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I am submitting herewith a dissertation written by Laura Ray Chittenden entitled “Molecular Characterization of the T(4;9)12Gso Mutation and Analysis of the Associated Fitness, Skeletal, and Lymphoproliferative Phenotypes.” I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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**Molecular Characterization of the T(4;9)12Gso Mutation and Analysis of the
Associated Fitness, Skeletal, and Lymphoproliferative Phenotypes**

A Dissertation Presented
for the
Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Laura R. Chittenden
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DEDICATION

This manuscript is dedicated to my parents, George and Rebecca Chittenden, whose motivational influences are the reason for my educational goals. I would also like to dedicate this manuscript to my wonderful fiancé, Colin Dreizin, whose encouragement, support, and patience have been a godsend throughout the latter portion of my graduate career. Thank you all for your love and support.

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ABSTRACT

The t(4;9)(B3;E3.2)12Gso reciprocal translocation is an autosomal recessive mouse mutation involving chromosome 4, band B3 (Mmu4B3) and chromosome 9, band E3.2 (Mmu9E3.2). The most striking phenotype of the 12Gso homozygote involves axial skeleton deformities, resulting in significant shortening of body length, scoliosis, displaced hips and kinky tails. Homozygous animals are significantly smaller than normal littermates and frequently exhibit early lethality, as many of the mutants perish by 1 day postnatal, and many others do not survive the three weeks to weaning age. Homozygous 12Gso mice also develop an unusual bone marrow defect that closely resembles leukemia. The penetrance of the mutation is variable with significant dependence on genetic background.

