Gulo*$^{-/+}$*cII*$^{+}$ mouse was crossed with a male *Gulo*$^{-/-}$ mouse. In order to obtained *Gulo*$^{-/-}$ *cII*$^{+}$ mouse and *Gulo*$^{-/+}$ *cII*$^{+}$ mice. Homozygous *Gulo*$^{-/-}$*cII*$^{+}$ mice were mated with Homozygous *Gulo*$^{-/-}$ *cII*$^{+}$ to obtain more homozygous *Gulo* mice carrying *cII*. These mice had free access to water with vitamin C and were kept in a level B room.

2.3 Genotyping

2.3.1 Tissue collection

First generation *Gulo*$^{-/+}$ *cII*$^{+}$ and second generation *Gulo*$^{-/-}$ *cII*$^{+}$ mice were weaned at four weeks. The mice were marked with an ear punch for identification. Tissue samples were collected from the tail of Big Blue *Gulo*$^{-/-}$ mice to detect if these mice were *cII*$^{+}$ and homozygous for *Gulo*$^{-/-}$. To collect tissue samples, mice were under gas anesthetic machine or injected with pain killer by using the optimum recommended dose for mice. The tissue samples were used for DNA extraction, and this DNA was used to confirm the *Gulo*$^{-/-}$ *cII*$^{+}$ genotype by PCR.

2.3.2 Primers

Table 1: List of *Gulo* and *cII* primers

Primer names	Sequence	Size of PCR Product (bp)	Genotype
Gulo -P2	CGCGCCTTAGCATCCCAGTGACTAAGGAT	230	$Gulo^{-/-}$
Gulo -P3	GTCGGTGACAGAATGTCTTGC	230and330	$Gulo^{-/+}$
Gulo -P4	GCATCCCAGTGACTAAGGAT	330	$Gulo^{+/+}$
cII forward	AATTAAACCACACCTATGGTG	381	*cII* positive
cII reverse	CCTCTGCCGAAGTTGAGTATTT		

2.3.3 DNA isolation

Tissues sample were collected from the tail of Big Blue mice and *Gulo* mice. To dissolve tail tissue, 500 µl of Proteinase PK buffer was added to the tissue, and then incubated at 50°C for overnight. 500 µl of phenol was added and vortexed for ten s. The samples were centrifuged at 13,000 x g for five min. The supernatant layer was placed into a new tube. 500 µl of chloroform was added, and vortexed for ten s. The samples were centrifuged at 13,000 x g for five min. The supernatant was placed into new tube. 1000 µl of 100 % ethanol was added, and the tube was inverted four or six times for even mixing. The samples were centrifuged at 12, 0000 x g for ten min. The ethanol was discarded, and then samples were left to dry. Once the sample completely dried, 50µl or 100µl of sterile water was used to re-suspend DNA. The DNA samples were stored at -20°C.

2.3.4 PCR

a) *Gulo* PCR

PCR was performed with a final reaction volume of 25 µl, containing 2.5 µl of 10XTaq buffer with KCl and without $MgCl_2$, 1.5 of $MgCl_2$ (25mM), 0.5 of dNTP mix (10mM), 1 µl of P2 Rv Primer (25µM), 1µl of P3 Fr Primer (25µM), 1µl of P4 Rv Primer (25µM) and 1 µl Taq Pol (1U/µl). As well, the reaction had 5µl of template (DNA sample). The DNA sample was diluted 1:10 for the PCR reaction. PCR steps were 94°C for 2 min, followed by 30 cycles of 94 °C for 20 s, 52 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 7 min for 1 cycle.

b) *cII* PCR

PCR was performed in a reaction volume of 25 µl which are 2.5 µl of 10 x Taq buffer with KCl and without $MgCl_2$, 1.5 of $MgCl_2$ (25mM), 0.5 of dNTP mix (10mM), 1 µl of *Gulo*-P2 Rv Primer (25µM), 1µl of *Gulo*- P3 Fr Primer (25µM), 1µl of *Gulo*- P4 Rv Primer (25µM) and Taq Pol (1U/µl). 5 µl of template (DNA sample) was added. The DNA sample was diluted 10 x for the PCR reaction PCR steps were 94 °C for 2 min, followed by 30 cycles of 94 °C for 20 s, 48 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 7 min for 1 cycle.

2.4 Western Blot

2.4.1 Collect tissue samples and protein extraction

Tissue samples were taken from wild type $Gulo^{+/+}$, heterozygote $Gulo^{-/+}$, homozygote $Gulo^{-/-}$ mice and guinea pig. The tissue samples were taken from liver, spleen, kidney and mammalian cells. Tissue were frozen in liquid nitrogen after excision and stored at -80 °C:

a) Tissue protein extraction

For this procedure, a ratio of tissue to CelLytic MT reagent of 1:20 (1gram of tissue/20 ml of reagent) is recommended. When a concentrated lysate is required the tissues were lysed in a lower volume of CelLytic MT reagent. Proteinase inhibitor Cocktail was added to the CelLytic MT reagent. The tissue samples were weighed (100mg from samples per 500 ul from reagent). The appropriate amount of CelLytic MT was added to the tissue sample. Centrifuge the lysed sample for 10 min at 13,000 x g to pellet the tissue debris. The lysed sample is re-centrifuge to get all tissue debris. The protein containing supernatant is transferred to a chilled test tube. We stored the lysate at -70 °C.

b) Cellular protein extraction:

Cells lysates was prepared by washing adherent cells from culture residual medium. Cells were rinsed once with DPBS being careful not to dislodge any of the cells. DPBS was discarded. Cells were lysed with appropriate volume of CelLytic MT reagent. Cells were incubated for 15 min on a shaker. Cell lysate were collected and centrifuged for 10 min at 13,000 x g to pellet the cellular debris. The protein-containing supernatant was transferred to a chilled test tube.

2.4.2 Bradford assay

Bradford dye (Bio-Rad Protein Assay Catalog #500-0006) concentrate was diluted with H_2O (2ml dye + 8ml d H_2O). Standard protein curve was used to determine the concentration of unknown samples. Calculated OD of each protein sample and OD curve using spectrophotometer (protein concentration OD 595), then analyzed by using standard curve to calculate the protein concentration (as shown in following Table 2):

Table 2: protein concentration of samples

Protein sample	Protein concentration µg/ul
1	9.6
2	9.7
3	9.1
4	8.8
5	9.1
6	8.8

2.4.3 Gel electrophoresis SDS-PAGE

Based on the protein extraction yield (as determined by Bradford Assay), we calculated the sample needed to be added to each lane, for a 10 well mini gel, appropriate 2 X SDS-PAGE dye mixes were added to the protein. Total volume of dye with protein was 50ul containing approximately 40ug of protein. The microtubes were heated at 70°C for 5 min, and then placed on ice. Samples were run at 100 V for 2 h.

2.4.4 Transfer PVDE membrane

The idea in this step is to transfer protein sample from gel to PVDF membrane. Filter paper (typical chromatography paper) in approximately 7 x 20cm pieces with PVDF membrane 7 x 20 cm was used. The PVAF membrane was welted using 100% methanol for five min and then immersed in transfer buffer. The filter paper pads were soaked in PVDF transfer buffer. The membrane sandwich was assembled from black foam, to three filter papers, gel, PVDF membrane, three papers, and white foam. The transfer was filled tank with transfer buffer and placed the sandwich into the transfer buffer and run the transfer at 90V for 1 hour.

2.4.5 Western blot protocol or immunodetection

After completing electrophoretic transfer, the membrane was blocked in 2.5% Non Fat Dry Milk (NFDM) in TBST for overnight. 30 ml of blocking solution was added to the

PVDF membrane. The membrane was incubated with primary antibody (Gulo-antibody) for sixteen h at 4°C. The Gulo-antibody was diluted (1:300 or 1:3000) with 5% Non Fat Dry Milk (NFDM) in TBST solution. The total antibody and diluents solution was 10 ml or 30 ml according to size of membrane. I re-blocked the membrane in 10% Non Fat Dry Milk (NFDM) in TBST solution for 10 min at room temperature. The PVDF membrane was washed three times by 1 X TBST for ten min then incubated with secondary antibody (goat-antibody) at room temperature for 1hour. The antibody was diluted (1:3000) in 5% Non Fat Dry Milk (NFDM) in TBST solution. The membrane was washed three times by 1 X TBST for ten min and the TBST solution was discarded.

2.4.6 Detection

ECL solution was prepared, added 3 ml from reagent 1, and 3 ml from reagent 2. The membrane was incubated with ECL reagents for one minute and then allowed to dry completely. The membrane was then placed in a film cassette with the appropriate film, and exposed in the darkroom with hyperfilm (RPN2114K) for the appropriate amount of time (30s-5min). The film was developed by the developer machine.

2.4.7 Coomassie Blue staining

Coomassie blue was used to stain protein gels. The glass plates were opened and the edges of the gel were released using a razor blade. Then the gel was transferred to a big petri dish. 30ml of Coomassie blue dye was discarded and left gel for 1h. The stain was discarded, destain solution was added to checker for 0.5h. Destaining solution was changed until protein band was clear and picture was taken.

2.5 Design of experiment

Ten groups of mice were used for experiment, there were eight male and two female as shown in Table 3. The genotype of these mice is homozygote *Gulo cll*

positive, they were around 35 days old. Group A, C, E, G, and I were on vitamin C deficiency diet while B, D, F, H, and J were on vitamin C. Table 4, 5 and 6 shown subdivision of each category were listed. As well, these tables have shown the treatment period, genotype and birth day.

Table 3: Groups and treatment condition of mice used

Group ID	Gender	Birth Day	Genotype	Treatment Period	Start Treatment	Harvest Data
A	Male	26-Jun	*Gulo-/-cII+*	Six months	02-Aug	
B	Male	26-Jun	*Gulo-/-cII+*	Six months	02-Aug	
C	Male	15-Jul	*Gulo-/-cII+*	Four months	17-Aug	
D	Male	15-Jul	*Gulo-/-cII+*	Four months	17-Aug	
E	Male	18-Aug	*Gulo-/-cII+*	Two months	19-Sep	20-Nov
F	Male	18-Aug	*Gulo-/-cII+*	Two months	19-Sep	20-Nov
G	Male	17-Sep	*Gulo-/-cII+*	one month	22-Oct	05-Dec
H	Male	17-Sep	*Gulo-/-cII+*	one month	22-Oct	05-Dec
I	Female	16-Sep	*Gulo-/-cII+*		09-Nov	
J	Female	16-Sep	*Gulo-/-cII+*		09-Nov	

Table 4: Group A and B and treatment condition

Group A & B ID	Gender	Birth Day	Diet	Genotype	Treatment Period
A1	Male	26-Jun	No vitamin C	Gulo-/-cII+	Six months
A2	Male	26-Jun	No vitamin C	Gulo-/-cII+	Six months
A3	Male	15-Jul	No vitamin C	Gulo-/-cII+	Six months
B1	Male	15-Jul	On vitamin C	Gulo-/-cII+	Six months
B2	Male	18-Aug	On vitamin C	Gulo-/-cII+	Six months
B3	Male	18-Aug	On vitamin C	Gulo-/-cII+	Six months

Table 5: Group C and D and treatment condition

Group C & D ID	Gender	Birth Day	Diet	Genotype	Treatment Period
C1	Male	26-Jun	No vitamin C	*Gulo-/-cII+*	Four months
C2	Male	26-Jun	No vitamin C	*Gulo-/-cII+*	Four months
C3	Male	15-Jul	No vitamin C	*Gulo-/-cII+*	Four months
D1	Male	15-Jul	On vitamin C	*Gulo-/-cII+*	Four months
D2	Male	18-Aug	On vitamin C	*Gulo-/-cII+*	Four months
D3	Male	18-Aug	On vitamin C	*Gulo-/-cII+*	Four months

Table 6: Group E and F and treatment condition

Group E & F ID	Gender	Birth Day	Diet	Genotype	Treatment Period	Harvest Data
E2	Male	26-Jun	No vitamin C	*Gulo-/-cII+*	Two months	20-Nov
E3	Male	26-Jun	No vitamin C	*Gulo-/-cII+*	Two months	20-Nov
E4	Male	15-Jul	No vitamin C	*Gulo-/-cII+*	Two months	20-Nov
F1	Male	15-Jul	On vitamin C	*Gulo-/-cII+*	Two months	20-Nov
F2	Male	18-Aug	On vitamin C	*Gulo-/-cII+*	Two months	20-Nov
F3	Male	18-Aug	On vitamin C	*Gulo-/-cII+*	Two months	20-Nov

*2.6 Lambda select-*cII *mutation detection system*

2.6.1 Collect blood samples and tissue samples such as liver, kidney, heart, muscle, and brain

For blood collection, mice were placed under anesthetic with isoflourane. The blood sample was collected by inserted 23 G needle on 1 ml syringe into heart on thorax area. The blood sample was transferred into 1.5 ml tubes with 20µl of heparin (20 units) then centrifuged at 2,000 x g. The plasma (top layer) was transferred into 1.5 ml tubes containing 10% of MPA-2mM EDTA solution and mixed well. Samples were centrifuged again for 2 min at 14,000 x g. The plasma was collected into new tubes placed into liquid nitrogen and stored at -80 °C.

For tissue collection, Group E and F were killed after two months treatment (Table 5). The mice were taken from level B room, and then killed after collected blood samples or

put them in CO_2 cage for five to ten min. Tissue samples were collected, wrapped completely in aluminum foil, inserted into liquid nitrogen and stored in -50 °C.