Fluorescent *in situ* hybridization and reverse transcription polymerase chain reaction were used to localize the chromosome 4 breakpoint to a 600 base pair region intrinsic to *ATP-binding cassette 1 (Abca1)*, a gene that encodes a large transmembrane protein involved in cholesterol efflux. The disruption of *Abca1* results in a truncated transcript. Aberrant expression of genes associated with lipid metabolism has been detected in 12Gso homozygotes, and previous data by others describing the effects of *Abca1* null mutations in humans and mice indicate the likely involvement of this gene in the early lethality of 12Gso homozygotes. Using polymerase chain reaction (PCR)-based methods, we have cloned and sequenced DNA surrounding the 9;4 chromosome junction in 12Gso/12Gso DNA. The chromosome 9 breakpoint maps to a relatively gene-poor region, lying in the intergenic region between an uncharacterized T-box gene, *Tbx18*, and a *ras*-like EST, *Rock1*. Both genes represent potential candidates for the skeletal anomalies present in 12Gso homozygotes. These studies lay a firm foundation for future studies with the unique model offered by the 12Gso mouse, aimed at identification of novel genes and previously unknown pathways required for skeletal development and associated with leukemia susceptibility in humans and mice.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION	1
THE ORIGINS OF THE T(4;9)12Gso MUTATION	1
THE NATURE OF THE 12Gso MUTATION AND THE ASSOCIATED HOMOZYGOUS PHENOTYPE	2
NORMAL SKELETAL DEVELOPMENT	8
THE MOLECULAR BASIS OF SKELETAL DEFECTS	12
ASPECTS OF HEMATOPOIESIS RELEVANT TO BONE MARROW COMPONENTS	17
COMMON FORMS AND GENETIC CAUSES OF INHERITED BONE MARROW HYPERPLASIA	19
THE ROLE OF GENES FOR SOMITIC DEVELOPMENT IN BOTH SKELETAL DEVELOPMENT AND HEMATOPOIESIS	23
THE GENETIC RELATIONSHIP BETWEEN TRANSLOCATION BREAKPOINTS AND GENE(S) ASSOCIATED WITH THE MUTANT PHENOTYPES	26
CHROMOSOME REARRANGEMENTS AS A TOOL FOR IDENTIFYING GENES ASSOCIATED WITH HUMAN DISEASE	28
CHROMOSOME REARRANGEMENTS AND INHERITED DISORDERS IN MICE	31
USING TRANSLOCATIONS AS A TOOL TO MAP AND CLONE MUTANT GENES	32
THE SEARCH FOR CANDIDATE GENES	37
METHODS OF LOCATING AND TESTING CANDIDATE GENES	38
THE AIMS OF THE 12Gso PROJECT	39
CHAPTER 2: MATERIALS AND METHODS	40
ALIZARIN RED STAINING OF THE SKELETON	40
COMBINED STAINING OF THE SKELETON AND CARTILAGE	40
PERFUSION OF THE MOUSE FOR SECTIONING	41
IMMUNOHISTOCHEMISTRY	41
DETERMINATION OF CARRIER STATUS BY BREEDING TESTS	42
DETERMINATION OF HETEROZYGOSITY BY WHOLE CHROMOSOME PAINTS	42
ISOLATION OF EMBRYOS FOR EXPRESSION STUDIES AND PRENATAL ANALYSIS	42
OUTCROSSES TO TEST EFFECTS OF GENETIC BACKGROUND	44
COMPLEMENTATION TESTING	44
AMPLIFICATION OF MICROSATELLITE MARKERS	44
ACRYLAMIDE GEL ELECTROPHORESIS TO RESOLVE AMPLIFIED PRODUCTS	45
ESTIMATION OF THE MAXIMUM MAP DISTANCE BETWEEN THE TRANSLOCATION AND THE MUTATION IN 12Gso	45
PROBES USED FOR GENETIC MAPPING	45
INTERSPECIFIC BACKCROSS MAPPING	46
ISOLATION OF PLASMID DNA	47
ISOLATION OF DNA INSERTS	47
ISOLATION OF BACTERIAL ARTIFICIAL CHROMOSOME (BAC) CLONES	48
CULTURE OF MOUSE SPLENOCYTES AND GENERATION METAPHASE CHROMOSOME SPREADS	49
DIRECT FLUORESCENT LABELING OF PROBES BY ENZYMATIC METHODS	50
FISH USING BIOTINYLATED PROBES GENERATED BY DEGENERATE OLIGONUCLEOTIDE-PRIMED PCR	50
PCR ANALYSIS OF THE 12Gso BREAKPOINT REGION	51
GENERATION AND SCREENING OF COMPARATIVE GENOMIC SOUTHERN HYBRIDIZATIONS	51
REVERSE TRANSCRIPTASE PCR (RT-PCR)	52
ISOLATION OF MOUSE TISSUES AND EXTRACTION OF TOTAL RNA FROM MOUSE TISSUES	52
PREPARATION OF POLY-A ENRICHED MRNA	52
EXPRESSION ANALYSIS USING NORTHERN BLOTTING METHODS	53
MICROARRAY ANALYSIS	53
CULTURE OF MOUSE FIBROBLASTS AND CORRESPONDING METAPHASE CHROMOSOME SPREADS	53
BREAKPOINT FRAGMENT ISOLATION AND SEQUENCING	54

CHAPTER 3: RESULTS	55
CHARACTERIZATION OF DEFECTS IN THE SKELETON AND CARTILAGE OF 12Gso MUTANT MICE	55
HISTOPATHOLOGY	55
PRELIMINARY STUDIES OF THE EFFECTS OF GENETIC BACKGROUND ON SURVIVAL AND EXPRESSION OF THE SKELETAL PHENOTYPE	67
CONCORDANCE BETWEEN SKELETAL PHENOTYPE AND INHERITANCE OF THE 12Gso TRANSLOCATION	69
ESTIMATION OF THE MAXIMUM MAP DISTANCE BETWEEN THE TRANSLOCATION AND THE MUTATION IN 12Gso	69
TESTING GENETIC BACKGROUND OF THE MMU4 BREAKPOINT REGION AND SURROUNDING DNA.	70
GENETIC MAPPING: LOCATING GENES ON MMU4 AND MMU9	70
MAPPING THE MMU 4 TRANSLOCATION BREAKPOINT USING FISH AND THE AVAILABLE PUBLIC DATABASES	72
EXAMINATION OF THE SEQUENCE OF THE BREAKPOINT-SPANNING BAC, RPCI-23-353G1	81
STRATEGY USING PCR AND GENOMIC SOUTHERN HYBRIDIZATION TO ANALYZE THE BREAKPOINT REGION OUTSIDE OF THE ABCA1 GENE	89
RESULTS OF RT-PCR WITH ABCA1	89
RESULTS OF COMPARATIVE MICROARRAY ANALYSIS	92
WILD TYPE MOUSE NORTHERN BLOT ANALYSIS	92
RT-PCR ANALYSIS OF NEARBY MMU 4 GENES	98
TESTING POTENTIAL ALLELISM BETWEEN 12Gso AND THE TK MUTATION	98
MAPPING THE MMU 9 CHROMOSOMAL BREAKPOINTS	106
ANALYSIS OF A FRAGMENT CONTAINING THE T(9;4) CHROMOSOMAL BREAKPOINT	111
CHAPTER 4: DISCUSSION	117
THE GOAL OF THE 12Gso PROJECT	117
ANALYSIS OF THE SKELETAL PHENOTYPE OF 12Gso HOMOZYGOTES	117
THE ABNORMAL BONE MARROW PHENOTYPE IN 12Gso/12Gso IS LEUKEMIC	120
PENETRANCE OF THE 12Gso HOMOZYGOUS PHENOTYPE	121
THE RELATIONSHIP BETWEEN TRANSLOCATION BREAKPOINTS AND GENE(S) ASSOCIATED WITH THE MUTANT PHENOTYPES	122
LOCALIZATION OF THE 12Gso CHROMOSOMAL BREAKPOINTS	125
MOLECULAR ANALYSIS OF BREAKPOINT REGION	126
PRELIMINARY EVIDENCE FOR ABCA1 AS THE CANDIDATE GENE ASSOCIATED WITH THE REDUCED FITNESS OF 12Gso/12Gso MICE	128
IDENTIFICATION OF A POSSIBLE CANDIDATE GENE FOR THE 12Gso SKELETAL PHENOTYPE ON MMU9	134
FUTURE EXPERIMENTS PROPOSED FOR STUDYING THE 12Gso MOUSE	135
FINAL CONSIDERATIONS	142
REFERENCES	145
APPENDIX	177
VITA	183

LIST OF TABLES

TABLE 1. MOUSE SKELETAL MUTATIONS MAPPING TO MMU4 AND MMU9	9
TABLE 2. KARYOTYPE ANALYSIS OF LIVEBORN OFFSPRING FROM 12Gso/+ X 12Gso/+ CROSSES	68
TABLE 3. MICROSATELLITE AND GENE MARKERS USED TO CHARACTERIZE THE 12Gso MMU4 BREAKPOINT REGION.	71
TABLE 4. RESULTS FROM THE PRELIMINARY COMPARATIVE MICROARRAY EXPERIMENT, USING 12Gso/12Gso AND B6C3 BONE TOTAL RNA: GENES WITH 10-FOLD OR GREATER CHANGE IN EXPRESSION BETWEEN THE RNA SAMPLES. -DENOTES A DECREASE IN EXPRESSION; + DENOTES AN INCREASE IN EXPRESSION.	97
TABLE 5. TARGETED ALLELES OF NOTCH-DELTA PATHWAY GENES INVOLVED IN MAMMALIAN SKELETOGENESIS.	119