2.6.2 Isolation of genomic DNA from homozygote *Gulo cII* positive mice (Refer to Stratagene manual)

A clean 7-ml Wheaton Dounce tissue grinder lysis buffer was chilled on ice, and liver samples were weighted (mass 50-80mg). The 500 µl lysis buffer was added into clean 7-ml Wheaton Dounce tissue grinder, and following by the tissue samples. The tissue samples were disaggregated by using pestle B-(loose) for 3-10 strokes until the sample appeared completely homogenized. The cell nuclei were released by used pestle A-(tight) for 8 strokes. The homogenate was poured through a sterile cell strainer into a sterile 50 ml conical tube and an additional 3 ml of ice cold lysis buffer were added through cell strainer into conical tube. The cell strainer was discarded and a cap was placed on the conical tube. The conical tubes were centrifuged at 1100 x g for 12 min at 4°C. Uncapped the tube and the supernatant were discarded, and then the uncapped tubes were inverted on paper towel for one minute. Sterile applicators were used to dry the wall of tube. 70-µl aliquot of proteinase K solution was warmed in 50°C water bath for 2-5 min to activate the enzyme. A digestion solution was prepared by adding 20 µl of RNase-It™ ribonuclease cocktail/ml of digestion buffer. 70-µl of prepared digestion solution was added to cell nuclei pellet and the conical tube was rocked gently to dislodge the pellet from bottom of tube. The conical tubes were placed in a 50 °C water bath and 70-µl of warmed proteinase K solution was added to the free-floating pellet. The conical tubes were incubated in a 50 °C water bath for 45 mins. 2 L TE buffer were poured in to a dialysis reservoir and floated a dialysis cup on surface of a buffer. The viscous genomic DNA was collected from conical tube to floating dialysis cup using a used wide-bore pipet tip. The genomic DNA was dialyzed at room temperature for 26 h

and buffer being stirred. The TE buffer was replaced once during the dialysis period. On completing dialysis, the dialysis cup was removed from TE buffer and the genomic DNA was transferred to sterile tube using a wide-bore pipet.

2.6.3 Performing the packaging reaction

A wide-bore pipet tip was used to transfer DNA ≥8µl of genomic DNA samples to red tube containing Transpack packaing extract. The packaging reaction was mixed by pipetting the reaction up and down three times and placed in a 30°C water bath for 90 min. 12 µl of transpack packaging extract were quickly transferred from blue tube to one of the red tubes containing a packaging reaction and mixed well by pipetting the reaction up down three to five times with a wide-bore pipet tip. The packaging reaction was incubated for 90 min in a 30°C water bath. The completed packaging reaction was diluted by adding sterile SM buffer to a final volume of 1 ml, then vortexed at maximum speed for ten 10 S and placed on ice. 50 µl of chloroform was added to per ml of packaged DNA sample, mixed gently and stored at 4°C.

2.6.4 Preparing the SCS-8 plating culture

Used a sterile inoculating loop, SCS-8 host strain was streaked onto an LB-tetracycline agar plate. Bacterial streak plates were incubated for overnight at 37°C and sorted at 4°C. I added 20 ml of NZY broth supplemented with 250 µl of a 20% (w/v) maltose and 1 M $MgSO_4$ solution onto a sterile conical tube and inoculated with several colonies from bacterial streak plate. The SCS-8 cells cultured in NZY broth supplemented with the maltose- $MgSO_4$ solution is referred to as the SCS-8 liquid culture. It was incubated at 37°C for 4-6 h with shaking at 300 rpm. The conical tubes were centrifuged at 1000 x g for 10 min to pellet the bacterial cells. I then discarded the

supernatant and re-suspended the pellet in 10 ml of 10 mM $MgSO_4$. SCS-8 bacterial cell suspensions were diluted to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$.

2.6.5 Titration of the packaged DNA samples

Each packaged DNA sample was vortexed briefly. 200 µl of prepared SCS-8 plating culture was transferred to a sterile 5 ml test tube in duplicate. The test tube containing the packaged DNA samples and SCS-8 plating culture were incubated at 37°C for 15 min to allow the phage to adsorb to host cells. I then added 3-4 ml of molten NZY top agarose cooled to 50°C to each tube. The contents of each tube were poured gently over a separate 100 mm NZY agar plate and agitated the plate to ensure a smooth and even distribution before the top agarose hardens. Then the agar plates were inverted and incubated overnight 37°C air incubator. The number of phage plaques was counted on each agar plate and the mean was determined. The actual number of plaques per microliter of packaged DNA was determined by multiplying the mean by 1.3 as follows:

Mean pfu/ µl x 1.3 = actual number pfu/ µl

Then the actual number of plaques per packaging reaction was calculated as follows: actual number pfu/ µl x 1000 µl/packaging reaction = actual number of plaques pfu/packaging reaction.

2.6.6 Preparing the G1250 liquid culture

Sterile tube inoculating loop was used to streak the bacterial glycerol stock (*E.coli* host strain G1250) onto a 100-mm TB 1-kanamycin agar plate. The bacterial streak plates were incubated in 30°C incubator for 28 h. Before plating, 10 ml of TB1 liquid medium was mixed with 100 µl of the 20% (w/v) maltose- M Mg_2SO_4 solution in a sterile 50 ml screw-cap conical tube. Several colonies were picked from the bacterial streak

plate and inoculated into liquid medium TB. The liquid culture was incubated overnight in a 30°C shaking incubator with vigorous shaking (300 rpm).

2.6.7 Preparing the G1250 plating culture on the day of plating

The G1250 liquid culture in conical tube was centrifuged at 1500 x g for 10 min to pellet the bacterial cells. The supernatant was discarded and the pellet was gently re-suspended in 10 ml of mM $MgSO_4$. The cell suspension was diluted to final OD_{600} of 0.5 with mM $MgSO_4$. The G1250 plating culture was stored on ice for further use.

2.6.8 Plating a packaged DNA sample

16 sterile 14 x 100-mm tubes were labeled and 16 TB1 agar plates as follows: label three tubes and three plates were titer 100, labeled three tubes and three plates were titer 20 and ten plates were screening. 0.2 ml of the G1250 plating culture was taken into each of the 16 labeled tubes (Table 7). The packaged DNA samples were diluted 1:100 by combining 10 µl of packaged DNA samples with 990 µl of SM buffer in each three 1.5-ml microcentrifuge tubes (Table 7):

Table 7: Dilutions of packaged DNA sample

1.5-ml Microcentrifuge tube	Undiluted packaged DNA sample volume	SM buffer volume
A	10 µl	990 µl
B	10 µl	990 µl
C	10 µl	990 µl

Using vortex mixer, the diluted packaged DNA samples were mixed. Each of the three 1:100 dilutions of the packaged DNA samples were divided into two tubes from step 2 that contain 0.2 ml of G1250 plating culture as follows: 100 µl of each 1:100 dilution were added to a tube labeled titer 100 and 20 µl added to a tube labeled titer 20 (six of the 16

tubes from step 2 will be used) (Table 1). 100 µl of undiluted packaged DNA samples was added to each of the tubes from steps 2 labeled screening (Table 1).

Table 8: A scheme of plating a packaged DNA

			Diluted packaged DNA sample volume				
Tube/plate number	Tube label	G1250 plating culture volume	From Tube A	From Tube B	From Tube C	Undiluted packaged DNA sample volume	protocol step
1	Titer 100	0.2 ml	100 µl	----	----	----	4
2	Titer 100	0.2 ml	----	100 µl	----	----	4
3	Titer 100	0.2 ml	----	----	100 µl	----	4
4	Titer 100	0.2 ml	20 µl	----	----	----	4
5	Titer 100	0.2 ml	----	20 µl	----	----	4
6	Titer 100	0.2 ml	----	----	20 µl	----	4
7-12	screening	0.2 ml	----	----	----	100 µl each	5

All tubes were agitated for few s to mix the contents and incubated at room temperature for 30 mins to allow the phage to adsorb to the host cells. Using a 10-ml pipet, 12.5 ml of molten TB1 top agar (cooled to 55°C) was taken and immediately pipet 2.5 ml into each of five of the tubes that contain phage-adsorbed host cells. I quickly but gently, poured the contents of each tube onto the appropriately labeled TB1 agar plate and agitated the plate to ensure the smooth, even distribution before the top agar hardens. The steps were repeated for all 16 plates. The six plates were placed upside down in a stationary 37°C incubator and incubated overnight. The 10 screening plates were place upside down in a stationary 24°C incubator and incubated the plates for 46-48 h.

CHAPTER 3: RESULTS

3.1 Crossing

The plan for crossing the mice was to start with homozygous *Gulo* mice and Big Blue mice. The first generation produced the expected results. There were four heterozygous *Gulo* and two *cII* positive mice, each confirmed by *Gulo* and *cII* genotyping. The body weight of these pups (heterozygote *Gulo cII* positive) was less than wild type Gulo after weaning, but pups (heterozygote *Gulo cII* positive) were maintained from the first crossing on vitamin C supplementation. Then the heterozygous *Gulo cII* positive mice were crossed with homozygous mice. After that, results of this cross were confirmed by genotyping in both cases. Four homozygote *Gulo cII* positive mice were obtained, two males and two females. In addition, some were heterozygous *Gulo cII* positive. For our experiment at least twelve mice homozygous for *Gulo* and *cII* positive were needed. Therefore, homozygous *Gulo cII* positive males were crossed with homozygous *Gulo cII* positive females, producing twenty homozygous *Gulo cII* positive mice. All homozygous *Gulo cII* positive mice were on vitamin C supplementation in drinking water (330 mg per liter) for protection. Seven heterozygous *Gulo cII* positive were also obtained. The homozygous *Gulo cII* positive were kept and crossed with other homozygous *Gulo cII* positive and confirmed by genotype. Thirty homozygous *Gulo cII* positive mice were used for the experiment. Five groups of homozygous *Gulo cII* positive were placed on vitamin C deficient diet while continuing another group of age, genotype and gender matched mice on ascorbic acid supplementation. Five groups were started on treatment at different times and also harvested at different times.

3.2 Genotype

Knockout *Gulo* mice were used to study the effect of ascorbic acid on preventing DNA mutation as a result of oxidative stress. Big Blue mice (carrying the *cII* gene) were used to analyze mutation frequencies. PCR was used to detect alleles of the *Gulo* gene as well as the *cII* gene. Wild type *Gulo cII* negative, wild type *Gulo cII* positive, heterozygote *Gulo cII* negative, heterozygote *Gulo cII* positive, homozygote *Gulo cII* negative and homozygote *Gulo cII* positive mice were obtained during the experiment (Figure 5). These results were confirmed by PCR results, which are 330 bp for wild type *Gulo* and 230 bp for a *Gulo* deletion, and 381bp if the mouse is *cII* positive but no band if *cII* negative (Figure 5: lane 1 and 2). In heterozygous *Gulo* mice there are two different bands: one for the deletion (230 bp) and one for the wild type allele (330 bp) (Figure 5: lane 3 and 4). Also, *cII* primers were used to detect the presence or absence of the *cII* gene in homozygote or heterozygote *Gulo* mice. The PCR amplificon for *cII* positive mice was 230bp in size (Figure 5: lane 5 and 6).

3.2.1 First generation results

Homozygous *Gulo* ($Gulo^{-/-}$) mice were crossed with Big Blue mice (cII^{+}) while on vitamin C supplemented water, and four mice were obtained: two males and two females, referred to as the first generation. Once these mice were weaned, tissue samples were taken from these mice for genotyping. Genotyping was done by PCR using *Gulo* primers and *cII* primers (Figures 6). All first generation mice were heterozygous for *Gulo* while two mice were positive *cII* (Figure 6 for Gulo and *cII* genotyping). For *Gulo*, Each mouse tissue sample shows two bands: one is 230 bp and the other is 330bp. The same DNA from these mice was then taken and used with *cII* primers to detect the *cII* gene from Big Blue mice. Two mice were carrying the *cII* gene, with one 381bp band (Figure 6). The

two mice were heterozygote *Gulo cII* positive (Referred as first generation Gulo$^{+/-}$ *cII*$^{+}$) (Figure 6: lane 1 and 3).

3.2.2 Second generation result

The first generation Gulo$^{+/-}$ *cII*$^{+}$ was re-crossed with homozygous *Gulo* mice, producing six mice. Tissue samples were collected from these mice for DNA extraction. DNA samples were used for PCR reactions (using *Gulo* primers and *cII* primers), and the result was two homozygote *Gulo cII* positive mice and one heterozygote *Gulo cII* positive mouse (Figure 7). The homozygote *Gulo* was re-crossed to obtained more homozygote *cII* positive mice. After DNA extraction and PCR, as seen in the result, the mice were homozygous *Gulo cII* positive (Figure 8).

3.3 Expression of Gulo

Western blot is based on both the antibody reaction and its reaction with the Gulo protein. The Gulo protein was separated on a denaturing SDS polyacrylamide gel and transferred to a PVDF membrane. The membrane was incubated in a solution containing the Gulo antibody (primary antibody) followed by goat antibody (secondary antibody). The membrane was then soaked in the developing solution, producing the obtained result (Figure 9). The summary of this result is that Wild type (*Gulo*$^{+/+}$) mice are capable of expressing the Gulo protein, and can produce ascorbic acid. However, in the knockout mouse (*Gulo*$^{-/-}$) there was no expression of the Gulo protein, which means there could be no ascorbic acid produced.