LIST OF FIGURES

FIGURE 1. THE 12GSO PHENOTYPE IS ASSOCIATED WITH A t(4;9)(B3;E3.2) TRANSLOCATION.	3
FIGURE 2. 12GSO HOMOZYGOUS MICE.	5
FIGURE 3. THE 12GSO HOMOZYGOTE SKELETAL PHENOTYPE STRONGLY RESEMBLES THAT OF MUTATIONS IN THE GENES FROM THE <i>NOTCH-DELTA</i> PATHWAY.	15
FIGURE 4. FISH-BASED STRATEGY FOR MAPPING THE CHROMOSOMAL BREAKPOINT.	34
FIGURE 5. SKELETAL ANOMALIES OF THE ADULT 12Gso HOMOZYGOUS MOUSE.	57
FIGURE 6. SKELETAL ANOMALIES ARE PRESENT IN E18.5 12Gso/12Gso MICE.	59
FIGURE 7. ABERRATIONS ARE PRESENT IN THE SKELETON OF E17.5 12Gso/12Gso EMBRYOS.	62
FIGURE 8. SECTIONS OF 6-WEEK OLD 12Gso/12Gso MICE INDICATE ABNORMAL BONE AND MARROW STRUCTURE.	64
FIGURE 9. THE BONE MARROW OF 12Gso HOMOZYGOUS MICE SHOWS A DIFFUSE PATTERN OF LYMPHOCYTE PROLIFERATION.	66
FIGURE 10. GENETIC MAP OF MOUSE CHROMOSOME 4.	74
FIGURE 11. GENETIC MAP OF MOUSE CHROMOSOME 9.	76
FIGURE 12. INITIAL MMU 4 FISH RESULTS.	78
FIGURE 13. USING THE MOUSE RADIATION HYBRID MAPS AND FINGERPRINTED CONTIGS TO NARROW THE BREAKPOINT REGION	80
FIGURE 14. ANALYZING THE HUMAN BAC CLONE CONTIGS FOR HOMOLOGOUS MOUSE BAC CLONES.	83
FIGURE 15. FRAMING THE 1 MB CRITICAL REGION AT THE 12Gso CHROMOSOMAL BREAKPOINT.	85
FIGURE 16. THE 12Gso CHROMOSOME 4 BREAKPOINT LIES IN THE REGION SPANNED BY BAC RPCI-23-353G1.	87
FIGURE 17. SCHEMATIC OF THE MMU 4 BREAKPOINT REGION ENCOMPASSED BY THE PUBLISHED <i>ABCA1</i> CONTIG (QUI <i>ET AL.</i> , 2001) AND THE METHODOLOGY USED TO FIND THE 12Gso BREAKPOINT.	91
FIGURE 18. RT-PCR ANALYSIS OF THE <i>ABCA1</i> GENE.	94
FIGURE 19. RT-PCR ANALYSIS OF THE <i>ABCA1</i> REGION NARROWS THE BREAKPOINT INTERVAL TO APPROXIMATELY 600 BASE PAIRS.	96
FIGURE 20. WILD TYPE <i>NIPSNAP3</i> EXPRESSION.	100
FIGURE 21. WILD TYPE EXPRESSION OF <i>NIPSNAP4</i> .	102
FIGURE 22. RT-PCR ANALYSIS OF 3' <i>NIPSNAP3</i> .	104
FIGURE 23. INITIAL 12Gso CHROMOSOME 9 FISH.	108
FIGURE 24. NARROWING THE 12Gso CHROMOSOME 9 BREAKPOINT.	110
FIGURE 25. RESULTS FROM THE GENOME WALKER EXPERIMENT.	113
FIGURE 26. DOUBLE-PASS SEQUENCE OF THE t(9;4) <i>PvuII</i> FRAGMENT GENERATED IN THE GENOME WALKER EXPERIMENT.	116

ABBREVIATIONS

Acute lymphocytic leukemia	ALL
Acute myelogenous leukemia	AML
Bacterial artificial clone	BAC
Base pairs	bp
Celsius	C
Centimorgan	cm
Chronic lymphocytic leukemia	CLL
Chronic myelogenous leukemia	CML
Days past coitus	dpc
Deoxyribonucleic acid	DNA
Diethylpyrocarbonate	depc
Distilled water	dH ₂ O
Fluorescent <i>in situ</i> hybridization	FISH
Heritable translocation test	HTT
Immunohistochemistry	IHC
<i>In situ</i> hybridization	ISH
Interspecific backcross	IB
Kilobase	kb
Lawrence Livermore National Laboratory	LLNL
Megabase	Mb
Methyl methanesulfate	MMS
Microcurie	μCi
Microgram	μg
Microliter	μL
Milligram	mg
Milliliter	ml
Mouse chromosome 4	Mmu 4