3.4 Problem with detecting Gulo expression

The first step that we had a problem with expressing the Gulo protein was by Western blot. We used different methods to optimize the Western blot, such as altering the antibody concentration (high or low primary antibody concentration and high or low secondary antibody concentration), the duration of running the gel, more blocking time, altering the efficiency of the transfer buffer, the concentration of the protein and more washing steps. A different idea to improve the Western blot, which leads to expression of the Gulo protein, is by increasing the duration of running the protein gel and the number of wash steps to reduce the amount of non-specific binding. To block the PVDF membrane, 5% non-fat dry milk with TBST buffer was used, and the same buffer was used to dilute the antibody. The PVDF membrane was incubated in blocking buffer for one hour. As well, the concentration of the first antibody was increased (Gulo-antibody 1:300). The membrane was incubated with Gulo-antibody (1:300) overnight, and the membrane was washed four times to minimize non-specific binding. Protein samples were taken from the liver of wild type *Gulo*$^{+/+}$ and heterozygous *Gulo*$^{+/-}$ mice and also from cell cultures as a positive control. Protein samples were also extracted from the kidneys of *Gulo*$^{+/+}$ (negative control), and the heart of *Gulo*$^{+/+}$ (negative control). A protein sample was taken from the liver of a homozygous *Gulo*$^{+/-}$ mouse to compare the expression of the Gulo protein between different genotypes of *Gulo* mice. A faint band was detected in the positive control, no band detected in wild type *Gulo*$^{+/+}$ heterozygote *Gulo*$^{+/-}$, homozygote *Gulo*$^{-/-}$ and negative control. There should have been a clear Gulo band (size of 53 kDa) in wild type *Gulo*$^{+/+}$ and heterozygote *Gulo*$^{+/-}$. But the results in lanes 2 and 3 show no expression of *Gulo*. When compared with the negative controls of lane 4 and 5 there is no specific band related to the Gulo protein (Figure 10).

According to the last result (Figure 10), we decided to use the same samples from different *Gulo* genotypes, including $Gulo^{+/+}$, $Gulo^{-/+}$ and $Gulo^{-/-}$ mice. After the gel was run, the protein was transferred onto PVDF membrane and the membrane was blocked with 2.5% dry milk in TBST (overnight). The PVDF membrane was incubated with primary antibody for 16 h at 4°C, and the Gulo antibody was diluted with 5% non-fat dry milk. Then the Gulo antibody was removed and the membrane was washed three times with 1X TBST. The membrane was incubated with the secondary antibody for 1 hour and the secondary antibody was diluted by 5% milk 1x TBST. The membrane was washed 3x for 10 min with 1x TBST, then was wrapped in cling film and exposed to x-ray film for one min and developed. The result shows protein samples from different genotypes which are $Gulo^{+/+}$, $Gulo^{-/-}$ and a negative control. This shows a decreased amount of non-specific binding, which means the amount of blocking time is important to minimize it. There is a faint protein band, which is expressed on $Gulo^{+/+}$ as shown in lane 2, and there is no band on $Gulo^{-/-}$ as shown in lane 3 and the negative control. As well, there is a band between 40kDa and 55 kDa, but this band is close to 40kDa. As the Gulo protein size should be 53 kDa, this protein is likely not be the Gulo protein (Figure 11).

3.5 Effect of vitamin C deficiency on weight of homozygote Gulo cII positive mice

The initial sign of scurvy is weight loss, as shown in guinea pigs (Hermreck, Kofender, and Bell, 1975;Liu and Lee, 1998). They start to lose weight after 2 weeks on a vitamin C deficient diet. To determine whether this weight loss is noticeable in the homozygous *Gulo cII* positive mice and investigate how long it take to start to be scurvy after both them on vitamin C deficient diet, six groups (A, B, C, D, E and F) of mice were used for treatment. The first group was three males on vitamin C deficient diet and three males

on vitamin C, as well, in a second and third group. These (six groups) homozygote *Gulo*$^{-/-}$ mice were under treatment at age 35 days and weighed once a week and results are discussed below.

3.5.1 Effect of vitamin C deficient diet on homozygote *Gulo cII* positive mice (Group A and B)

Group A (*Gulo*$^{-/-}$ *cII*$^{+}$) and group B (*Gulo*$^{-/-}$ *cII*$^{+}$) were 86 days old when first weighed. They were under treatment for four months. Figure 12 shows weight of mice in cage A and B. Mice A1 (*Gulo*$^{-/-}$ *cII*$^{+}$), A2 (*Gulo*$^{-/-}$ *cII*$^{+}$) and A3 (*Gulo*$^{-/-}$ *cII*$^{+}$) are under treatment of water without vitamin C. Mice B1 (*Gulo*$^{-/-}$ *cII*$^{+}$), B2 (*Gulo*$^{-/-}$ *cII*$^{+}$) and B3 (*Gulo*$^{-/-}$ *cII*$^{+}$) are under treatment of water with vitamin C, which is a control of group A. There is a difference between the weights of mice under water with and those without vitamin C. The mice B1 (*Gulo*$^{-/-}$ *cII*$^{+}$), B2 (*Gulo*$^{-/-}$ *cII*$^{+}$) and B3 (*Gulo*$^{-/-}$ *cII*$^{+}$) are heavier than mice A1 (*Gulo*$^{-/-}$ *cII*$^{+}$), A2 (*Gulo*$^{-/-}$ *cII*$^{+}$) and A3 (*Gulo*$^{-/-}$ *cII*$^{+}$). However mouse B1 in (control) has a high weight compared to the others. It has a high variation as shown in figure 12. Overall, the mice maintained on water with vitamin C (B1, B2 and B3) gain weight more than mice without vitamin C treatment (A1, A2 and A3). Mice A2 and A3 had a rise and fall in their weight, followed by a big drop in weight after being given water without vitamin C. However, Mouse A1 started to gain weight (Figure 12).

3.5.2 Effect of vitamin C deficient diet on homozygote *Gulo cII* positive mice (Group C and D)

Six male mice were 66 days old when first weighed. Figure 13 shows the weight of mice in groups C and D. Mice C1, C2 and C3 are under treatment of water without vitamin C, and mice D1, D2 and D3 are supplied with vitamin C as a control of group C. There is no significant weight loss observed until two months after taking away vitamin C. Then, after three months of absence of vitamin C, they started losing weight. The mice

on C1, C2 and C3 are between 28 g and 31 g. Also, during the treatment mice on a vitamin C deficient diet were in stable weight as in lane C3. But mice in the control group have variation as shown by the weight of mouse D2, and mouse D3 (*Gulo*$^{-/-}$ *cII*$^{+}$) had a low weight compared with other mice on vitamin C (Figure 13).

3.5.3 Effect of vitamin C deficient diet on homozygote *Gulo cII* positive mice (Group E and F)

As mentioned before, the difference between homozygote *Gulo*$^{-/-}$ *cII*$^{+}$ mice on vitamin C deficient diet and on vitamin C, group E (*Gulo*$^{-/-}$ *cII*$^{+}$ on vitamin C deficient diet) mice and F (*Gulo*$^{-/-}$ *cII*$^{+}$ on vitamin C), was significant in weight loss. Group E mice started to lose in their weight one month after vitamin C withdrawal. After nineteen days of losing weight, they lost 22% of their weight and the experiment was discontinued. However, mice in Group F gained weight and were still healthy until the end of the experiment (Figure 14).

3.6 Estimated cII mutant frequencies in the liver of homozygote Gulo cII positive mice on vitamin C deficient diet

Spontaneous production of hydrogen peroxide has been recognized as a mutagenic factor (Glaab, Hill, and Skopek, 2001). Ascorbic acid is an antioxidant that can reduce the harmful effect of oxidative stress (Glaab, Hill, and Skopek, 2001;Mure and Rossman, 2001). Knockout *Gulo*$^{-/-}$ mice have been used to investigate the role of ascorbic acid on free radicals and 7,8-dihydro-8-oxoguanine (8-oxoG)(Gaut *et al.*, 2006). Big Blue mice have been used to detect spontaneous mutations (Moore *et al.*, 1999). To determine whether ascorbic acid can suppress production of free radicals following oxidative stress, we placed one group of *Gulo*$^{-/-}$ *cII*$^{+}$ mice on water without ascorbic acid for forty eight days while maintaining another group of same age and gender mice on ascorbic acid

supplementation. After 48 days the ascorbic acid deficient mice had lost 22% of their weight. The mutation frequency was calculated (Table 8). The total plaques in an ascorbic acid deficient (E3) mouse were an insufficient plaque which means no significant number to calculate the mutation frequency (Table 8 and 9). But, the total plaques in another ascorbic acid deficient mouse (E2) were 108,750 pfu and a total of 13 mutant plaques. The mutation frequency in this mouse (E2) was 1.25×10^{-4}. Also, in mouse E4 the total number of plaques screened was 108,750 pfu and 7 were mutant plaques, making the mutation frequency in this mouse (E4) 6.5×10^{-5}. The total number of plaques measured in the ascorbic acid supplemented (F1, F2 and F3) group included one mouse with no significant result (F3). However, the F1 mouse had a mutation frequency of 1.96×10^{-5} and mouse F2 had 6.9×10^{-5} (Table 9). The level of mutation frequencies of $Gulo^{-/-}$ cII^{+} (untreated) was 1.25×10^{-4} mutation frequency (MF) and of (treated) was 6.9×10^{-5} mutation frequency (MF). Therefore the number of spontaneous mutations was greater in an ascorbic acid deficient diet than in the group on ascorbic acid supplementation. To this point, the level of mutations in other groups must be confirmed as well as in the rest of mice (E2 and E3) because a low number of plaques were obtained in tittering. Furthermore, the Package DNA sample will be amplified and then sequenced to identify mutation type.

Table 9: Titration of the packaged DNA samples of *Gulo*$^{-/-}$ *cII*$^{+}$ treated and untreated.

			Mean number of plaques	Total Plaques Screened
Group E &F	Genotype	Treatment Ascorbic Acid (AA)	Titer 20	Titer 20
E2	*Gulo*$^{-/-}$*cII*$^{+}$	No AA	69	103,500
E3	*Gulo*$^{-/-}$*cII*$^{+}$	No AA	0	0
E4	*Gulo*$^{-/-}$*cII*$^{+}$	No AA	108.75	108,750
F1	*Gulo*$^{-/-}$*cII*$^{+}$	On AA	34	51000
F2	*Gulo*$^{-/-}$*cII*$^{+}$	On AA	106.25	159,375
F3	*Gulo*$^{-/-}$*cII*$^{+}$	On AA	0	0

Table 10: Measurement of mutation frequency on *Gulo*$^{-/-}$ *cII*$^{+}$ treated and untreated.

Group E &F	Genotype	Treatment Ascorbic Acid (AA)	Total Mutant (screened)	Mutant Frequencies	Average	Standard Error
E2	*Gulo*$^{-/-}$*cII*$^{+}$	No AA	13 plaques	1.25×10^{-4}	6.30×10^{-5}	3.60879×10^{-5}
E3	*Gulo*$^{-/-}$*cII*$^{+}$	No AA	0 plaques	0		
E4	*Gulo*$^{-/-}$*cII*$^{+}$	No AA	7 plaques	6.4×10^{-5}		
F1	*Gulo*$^{-/-}$*cII*$^{+}$	On AA	1 plaques	1.96×10^{-5}	2.95×10^{-5}	2.05285×10^{-5}
F2	*Gulo*$^{-/-}$*cII*$^{+}$	On AA	11 plaques	6.9×10^{-5}		
F3	*Gulo*$^{-/-}$*cII*$^{+}$	On AA	0 plaques	0		

List of Figures:

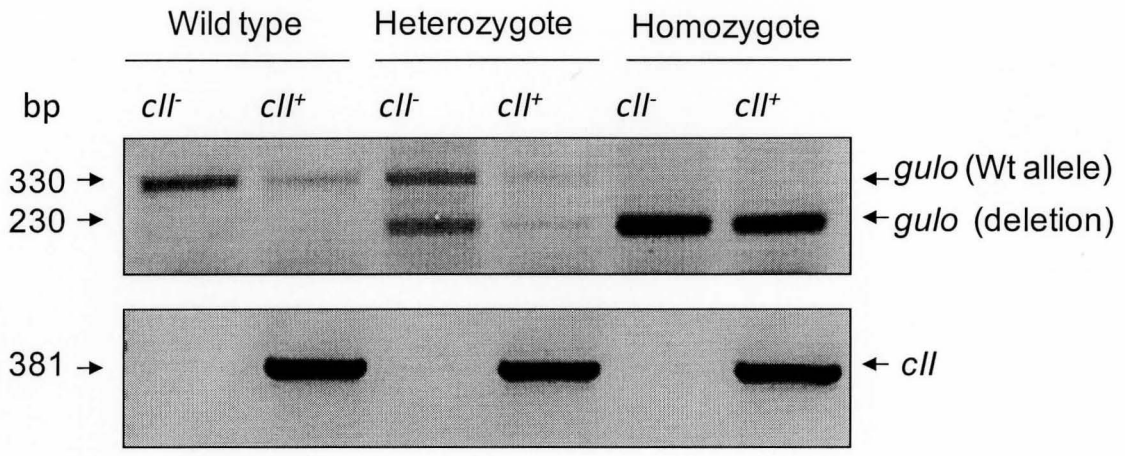

Figure 6: Gel photo showing PCR products from amplification of genomic DNA isolating from mice using *Gulo* and *cII* primers. As well, the different genotypes resulting from crossing Gulo and Big blue mice are shown, such as Wild type *Gulo cII*$^{-}$ (lane: 1), Wild type *Gulo cII*$^{+}$ (lane: 2), Heterozygote *Gulo cII*$^{-}$(lane: 3) , Heterozygote *Gulo cII*$^{+}$ (lane: 4), Homozygote *Gulo cII*$^{-}$ (lane: 5), Homozygote *Gulo cII*$^{+}$(lane: 6).

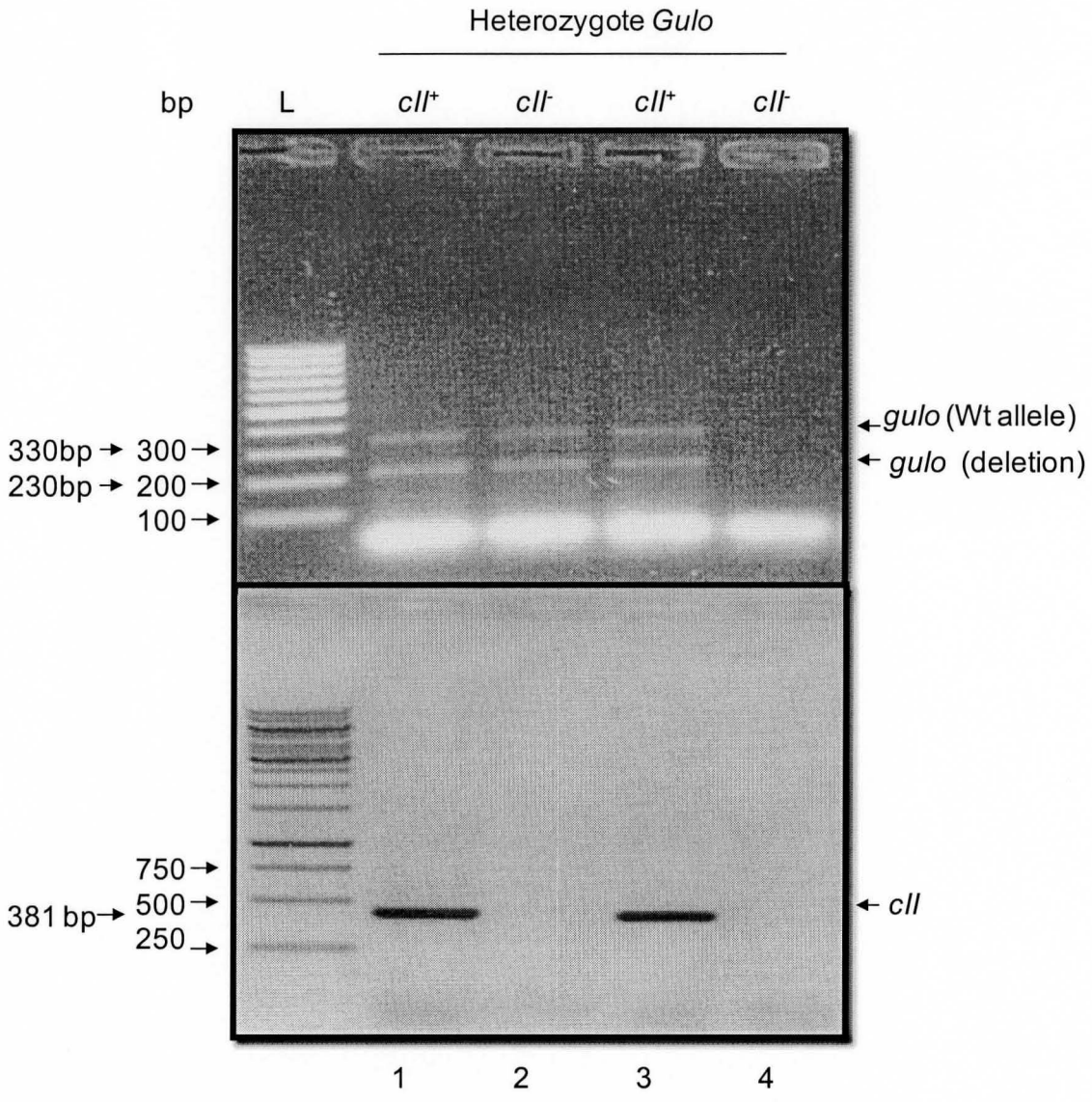

Figure 7: First generation results of PCR genotyping from amplification of genomic DNA isolated from first generation mice (crossed *Gulo*$^{-/-}$ with *Gulo*$^{+/+}$ *cII*$^{+}$). The results were two heterozygote *Gulo* and two heterozygote *Gulo cII*$^{+}$ mice (lane: 1 and 3).

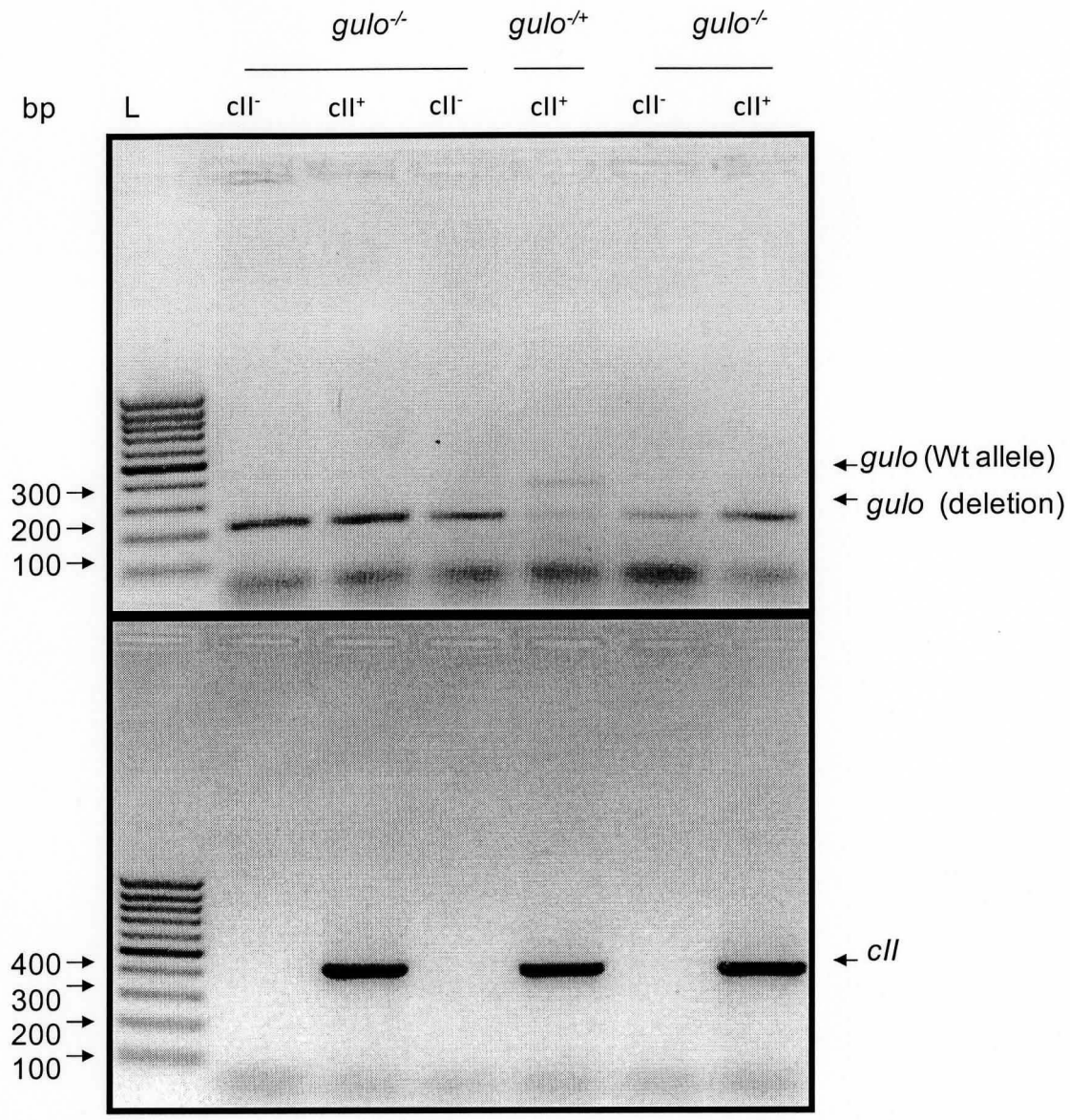

Figure 8: Genotype results of second generation mice (F2). Genomic DNA was extracted from F2 (crossed *Gulo*$^{+/-}$ *cII*$^{+}$ with *Gulo*$^{-/-}$), and the results were three mice homozygote *Gulo* (lane: 1, 3 and 5), two homozygote *Gulo*$^{-/-}$ *cII*$^{+}$ (lane: 2 and 6) and one heterozygote *Gulo*$^{+/-}$ *cII*$^{+}$ mouse (lane: 4).

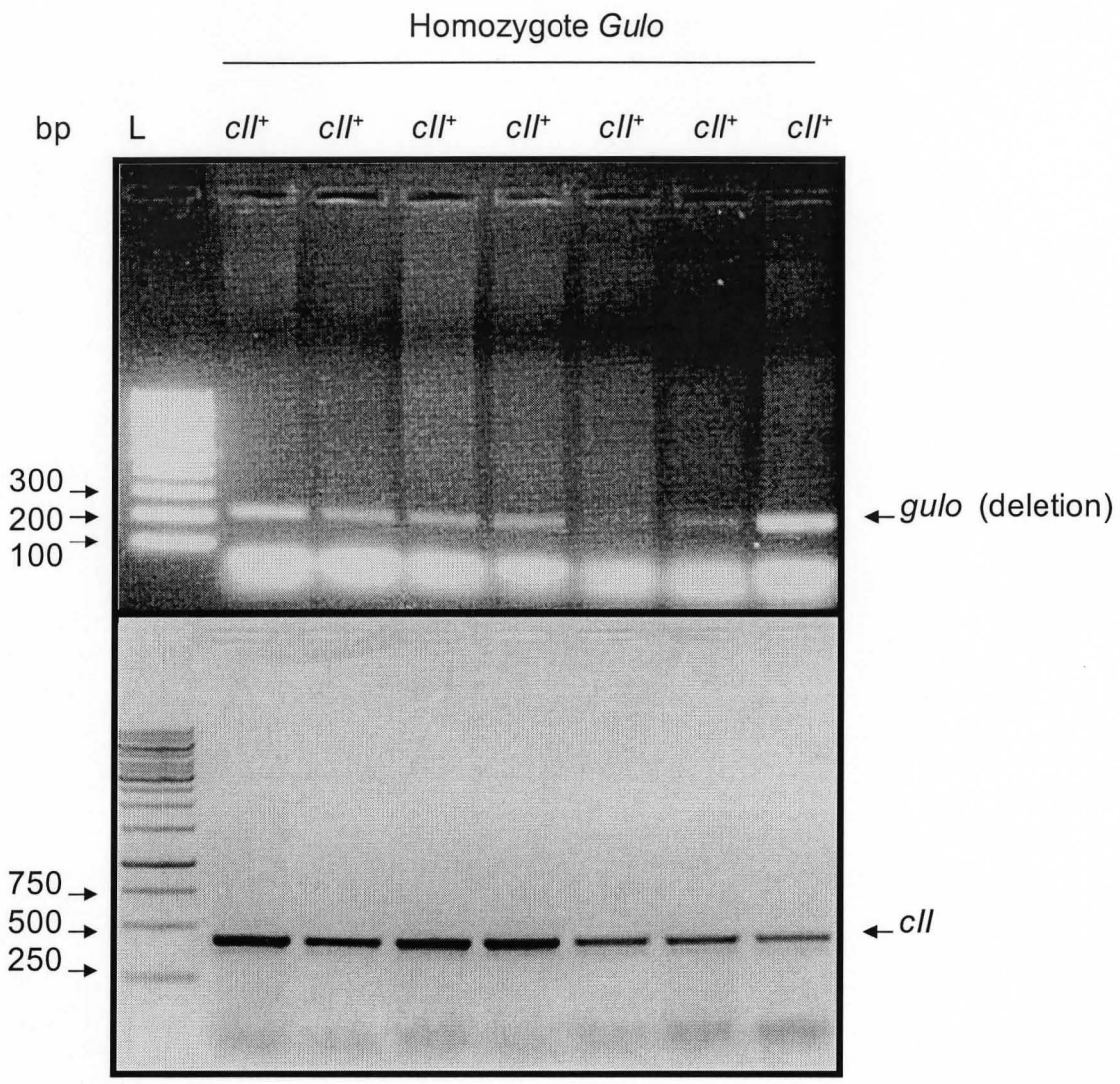

Figure 9: Image of PCR amplification of genomic DNA from mice with *Gulo*$^{-/-}$ *cII*$^{+}$ parents. All mice shown were homozygote *Gulo*$^{-/-}$ *cII*$^{+}$.