Mouse chromosome 9	Mmu 9
N,N'-methylene bisacrylamide	MBA
Oak Ridge National Laboratory	ORNL
Polymerase chain reaction	PCR
Reverse transcription polymerase chain reaction	RT-PCR
Ribonucleic acid	RNA
Theiler stage	TS

CHAPTER 1: INTRODUCTION

The origins of the t(4;9)12Gso mutation

The recessive t(4;9)12Gso mutation (hereafter referred to as 12Gso) arose in studies designed to determine the mutagenic effects of radiological and chemical clastogens on chromosome structure in mice. These studies were centered on the heritable translocation tests (HTT). HTT are used to detect large chromosomal rearrangements passed to viable offspring, specifically reciprocal translocations, based on the reduction in the number of viable gametes produced by translocation carriers (Generoso *et al.*, 1980). Approximately 50% of the gametes produced by translocation carriers carry partial duplications or deletions of chromosomal regions located telomeric to the sites of reciprocal exchange because of the disjunctional activity of unbalanced gametes in the first meiotic division. The embryos carrying these unbalanced chromosome complements die early in gestation. As a result, matings of translocation carrier mice generate litters that are statistically half the size of that produced by normal mice of similar genetic backgrounds, thus these carrier mice are termed “semisterile”. Semisterility, as measured by the size of litters produced by a normal inbred or hybrid female when mated to a translocation carrier relative to the litter size produced in matings to a normal male, has been used extensively since the 1940s as a test for the presence of reciprocal translocations in mutagenesis studies. The reliability of this simple breeding test in establishing carrier genotype has been confirmed by cytogenetics (Russell and Shelby, 1985; Generoso *et al.*, 1980; refs. therein). Litter-size measurements have also been used in the maintenance of translocation stocks, as a method for selecting carrier males and females that precludes the need for cytogenetics (review, see Stubbs *et al.*, 1997). Most of the Generoso (abbreviated Gso) collection of translocation stocks have therefore been maintained by crossing tested heterozygous carriers to normal inbred or hybrid mice, identifying translocation carriers for future generations by semisterility rather than by association with any abnormal phenotype.

However, a small number of lines displayed dominant mutations that clearly segregated with the inheritance of the translocation; one of these lines, 1Gso, was

described in an early publication (Rutledge *et al.*, 1987). The discovery of dominant phenotypes in translocation carriers inspired later studies in which the carrier animals were bred to generate offspring homozygous for the translocation to examine potential recessive phenotypes associated with the reciprocal exchange events. The recessively inherited phenotypes that were identified included developmental anomalies, neurological aberrations, and diseases such as cancer, leukemia, and polycystic kidney disease (reviewed by Culiati *et al.*, 1996; Stubbs, 1997).

One compound used in an HTT at Oak Ridge National Laboratory (ORNL) was *N,N'*-methylene bisacrylamide (MBA), the chemical from whose application 12Gso was derived. Dominant lethal (DLT) and HTT performed on males treated with this chemical indicate that the maximum genetic effect is seen in maturing sperm (Rutledge *et al.*, 1990). MBA is a strong clastogen, and is unlikely to cause point mutations. For this reason, the frequency of mutational events that are heritable is not expected to be great. Indeed, very few specific locus mutations were generated in treatment with MBA, as expected from the nature of the mutation and the cellular stage affected (unpublished; W. Generoso, pers. comm.). However, because of the lack of available data on the mutagenic ability of MBA, which could be identified in the specific locus test, estimation of genomic mutation frequency induced by MBA is difficult to determine.

The nature of the 12Gso mutation and the associated homozygous phenotype

The 12Gso mutant phenotype was discovered in the early stages of the breeding studies used to find recessive phenotypes in the Gso stocks. The balanced reciprocal translocation associated with 12Gso was mapped to chromosome 4, band B3 (Mmu 4B3), and chromosome 9, band E3.2 by cytogenetic methods (Mmu 9E3.2; N. L. A. Cacheiro, pers. comm.; Figure 1). All mice karyotyped as homozygous for the 12Gso translocation exhibited clear vertebral deformities which resulted in significant shortening of body length, scoliosis, displaced hips and kinked tails (Figure 2). Early lethality has been common to 12Gso homozygotes; many of the mutants have perished between 1 day postnatal and the three weeks to weaning