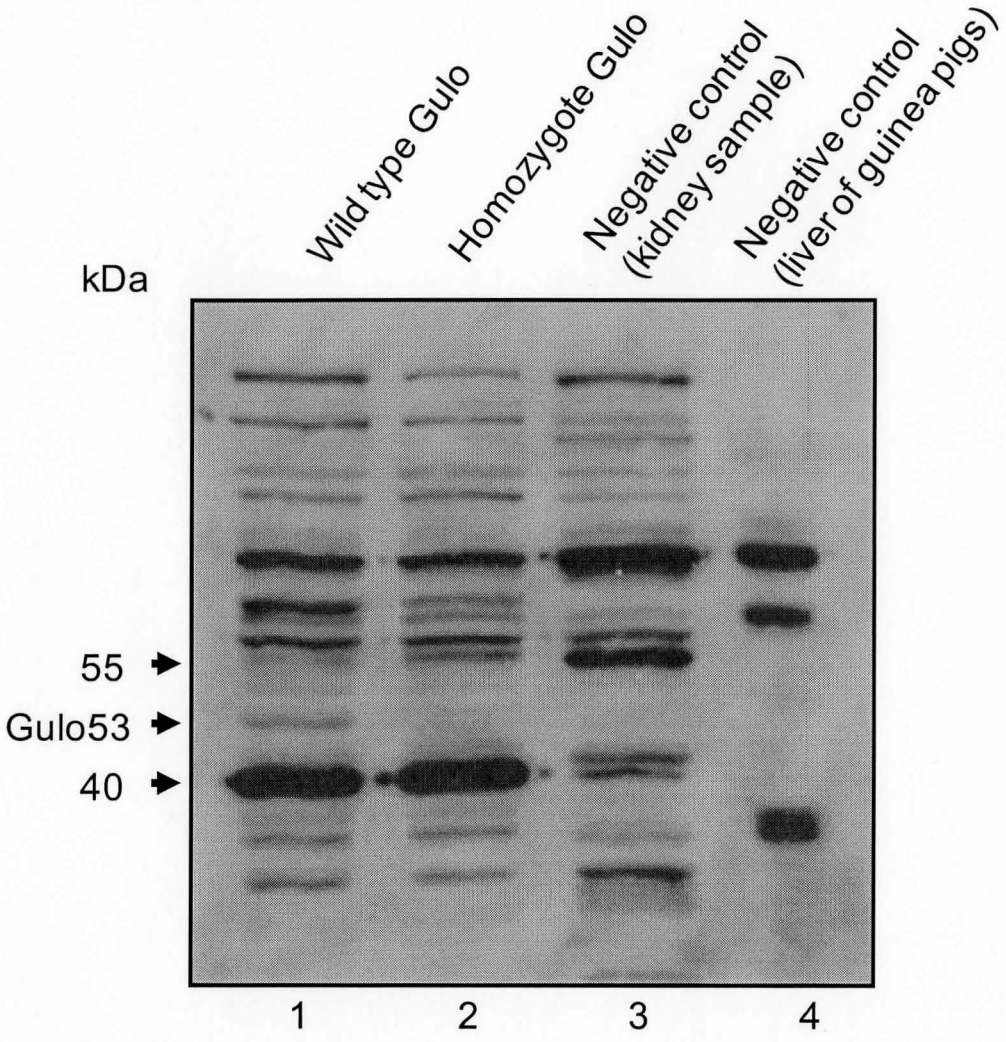

Figure 10: Western blot analysis of *Gulo* levels using Gulo-antibody protein (20-30mg) of wild type $Gulo^{+/+}$ and homozygote $Gulo^{-/-}$ mice. Liver samples were collected from mice, then protein samples were extracted from liver, spleen, and mammalian cells. Gulo-antibody used as primary antibody followed by Goat-antibody as secondary antibody. Lane 1: wild type $Gulo^{+/+}$, Lane 2: homozygote $Gulo^{+/+}$, Lane 3 and 4 were extracted from liver of guinea pigs and kidney, used as negative control.

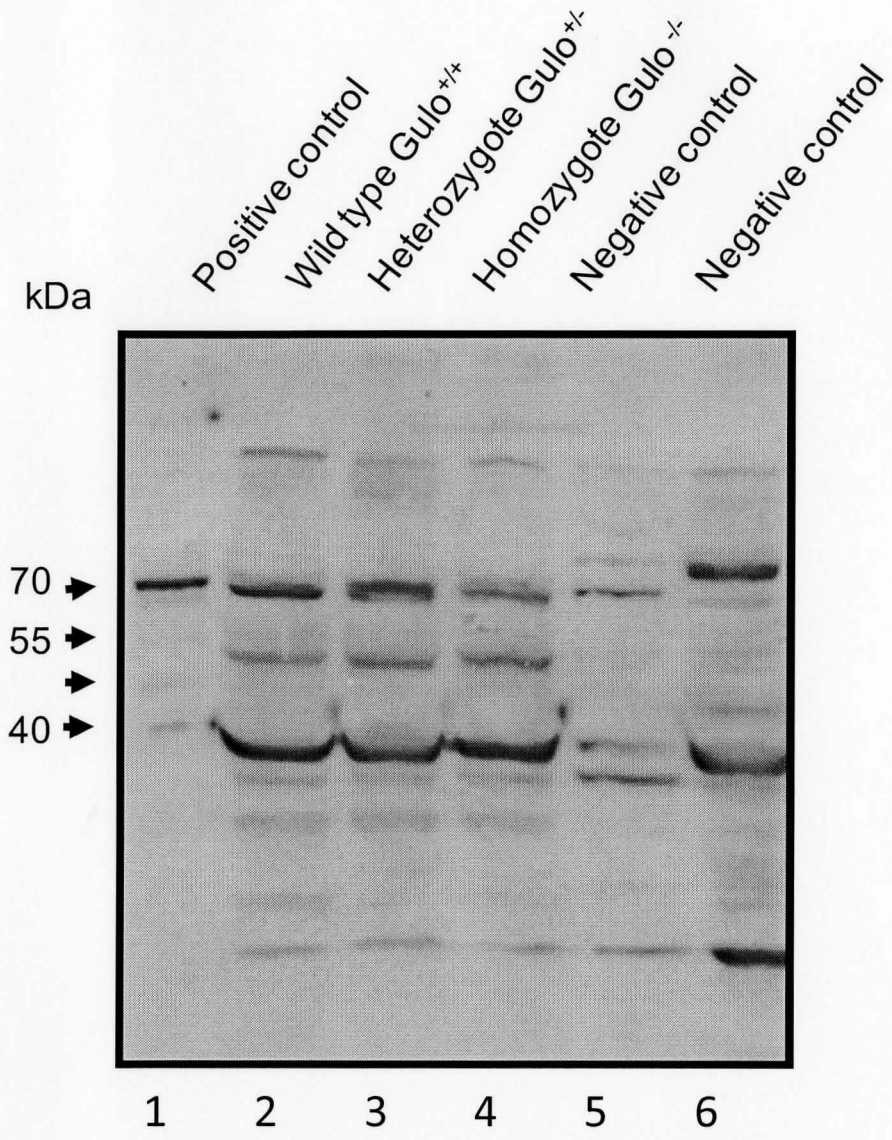

Figure 11: Western blot of Gulo protein expression in wild type *Gulo* and knockout *Gulo*$^{-/-}$ mice. Liver samples were collected from mice, then protein sample were extracted from liver, spleen, and mammalian cells. Gulo-antibody used as primary antibody followed by Goat-antibody as secondary antibody. Lane 1: positive control, Lane 2: wild type wild type *Gulo*$^{+/+}$, Lane 3 heterozygote *Gulo*$^{-/+}$, Lane 4: homozygote *Gulo*$^{-/-}$, Lane 5 and 6 extracted from different tissue, used as negative control.

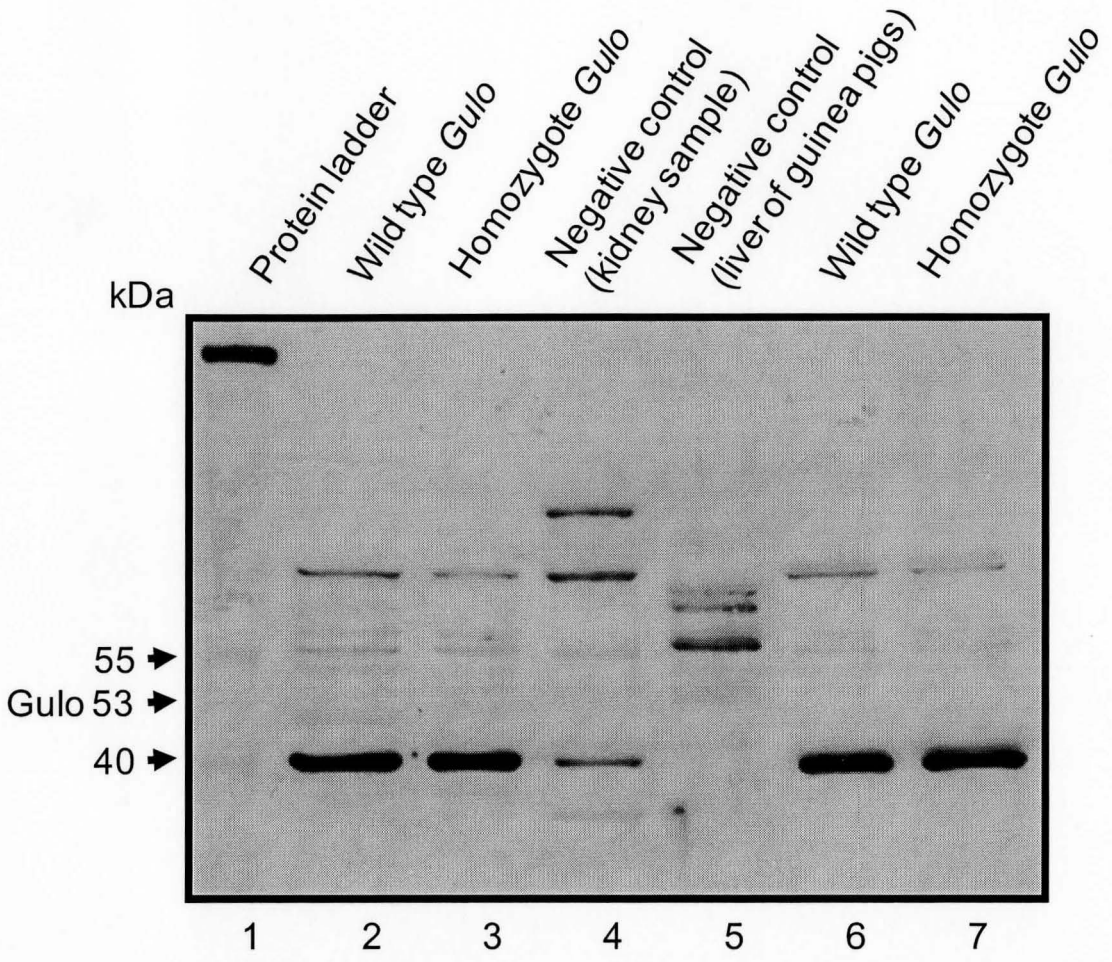

Figure 12: Western blot of Gulo protein expression in wild type *Gulo*$^{+/+}$ and knockout *Gulo*$^{-/-}$ mice. Liver samples were collected from mice, and then protein samples were extracted from the liver and spleen. Gulo-antibody was used as primary antibody followed by Goat-antibody as secondary antibody. Lane 1: protein ladder, Lane 2: wild type *Gulo*$^{+/+}$, Lane 3: homozygote *Gulo*$^{-/-}$, Lane 4: negative control (kidney sample), Lane 5: negative control (liver sample of guinea pigs), Lane 6: wild type *Gulo*$^{+/+}$ Lane7: homozygote *Gulo*$^{-/-}$.

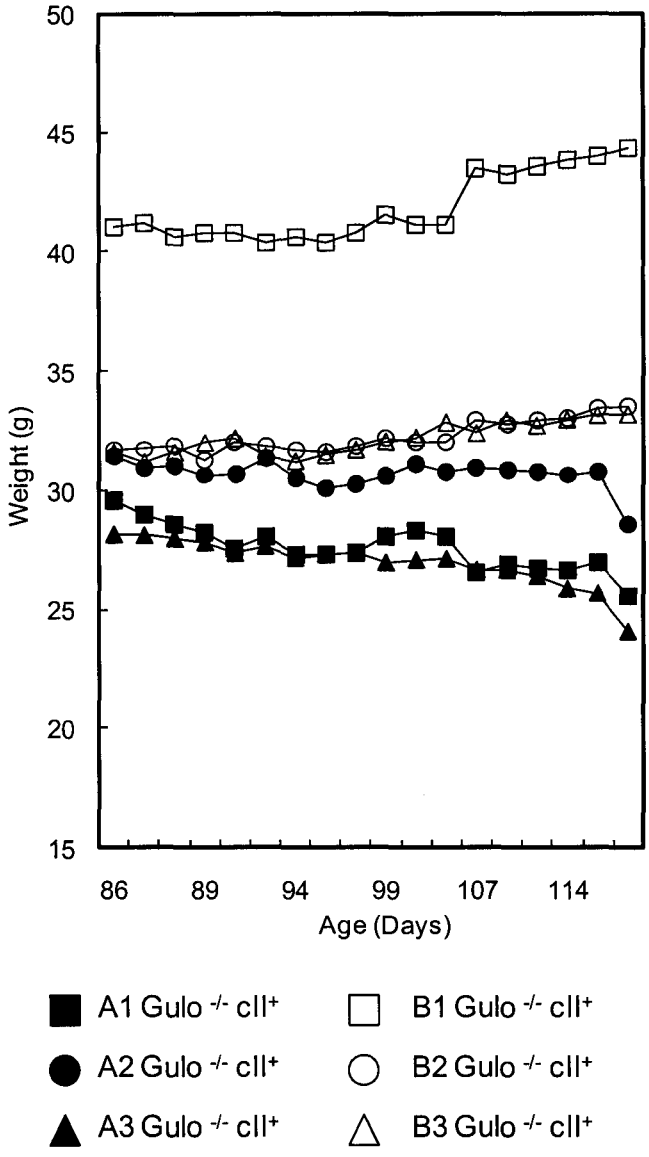

Figure 13: Weight data of mice in groups A and B. A1, A2 and A3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water without vitamin C. B1, B2 and B3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water with vitamin C (control). There is a difference between mice on water without vitamin C and on vitamin C.

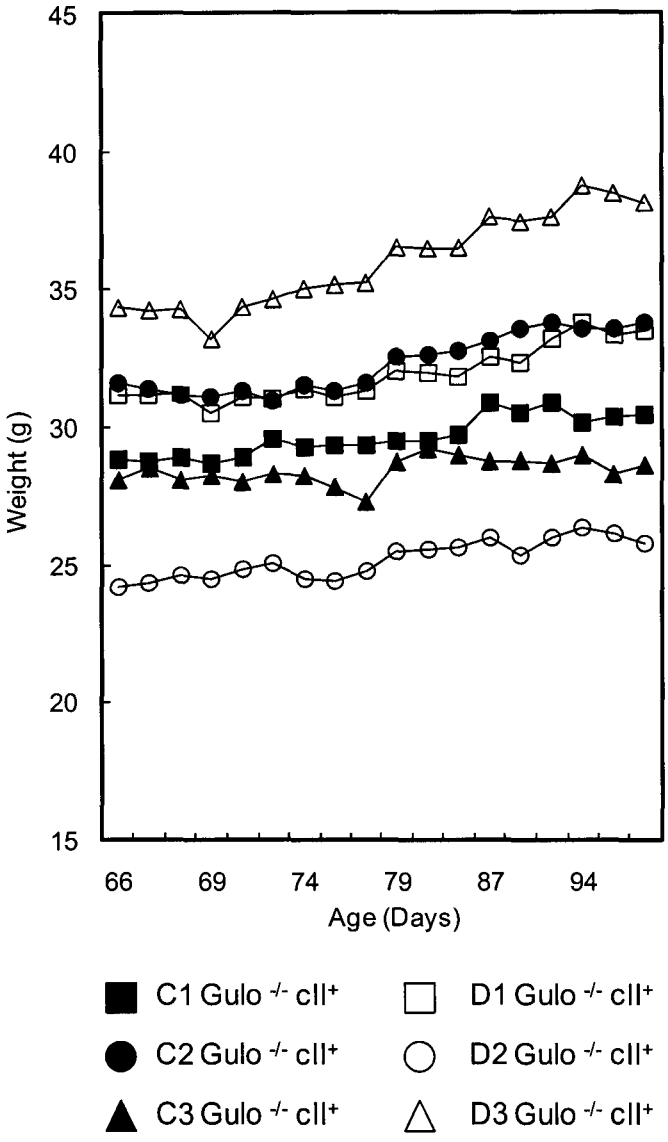

Figure 14: Weight data of mice in groups C and D. C1, C2 and C3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water without vitamin C. D1, D2 and D3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water with vitamin C (control). There is a different between mice on water without vitamin C and on vitamin C.

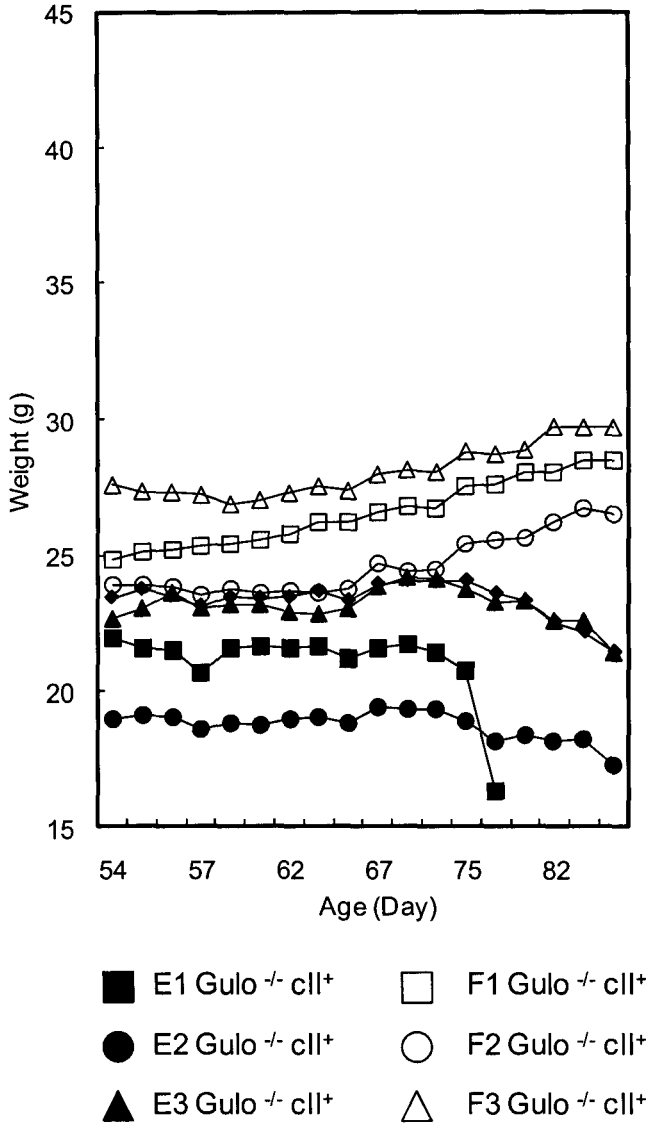

Figure 15: Weight data of mice in groups E and F. E1, E2 and E3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water without vitamin C. F1, F2 and F3 are *Gulo*$^{-/-}$ *cII*$^{+}$ and under water with vitamin C (control). Figure shown; there is a difference between mice on water without vitamin C and on vitamin C.

CHAPTER 4: DISCUSSION

Vitamin C is required for several important biological processes in the body. It minimizes mutations by reducing the effect of free radicals during oxidative stress (Montecinos *et al.*, 2007;Rodriguez *et al.*, 2005). Ascorbic acid has been implicated to be important in processes such as iron absorption, spermatogenesis, wound healing, blood formation and in boosting the immune system (Chen *et al.*, 2003;De la Fuente *et al.*, 1998;Fraga *et al.*, 1991;Parsons *et al.*, 2006). It acts as a co-factor to help in hydroxylation of proline to protect against cardiac vascular diseases, cancer and common colds (De la Fuente *et al.*, 1998;Deicher *et al.*, 2005;Pauling, 1971). In this study, we investigated 1) whether vitamin C acts as an antioxidant and 2) the ability of vitamin C to eliminate the harmful effects of free radicals, and therefore the development of certain diseases.

Reactive oxidative species (ROS) are a byproduct of normal processes that occur in the body, and these free radicals lead to DNA damage (Burk *et al.*, 2006;Duarte and Lunec, 2005;Evans and Halliwell, 1999). There is an increase in the level of 8-oxoG during any oxidative stress (Tarng, Liu, and Huang, 2004). In addition, one study reported that vitamin C acts as antioxidant, and described the damage that can happen during vitamin C deficiency (Montecinos *et al.*, 2007;Rodriguez *et al.*, 2005). Animal models are used to investigate the role of ascorbic acid in oxidative stress in vivo (Gaut *et al.*, 2006). Knockout *Gulo*$^{-/-}$ mice, guinea pigs, and Osteogenic Disorder Sionogi rats are common animal models used to study vitamin C (Alcantara-Martos *et al.*, 2007;Burk *et al.*, 2006;Li, Maeda, and Beck, 2006). These model organisms cannot form L-gulonolactone oxidase, which is the main enzyme for the production of vitamin C (Nishikimi, Kawai, and Yagi, 1992;Togari *et al.*, 1995). In this study, we utilized knockout *Gulo*$^{-/-}$ mice to investigate the function of ascorbic acid in decreasing the mutation rate

and Big Blue mice to quantify and identify the type of mutations caused during vitamin C deficiency. Big Blue mice can detect DNA mutations in any part of the body because they have prokaryotic shuttle vectors carrying a reporter gene, such as the *cII* and the *lacI* genes (Jakubczak *et al.*, 1996;Kohler *et al.*, 1991). These reporter genes are integrated into chromosome 4 in Big Blue mice (Kohler *et al.*, 1991). Here, we crossed Big Blue mice with homozygote $Gulo^{-/-}$ mice. The first generation, which was heterozygote $Gulo^{+/-}$ *cII* positive, was then re-crossed with homozygote $Gulo^{-/-}$ mice to produce homozygote $Gulo^{-/-}$ *cII* positive mice. We had several problems with the first cross. It took a long time to breed, pups often died soon after birth, litter sizes were small, and pups were skinny. Pups from the first generation were weak, and we had to keep them more than four weeks with their parent. The second generation mice were healthy. Their weights, once weaned as normal mice, were between 18 g and 19 g. The main symptom of knockout *Gulo* mice on a vitamin C deficient diet is weight loss. This symptom is apparent in guinea pigs when also on a vitamin C deficient diet. Vitamin C deficiency in the guinea pig may affect the digestion of food (Hermreck, Kofender, and Bell, 1975;Liu and Lee, 1998), which may also affect their weight.

In homozygous $Gulo^{-/-}$ mice, animals were fed without vitamin C, and control animals were fed with vitamin C in water. Our results show that vitamin C deficiency affects the weight of homozygous $Gulo^{-/-}$ mice. The knockout $Gulo^{-/-}$ *cII* positive mice were observed for more than five months for weight loss while on a vitamin C deficient diet. Some of these mice, after two months, lost more than 22% from their original weight. Post-mortem inspection showed some hemorrhaging in the muscles and an emaciated carcass. The control mice matches with vitamin C water were healthy, and showed no sign of weight loss, and in fact gained weight. Another group of mice on a vitamin C deficient diet had fluctuated in their weight. These mice remained healthy, and did not show signs of scurvy. Their overall weight, however, was less than the control mice on

vitamin C. Our results show two general trends in mice without vitamin C: mice losing weight after two months of treatment and mice a fluctuation in weight. Our investigation was to determine the difference in weight of knockout *Gulo*$^{-/-}$ *cII* positive mice, but how these mice can survive for a long time and not show signs of scurvy? Also, why did one group have early signs of scurvy and lose 22 % of their weight in a short period? To answer the questions, the level of vitamin C will be measured in their blood by HPLC and the food and shelter will be reanalyzed in the future.

A Western blot was done to determine the expression of Gulo protein. Protein was extracted from tissue samples and Gulo antisera were used to detect the Gulo protein. We optimized the Western blot because there was considerable nonspecific Gulo protein binding, which blocks the expression of the Gulo protein. We used a low Gulo antibody concentration, and subjected the film for two h of blocking. Once we increased the Gulo-antibody concentration and increase blocking time, we could more easily identify the Gulo protein. No *Gulo* signal was found in knockout *Gulo*$^{-/-}$ mice and the negative control. Guinea pig protein was used as a negative control because they do not have the *Gulo* gene. As well, to compare between wild type *Gulo*$^{+/+}$ mice and knockout *Gulo*$^{-/-}$ mice, a Western blot was used to identify any expression to Gulo in wild type *Gulo*$^{+/+}$ mice. We expressed Gulo in wild type *Gulo*$^{-/-}$ mice and compared the Gulo protein levels in samples collected of homozygote *Gulo*$^{-/-}$ and wild type *Gulo*$^{+/+}$ mice. As expected we found were that, in wild type mice there is expression of Gulo protein, and in homozygous mice there is no expression of the Gulo protein.

We then investigated the level of spontaneous mutation frequency in knockout *Gulo*$^{-/-}$ cII positive mice in a vitamin deficient diet. After a period of weight observation, group E and group F were killed and different tissue samples were collected. After DNA extraction, the DNA was packaged into phage and infected into an *hfl*$^{-}$ strain (*E.coli* G1250). Cells were grown at two different temperatures (24°C and 37°C). Only mutant

phage can grow at 24°C and make plaques at low temperature. On the other hand, all infected bacteria could make plaques at 37°C. The mutant frequency that resulted from vitamin C deficiency can be determined by the ratio of lambda *cII* plaques to the total number of plaques screened. Mutant plaques can be sequenced, and the type of mutation determined. The results show a high level (10^{-4}) of mutation in the vitamin C deficient group - more than those on vitamin C. Similarly, Big Blue mice were used to detect spontaneous mutations during vitamin E deficiency (10^{-5}) of mutation frequency (Moore *et al.*, 1999). However, our result was a higher level of mutation which means that there is something stressful that may increase the mutation frequency. It could be that the absence of vitamin C results in a greater frequency of DNA mutation. To confirm this result we need to obtain data from all mice and all groups. In addition, analysis of the mutation frequency for all the groups at that time can be compared to make conclusions of the final result and to see any differences. We can then analyze and confirm the result by DNA sequencing and identify the types of mutation (spontaneous or transition). In addition, we could detect the level of 8-oxo-7, 8-dihydro-2-deoxtguanosine (oxo^8dG) that result from oxidative stress and cause spontaneous mutation (Cadenas *et al.*, 1997;Moore *et al.*, 1999).

Moreover, the results show that vitamin C is important as a nutrient, in both spontaneous mutation and body weight experiments. This study demonstrated that body weight in mice decreased during a vitamin C deficient diet, compared with a control group which received vitamin C. But the variation between these groups was large, which means that there could be other variables. These mice may have been in a stress condition or been infected which could affect body weight. Also the level of mutations was high, making it surprising that mice can survive with such a high level of mutations.

CHAPTER 5: FUTURE WORK

a) PCR amplification and sequence analysis of lambda *cII*⁻ mutants:

The *cII*⁻ mutant will be amplified and sequenced by PCR by using *cII* primers. Primer sequences are listed in table 1. As well, we can analyze the sequence of the PCR product and compare to the wild type lambda *cII* gene and surrounding regions in Big Blue shuttle vector.

b) Group A, C, G, and I will be tested by using lambda select-*cII* mutation detection system and compared with results of group E.

c) Measurement of vitamin C concentration in blood by using HPLC.