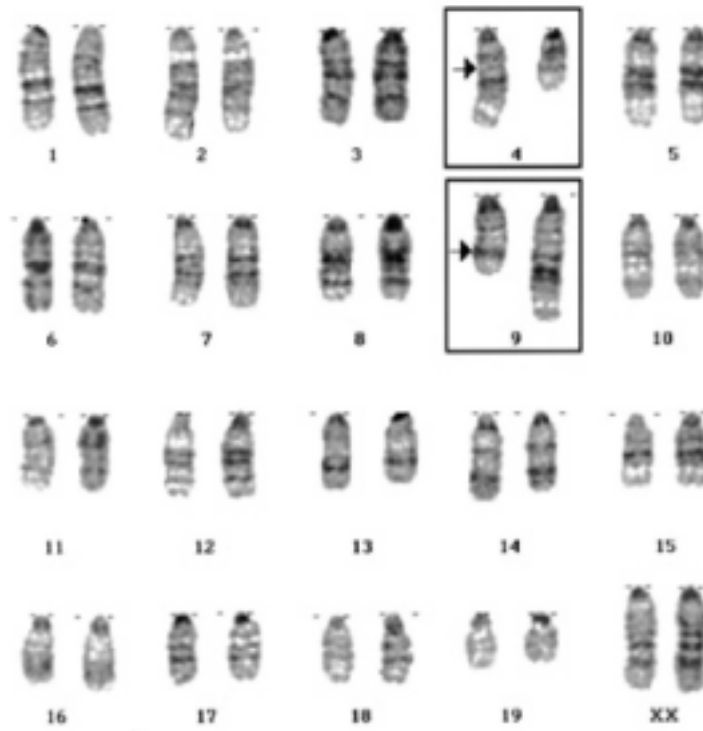
Figure 1. The 12Gso phenotype is associated with a t(4;9)(B3;E3.2) translocation.

A. 12Gso heterozygote karyotype. Arrows represent the location of the chromosomal breakpoint on the normal mouse chromosomes. Normal chromosomes (left) and translocation chromosomes (right) are boxed for size and banding pattern comparison.

B. Ideotype of mouse chromosomes 4 and 9, highlighting the band in which the chromosomal translocation occurred.

A.

O.R.N.L. BIOLOGY-MAMMALIAN GENETICS SECTION



B.