CHAPTER 6: APPENDICES

6.1 Point Form Methods

Polymerase Chain Reaction with Gulo primers

Combine reaction components in a 0.5 ml PCR tube in the following order:

PCR - Reagent	Volume (µl)	x5	x10	x20
ddH_2O	12.5	62.5	125	250
10x PCR buffer w/o $MgCl_2$	2.5	12.5	25	50
$MgCl_2$ (25 mM)	1.5	7.5	15	30
dNTP mix (10 mM)	0.5	2.5	5	10
P2 Rv Primer (25 µM)	1	5	10	20
P3 Fr Primer (25 µM)	1	5	10	20
P4 Rv Primer (25 µM)	1	5	10	20
DNA	5	5	5	5
Taq Pol (1 U/µl)	1	5	10	20

PCR thermocycler conditions

PCR Step	Temperature (°C)	Duration	Number of Cycles
Initial denaturation	94	2 min	1
Cycled denaturation	94	20 s	
Annealing	52	30 s	30
Extension	72	30 s	
Terminal extension	72	7 min	1

Polymerase Chain Reaction with *cII* primers

PCR - Reagent	Volume (µl)	x5	x10	x20
ddH_2O	13.5	67.5	135	270
10x PCR buffer w/o $MgCl_2$	2.5	12.5	25	50
$MgCl_2$ (25 mM)	1.5	7.5	15	30
dNTP mix (10mM)	0.5	2.5	5	10
P2 Rv Primer (25 µM)	1	5	10	20
P4 Rv Primer (25 µM)	1	5	10	20
DNA	5	5	5	5
Taq Pol (1 U/µl)	1	5	10	20

PCR thermocycler conditions

PCR Step	Temperature (°C)	Duration	Number of Cycles
Initial denaturation	94	2 min	1
Cycled denaturation	94	20 s	
Annealing	48.8	30 s	30
Extension	72	30 s	
Terminal extension	72	7 min	1

Identification of DNA on Agarose Gel

1. Weight 0.5 or 1 g of agarose (1%) and put in a 250 ml flask. Make volume to 50 or 100 ml from 1 x TAE.
2. Heat the mix in microwave for approximately 1 min. (or until the agarose is dissolved).
3. Leave in the fume hood to cool then add 25 µl (if using big gel) or 10 µl (if using small gel) of ethidium bromide.
4. Pour into a clean tray, insert comb and leave it to dry.
5. Once set, remove the comb and put gel in the gel box and fill it with 1 x TAE.
6. For sample preparation, add 2 µl of 6 x loading dye to 25 µl of the PCR reaction.
7. Load your samples (15 µl) and the 100bp ladder (5µl).
8. Run the gel for 35-40min.
9. Visualize the gel by placing on a UV light box and take a picture.

Western Blot

Tissue sample collection

Tissue samples were taken from each of wild type Gulo, heterozygote Gulo, homozygote Gulo mice and guinea pigs. The tissue samples were taken from the liver, spleen, kidney, and from mammalian cells. Freeze tissue in liquid nitrogen after excision and store at -80°C.

Procedure:

A- Tissue protein extraction

For this procedure, a ratio of tissue to CelLytic MT reagent of 1:20 (1 gram of tissue to 20 ml of reagent) is recommended. When a concentrated lysate is required, you can lyse the tissues in a lower volume of CelLytic MT reagent. The proteinase inhibitor Cocktail may be added to the CelLytic MT reagent.

1. Weigh tissue samples 1:20.
2. Add the appropriate amount of CelLytic MT to the tissue sample.
3. Transfer the sample (with lysis/extraction reagent) to pre-chilled microhomogenize.
4. Centrifuge the lysed sample for 10 min at 12 000-20 000 x g to pellet the tissue debris.
5. Re-centrifuge the lysed sample for 15 min at 12 000-20 000 x g to get all tissue debris, also to get clear lysate.
6. Transfer the protein containing supernatant to a chilled test tube.
7. If necessary, store the lysate at -70°C (lysate preservation requires low temperatures).

B- Cellular protein extraction:

The volume of CelLytic MT reagent to be added to the cells varies according to cell size and protein concentration required. In general, 125 µl of CelLytic MT is recommended for 10^6-10^7 cells. For adherent cells the plate size will dictate the amount of reagent used to cover the plate surface. Suggested working volumes are: 500-1000 µl for a 100 mm plate and 200-400 µl for a 35 mm plate.

1. Wash cells and lyses, and for adherent cells remove the growth medium from the cells to be assayed. Rinse the cells once with DPBS, being careful not to dislodge any of the cells. Discard DPBS. Lyse cells with appropriate volume of CelLytic MT reagent.
2. Incubate the cells for 15 min on a shaker.
3. Collect cell lysate.
4. Centrifuge the lysate cells for 10 min at 12 000-20 000 x g to pellet the cellular debris.
5. Transfer the protein-containing supernatant to a chilled test tube.

Bradford assay

1. Dilute Bradford dye concentrate with H_2O (2 ml dye + 8 ml d H_2O)
2. add 1 ml of diluted dye to appropriate number of disposable test tubes
3. Add 0, 2, 4, 6, 8, and 10 µl of Bio-Rad protein assay standard II (BSA) to the first 6 test tubes.
4. Add appropriate amount of unknown protein samples to the rest of the tubes. (1-5 µl).
5. Mix each tube well and transfer to semi-micro 1 ml cuvettes.
6. Measure the absorbance at 595 nm.

7. Used Microsoft Excel to calculate the concentration of protein and make a protein standard curve (protein concentration vs. OD 595).
8. Estimate the protein concentration in each of the unknown samples.

Stacking gel preparation

1. Prepare the running gel (10%) based on the following recipe (total = 10 ml):
    - 3.3 ml of 30% Bis/Acrylamide
    - 2.5 ml of 1.5 M Tris-HCl pH 8.8
    - 0.1 ml of 10% SDS
    - 4.0 ml of ddH_2O
    - 0.1 ml of 10% APS
    - 0.01 ml of TEMED
2. Set up the gel apparatus. Remember to clean the glass plates with kim wipes.
3. Pipet about 4 ml of the running gel into the plate.
4. Slowly add ddH_2O up to the top to avoid dehydration. Do not disturb the gel layer.
5. Start preparing the upper gel (4%) after 0.5 h. Use the following (total = 5ml)
    - 0.66 ml of 30% Bis/Acrylamide
    - 0.63 ml of 1.5 M Tris-HCl pH 6.8
    - 0.5 ml of 10% SDS
    - 3.6 ml of ddH_2O
    - 0.5 ml of 10% APS
    - 0.05 ml of TEMED
6. After solidification of the running gel, discard the water.
7. Slowly add the upper gel up to the edge of the plate.
8. Insert the comb. Wait 0.5 h for the gel to completely solidify.

9. Remove the comb. Transfer the plate to the electrophoresis tank.

Gel electrophoresis SDS-PAGE

SDS-PAGE is a commonly used molecular biology protocol that allows for the separation of molecules (proteins for Western Blotting).

Sample preparation

1. Based on the protein extraction yield (as determined by Bradford Assay), calculate what volume of sample needs to be added to each lane.
2. Mix appropriate amount of the protein extract with 2x SDS-PAGE dye to have final protein concentration of 1 µg/µl (total volume= 50 µl).
3. Put the samples in boiling water for 5 min to denature the proteins.
4. Load 10 µl of each sample on the gel. Also load 3 µl of the Page Ruler pre-stained protein ladder.

Electrophoretic Separation (SDS-PAGE):

1. Pour 1x SDS-PAGE buffer into the Western blot tank.
2. Position the acrylamide gels in the gel holder assembly and immerse into tank.
3. Fill the inner compartment (between two gels) with SDS-PAGE Buffer.
4. Carefully load the samples in the wells (using a fine-tipped pipette). Used a pre-stained molecular weight marker to determine the end point of the electrophoresis.
5. Place the lid on the tank and plug it into the power source.
6. Run the apparatus at 50 V until the sample passes the stacking gel or for 25 min.
7. Turn the voltage up to 100 V and allow the samples time to separate.

Protein gel staining

1. Gently open the glass plates and release the edges of the gel using a blade.
2. Transfer the gel to a big petri dish (gently).
3. Add enough Coomassie brilliant blue dye solution to cover the gel completely. Stain for 1h.
4. Discard the dye and add destaining solution. Change the destaining solution again after 0.5 h. Destain until the clear bands of protein are clear.

Transfer PVDF membrane

1. Cut filter paper (typical chromatography paper) in approximately 7x 20cm pieces.
2. Cut PVDF membrane to 7 x 20 cm. Make sure to handle the membrane at all times with clean forceps (never touch it).
3. Pre-wet the PVDF membrane using 100% methanol for five min and then immerse in transfer buffer.
4. Soak the filter paper pads in PVDF transfer buffer.
5. Release the gel gently from the plate and incise the extra parts.
6. Assemble the membrane sandwich: start with black foam, then three filter papers, the gel, the PVDF membrane, three more papers, and the white foam.
7. Close the sandwich and press on it to get bubbles out.
8. Fill the transfer tank with transfer buffer.
9. Place the sandwich into the transfer buffer.
10. Run the transfer at 90 V for 1 h.

Immunodetection

1. After completing electrophoretic transfer, take the membrane out of sandwich.
2. Block the membrane in 2.5% Non Fat Dry Milk (NFDM) in TBST for overnight. Use 30 ml of blocking solution for 1 small membrane.
3. Incubate the membrane with the primary antibody (Gulo-antibody) for sixteen h at 4°C. Dilute the Gulo-antibody (1:300 or 1:3000) with 5% Non Fat Dry Milk (NFDM) in TBST solution.
4. Re-block the membrane in 10% Non Fat Dry Milk (NFDM) in TBST solution for 10 min at room temperature.
5. Wash the PVDF membrane three times with 1 x TBST for ten min.
6. Incubate the membrane with the secondary antibody (goat-antibody) at room temperature for 1h. Dilute antibody (1:3000) in 5% Non Fat Dry Milk (NFDM) in TBST solution.
7. Wash the membrane three times with 1 x TBST for ten min.
8. Discard the TBST solution.

Detection

1. Prepare ECL solutions, add 3 ml of reagent 1, and then add three ml of reagent 2. Incubate the membrane with ECL reagents for one minute.
2. Leave the membrane to dry completely.
3. Place the membrane in a film cassette with the appropriate film. Exposure times are extremely variable and some care should be taken to determine the optimal exposure parameters.
4. Develop the film and get results.

Lambda select-*cII* mutation detection system

Preparation of tissue samples (liver samples)

1. Chill a clean 7 ml Wheaton Dounce tissue grinder and the lysis buffer on ice.

2. Add 5 ml of ice-cold lysis buffer to the tissue grinder.

3. Transfer the appropriate amount of excised tissue sample (see Table I) to the tissue grinder containing the ice-cold lysis buffer.

Table I

Guideline for the required amount of tissue

Type of tissue	Starting mass (mg)	Average final yield(μg)
Liver	50-80	175
Kidney	100 (approximately one-half of a whole mouse kidney)	200
Lung	175 (approximately a whole mouse lung)	125
Heart	185 (approximately a whole mouse heart)	75
spleen	40 (approximately a whole mouse spleen)	400

4. Disaggregate the tissue sample with pestle B for 3-10 strokes until the sample appears completely homogenized.

5. Release the cell nuclei within the homogenate using pestle A for 8 strokes.

6. Pour the homogenate through a sterile cell strainer into a 50 ml conical tube.

7. Add an additional 3 ml of ice-cold lysis buffer to the tissue grinder and pour the wash through the cell strainer to bring the total volume in the conical tube to 8 ml. Discard the cell strainer and place a cap on the tube.

8. Store the conical tube on ice until ready for further use and repeat steps 1-7 for any remaining tissue samples.

10. Uncap the tube and carefully discard the supernatant to avoid losing the cell nuclei pellet.

11. Carefully invert the uncapped conical tube on a paper towel for 1 minute to drain any excess liquid from pellet.

12. Dry any residual droplets from the inside walls of the tube using one of the sterile applicators provided. Avoid touching the actual pellet with the applicator because the pellet will adhere to the tip of applicator.

13. Warm a 70 µl aliquot of the proteinase K solution in a 50°C water bath in advance (i.e. 2-5 min prior to use) to activate the enzyme.

14. Prepare the digestion solution by adding 20 µl of RNace-It™ribonuclease cocktail per ml of digestion buffer.

15. Add 70 µl of the prepared digestion solution to the cell nuclei pellet and rock the conical tube gently to dislodge the pellet from the bottom of the tube.

16. Place the conical tube in a 50°C water bath and add 70 µl of the warmed proteinase K solution to the free-floating pellet. Swirl the conical tube gently every 10-15 min.

18. Pour at least 500 ml of TE buffer into a dialysis reservoir and float a dialysis cup on the surface of the buffer. Check the dialysis cup to ensure direct contact of the membrane surface with the TE buffer and then, if necessary, tilt the dialysis cup slightly to remove any large air bubbles from beneath the membrane surface.

19. Carefully transfer the viscous genomic DNA from the conical tube to the floating dialysis cup using a wide-bore pipet tip.

20. Dialyze the genomic DNA at room temperature for 16 to 48 h while stirring the buffer gently with a magnetic stir bar. If desired, replace the TE buffer once or twice during the dialysis period to maximize the purity of the recovered DNA.

21. On completion of dialysis, remove the dialysis cup from the TE buffer and immediately transfer the genomic DNA to a sterile microcentrtrifuge tube using a wide-bore pipet tip.

Performing the packaging reaction

1. Remove five red tubes of Transpack packaging extract from a -80°C freezer and quickly place each red tube on dry ice until ready for use.
2. Quickly thaw one red tube of Transpack packaging extract between your fingers or hands until the contents of the tube are approximately three-quarters thawed. Leave the remaining tubes on dry ice until ready for further use.
3. Using a wide-bore pipet tip, transfer ≥ 8 µl of the genomic DNA sample to the red tube containing the Transpack packaging extract. Mix the packaging reaction by pipetting the reaction up and down three times with the wide-bore pipet tip and place the tube in a 30°C water bath. (If necessary, pulse the tube in a microcentrifuge to force the contents to the bottom of the tube before placing the tube in the contents to the bottom of the tube before placing the tube in the water bath.)
4. Repeat steps 2 and 3 to package the remaining samples in the set.
5. Incubate each packaging reaction at 30°C for 90 min.
6. Remove one blue tube of Transpack packaging extract from the -80°C freezer for every set of five packaging reactions performed and quickly place the blue tube on dry ice until ready for use.
7. Thaw the blue tube of Transpack packaging extract by holding the tube between your fingers or hands until the contents of the blue tube are completely thawed. Quickly transfer 12 µl of Transpack packaging extract from the blue tube to one of red tubes containing a packaging reaction. Mix the packaging reaction by pipetting the reaction up

and down three to five times with the wide-bore pipet tip and return the tube to the 30°C water bath.

8. Repeat step 7 for the remaining samples.

9. Incubate each packaging reaction at 30°C for an additional 90 min.

10. Dilute each completed packaging reaction by adding sterile SM buffer to a final volume of 1 ml then vortex at maximum speed for 10 s and place on ice. Multiple reactions for a particular DNA sample may be pooled at this time. The completed packaging reaction, diluted with SM buffer, is referred to as packaged DNA sample.

11. If the entire packaged DNA sample will not be plated on the same day it was packaged, add 50 µl of chloroform per ml of packaged DNA sample, gently vortex and store at 4°C for up to 2 weeks.

Preparation of SCS-8 plating culture

1. Using a sterile inoculating loop, streak splinters of sold ice from the bacterial glycerol stock containing the SCS-8 host strain onto an LB-tetracycline agar plate.

2. Incubate the bacterial streak plate overnight in a 37°C air incubator. (Bacterial streak plates can be stored at 4°C for up to one week.)

3. In a sterile 50 ml conical tube, inoculate 20 ml of NZY broth (supplemented with 250 µl of a 20% (w/v) maltose and 1 M $MgSO_4$ solution), with several colonies from the bacterial streak plate. The SCS-8 cells cultured in NZY broth supplemented with the maltose and 1 M $MgSO_4$ solution is referred to as the SCS-8 liquid culture.

4. Incubate the SCS-8 liquid culture at 37°C for 4-6 h with shaking at 250-300 rpm.

5. Centrifuge the conical tube at 1000 x g for 10 min to pellet the bacterial cells.

6. Discard the supernatant and gently re-suspend the pellet in 10 ml of sterile 10 mM $MgSO_4$.

7. Dilute the SCS-8 bacterial cell suspension to an OD_{600} of 0.5 with sterile 10 mM $MgSO_4$. The prepared suspension of SCS-8 cells at an OD_{600} of 0.5 is referred to as the SCS-8 plating culture.

Titration of the packaged DNA samples

1. Vortex each packaged DNA sample briefly.

2. For each packaged DNA sample to be titrated, transfer 200 µl of prepared SCS-8 plating culture to sterile 5 ml test tubes in duplicate.

3. Carefully transfer 1 µl of each packaged DNA sample to each of the duplicate test tubes that contain the 200 µl aliquots of SCS-8 plating culture.

4. Incubate each test tube containing the packaged DNA sample and SCS-8 plating culture at 37 °C for 15 min to allow phage to adsorb to host cells.

5. Add 3-4 ml of molten NZY top agarose cooled to 50 °C to each tube.

6. Quickly but gently pour the contents of each tube over a separate 100 mm NZY agar plate and agitate the plate to ensure a smooth and even distribution before the top agarose hardens.

7. Invert and incubate each agar plate overnight in a 37 °C air incubator.

8. Count the number of the phage plaques on each agar plate and determine the mean.

Actual number of plaques = (mean of plaques / µl) x 1.3 = actual number pfu / µl

Next, calculate the actual number of plaques per packaging reaction as follows:

Actual number of plaques per packaging reaction = Actual number pfu / µl x 1000 µl

Preparation of the G1250 liquid culture

1. At least two days before plating, use a sterile inoculating loop to streak splinters of solid ice from the bacterial glycerol stock containing the *E.coli* host strain G1250 onto a 100 mm TB1 kanamycin agar plate.

2. Incubate the bacterial streak plate in a 30°C incubator for at least 24 h.

3. On the day before plating, combine 10 ml of TB1 liquid medium with 100 µl of the 20% (w/v) maltose and 1 M $MgSO_4$ solution in a sterile 50 ml screw-cap conical tube.

4. Inoculate the liquid medium with several colonies from the bacterial streak plate.

5. Incubate the liquid culture overnight in a 30°C shaking incubator with vigorous shaking (250-300) rpm.

Preparing the G1250 plating culture on the day of plating

1. Centrifuge the conical tube containing the G1250 liquid culture at 1500 x g for 10 min to pellet the bacterial cells.

2. Discard the supernatant and gently re-suspend the cell pellet in 10 ml of 10 mM $MgSO_4$.

3. Measure the absorbance of a 1:10 dilution (0.1 ml of cells and 0.9 ml of 10 mM $MgSO_4$) at 600 nm and calculate the OD_{600} of the undiluted cell suspension.

4. Dilute the cell suspension to a final OD600 of 0.5 with 10 mM $MgSO_4$.The prepared suspension of G1250 cells at an OD600 of 0.5 is referred to as the G1250 plating culture.

5. Store the G1250 plating culture on ice until ready to use.

Plating a packaged DNA sample

1. Label 12 sterile 14 x 100 mm tubes and 12 TB1 agar plates as follows: Label three tubes and three plates titer 100, label three tubes and three plates titers 20, and label six tubes and six plates screening.

2. Make 1:100 dilutions of the packaged DNA sample in triplicate by combining 10 µl of the packaged DNA sample with 990 µl of SM buffer in each of three 1.5 ml microcentrifuge tubes (see the Table 1). Mix the diluted packaged DNA samples thoroughly using a vortex mixer.

Table II

1.5- ml Microcentrifuge tube	Undiluted packaged DNA sample volume	SM buffer volume
A	10 µl	990 µl
B	10 µl	990 µl
C	10 µl	990 µl

3. Pipet 0.2 ml of the G1250 plating culture into each of the 12 labeled tubes (see table 2).

5. Add 100 µl of undiluted packaged DNA sample to each of the 6 tubes from steps 2 labeled screening (see Table 2)

Table III

Tube/plate number	Tube label	G1250 plating culture volume	Diluted packaged DNA sample volume			Undiluted packaged DNA sample volume	protocol step
			From Tube A	From Tube B	From Tube C		
1	Titer 100	0.2 ml	100 µl	----	----	----	4
2	Titer 100	0.2 ml	----	100 µl	----	----	4
3	Titer 100	0.2 ml	----	----	100 µl	----	4
4	Titer 20	0.2 ml	20 µl	----	----	----	4
5	Titer 20	0.2 ml	----	20 µl	----	----	4
6	Titer 20	0.2 ml	----	----	20 µl	----	4
7-16	screening	0.2 ml	----	----	----	100 µl each	5

6. Agitate all the tubes for a few s to mix the contents. Incubate the tubes at room temperature for 30 min to allow the phage to adsorb to the host cells.

7. Using a 10 ml pipet, take up 12.5 ml of molten TB1 agar (cooled to 55°C) and immediately pipet 2.5 ml into each of five of the tubes that contain phage adsorbed host cells.

8. Quickly but gently, pour the contents of each tube onto the appropriately labeled TB1 agar plate and agitate the plate to ensure a smooth, even distribution before the top agar hardens.

9. Repeat steps 7 and 8 until all 12 plates are completed.

10. Place the six titer plates upside down in 37°C incubator and incubate the plates overnight.

11. Within 30 min of plating, place the 10 screening plates upside down in a 24°C incubator and incubate the plates for 46-48 h.

Estimation of the total plaques screened

Following the 37°C overnight incubation, examine the two groups of triplicate titer plates (labeled titer 100 and titer 20). Count the number of plaques on the group of titer plates that falls closest to the range of 50-200 plaques per plate. Determine the mean number of plaques for the triplicate plating and calculate the total number of plaques screened over the 6 plates as following:

$$\text{Total plaques screened} = \left(\frac{\text{Mean number of plaques/titer plate}}{\text{Number of µl of dilution/titer plate}} \right) \times \text{dilution} \times (100\ \mu l / \text{plate}) \times 6 \text{ plates}$$

Mutation frequency (MF) = Total of mutant plaques / Total plaques screened

Verifying the putative lambda *cII* mutants

1. Core the plaque in question with a sterile wide-bore pipet tip and expel the core into a sterile microcentrifuge tube containing 500 of sterile SM buffer.

2. Incubate the tube for at least 2 h at room temperature, or overnight at 4, to allow the phage particles to elute from the agar plug.

3. In a sterile 14 x 100 mm tube, combine 0.2 ml of prepared G1250 plating culture with 1 µl of cored phage solution and incubate the tube at room temperature for 30 min.

4. Plate the sample using 2.5 ml of 55°C molten top agar and incubate the plate at 24°C for 46-48 h (selective conditions) as describe in plating the packaged DNA samples.

5. If the lambda *cII* phenotype is verified by plaque formation on the secondary plate incubated at 24°C, core an isolated plaque from the plate and store this secondary core in 500 µl of SM buffer at 4°C.

6. Count all the verified lambda *cII* plaques observed. Calculate the lambda cII mutant frequency, which is the ratio of lambda *cII* mutant to the total number of plaques screened.

Preparation of Media and Reagents

SDS-PAGE 10X running Buffer

- 30 g of Tris base
- 144 g of Glycine
- 1 L ddH_2O
- Store at 4°C

1X SDS-running Buffer

- 10 ml of 10% SDS
- 100 ml of 10X running buffer
- 900 ml of ddH_2O

4X SDS dye

- 32 g of Glycerol
- 24 ml of 1M Tris-HCl, pH = 6.8
- 16 mg of Bromophenol blue
- 8 g of SDS
- ddH_2O up to 80ml
- 20 ml of β-mercaptoethanol
- Store at -20°C aliquoted and for long term at -80°C.

10% APS

- 1g of Ammonium persulphate
- 10 ml of dH_2O

- Store at 4°C (pour it into a glass dark bottle).

Protein gel staining solution

- 0.5 g of Coomassie blue
- 200 ml of Methanol
- 50 ml of Glacial acetic acid
- 250 ml of ddH_2O
- Used filter paper for sterilize

Protein gel de-staining solution

- 450 ml of Methanol
- 100 ml of Glacial acetic acid
- dH_2O up to1L

Transfer Buffer

- 57.6 g of Glycine
- 12.1 g of Tris base
- 800 ml of Methanol
- ddH_2O up to 4L

10 X TBST

- 26.1 g of NaCl
- 30 ml of Tris-HCl, pH=8.0
- 1.5 ml of Tween 20
- 300 ml of ddH_2O

5% milk solution

- 5 g of Non-fat dry milk powder
- 100 ml of 1X TBST

2.5% milk solution

- 2.5 g of Non-fat dry milk powder
- 100 ml of 1X TBST

Gulo antibody stock

- Add 200 µl of ddH_2O/PBS to the stock tube of powdered antibody.
- Dilute Gulo antibody (1:300 or 1:3000) into 5% non-fat dry milk + 1X TBST

Secondary antibody

- Antibody stock (Biorad Goat anti-rabbit)
- 30 ml of 5% milk solution

Maltose (20 % (w/v)) and $MgSO_4$ (1 M) Solution (per 100 ml)

- 20.0 g of maltose
- 24.6 g of $MgSO_4 \cdot 7H_2O$
- Add ddH_2O to final volume of 100 ml
- Filter sterilize
- Store at 4°C

SM Buffer (per liter)

- 5.8 g of NaCl
- 2.0 g of $MgSO_4 \cdot 7H_2O$
- 50.0 ml of 1 M Tris-HCl (pH7.5)
- 5.0 ml of 2% (w/v) gelatin
- Add deionized H_2O to final volume of 1 L
- Adjust the pH to 7.5
- Autoclave for 30 min
- Store at room temperature for up to 1 year

$MgSO_4$ (10mM) (Per liter)

- 2.46 g $MgSO_4 \cdot 7H_2O$
- Add ddH_2O to final volume of 1 L
- Autoclave for 30 min
- Store at room temperature for up to 1 year

TE Buffer

- 10 mM Tris-HCl (pH7.5)
- 1 mM EDTA
- Autoclave for 30 min
- Store at room temperature for up to 1 year

Preparation of Media

NZY Broth (per Liter):

- 5.0 g of NaCl
- 2.0 g of $MgSO_4 \cdot 7H_2O$
- 5.0 g of yeast extract
- 10.0 g of Bacto-tryptone
- Add 800 ml of ddH_2O
- Adjust the pH to 7.0 with 1 N NaOH
- Add ddH_2O to a final volume of 1 liter
- Autoclave for 30 min

NZY Top Agraose (per Liter)

- 5.0 g of NaCl
- 2.0 g of $MgSO_4 \cdot 7H_2O$
- 5.0 g of yeast extract
- 10.0 g of Bacto-tryptone
- Add 800 ml of ddH_2O
- Adjust the pH to 7.0 with 1 N NaOH
- Add 7.0 g of agarose

- Add dH_2O to a final volume of 1 L
- Stir on a hot plate until the agarose dissolves
- Pour into 100 ml bottles
- Autoclave for 30 min
- Store at room temperature for up to 1 year

NZY Agar Plates (per Liter)

- 5.0 g of NaCl
- 2.0 g of $MgSO_4 \cdot 7H_2O$
- 5.0 g of yeast extract
- 10.0 g of Bacto-tryptone
- Add 15 g of agar
- Add 800 ml of ddH_2O
- Adjust the pH to 7.0 with 1 N NaOH
- Add ddH_2O to a final volume of 1 liter
- Autoclave for 30 min
- Mix well and allow to cool to 55°C
- Pour into sterile petri dishes (25-50 ml/100 mm plate)
- Store plates at 4°C

LB-Tetracycline Agar Plates (per Liter)

- 10 g of NaCl
- 10 g of Bacto-tryptone
- 5 g of yeast extract
- 20 g of agar

- Add 800 ml of ddH_2O
- Adjust the pH to 7.0 with 1 N NaOH
- Add dH_2O to a final volume of 1 L
- Autoclave for 30 min
- Mix well and allow to cool to 55°C
- Add 12.5 mg of tetracycline (dissolved in 10ml of 50% ethanol and filter-sterilized)
- Pour into sterile petri dishes (25-50 ml/ 100 mm plate)
- Store plates at 4°C protected from light (tetracycline is light-sensitive)

CHAPTER 7: REFERENCES

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