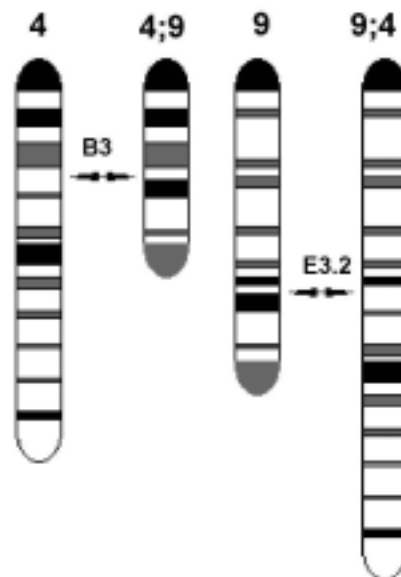
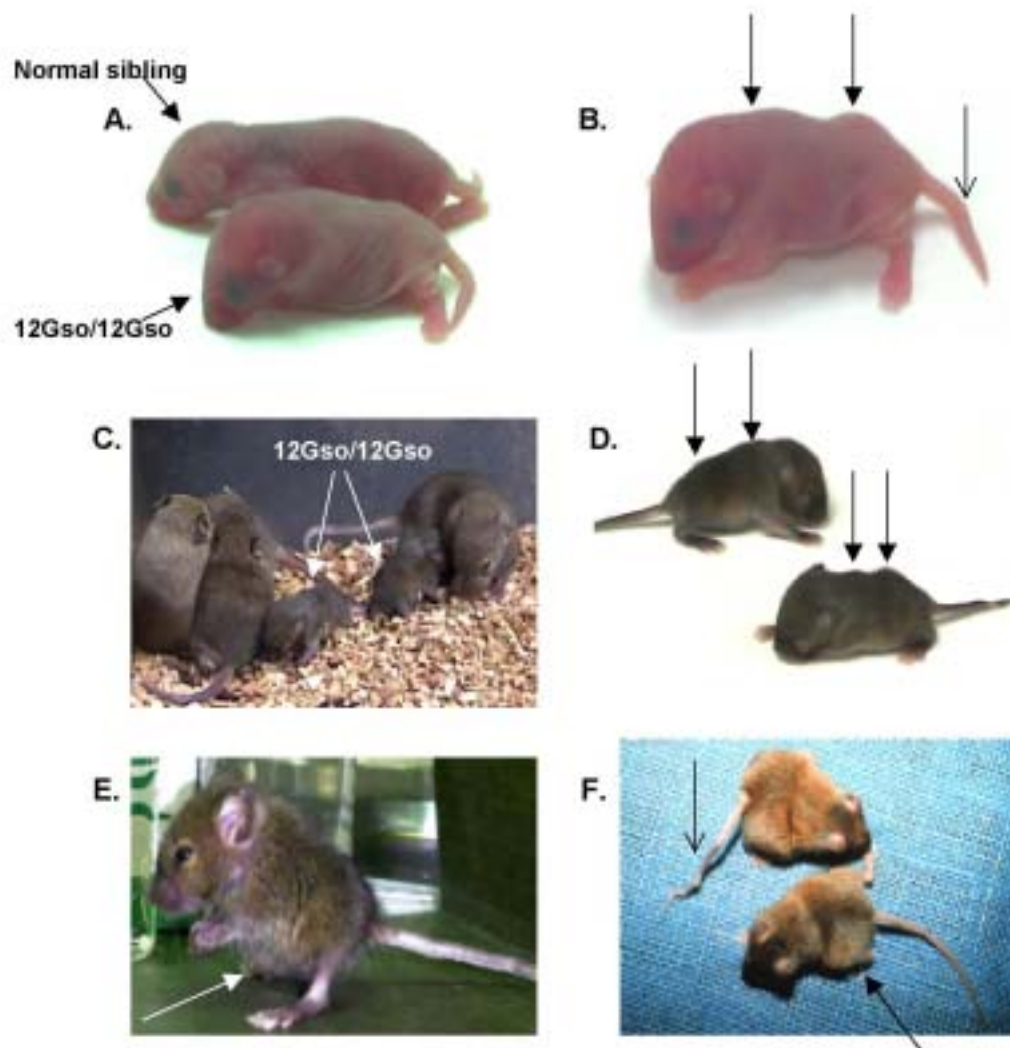


Figure 2. 12Gso homozygous mice. Skeletal malformations are visually identifiable in newborn 12Gso homozygotes. **A.** One-day postnatal 12Gso/12Gso (bottom) with sibling (top). **B.** One-day postnatal 12Gso/12Gso. Closed arrows indicate the curvature of the backbone, and the open arrow points to a kink in the tail. **C.** Two week old litter from a 12Gso/+ X 12Gso/+ mating. The two 12Gso/12Gso mice (arrows) are much smaller than their siblings. **D.** Magnified picture of the two 12Gso/12Gso mice from **C.**, with closed arrows indicating the curvature of the backbone. **E.** One month old 12Gso homozygote. Note the bulging abdomen caused by the curvature of the spine (white arrow). **F.** Two adult 12Gso/12Gso mice. The closed arrow indicates the bulging appearance of the rear of the mouse from the displaced hips; the open arrow indicates the severely kinked tail.



age. None of the matings between 12Gso/12Gso mice and normal, carrier, or homozygous mutant mice have produced any litters, although it is still not certain that the sterility is due to true reproductive defects rather than physical limitations imposed by the skeletal malformations. Unpublished studies from this project have also revealed that homozygous 12Gso mice have also developed an unusual bone marrow defect that closely resembles leukemia (L. Chittenden, X. Lu, unpublished; see details in chapter 3).

The 12Gso heterozygotes are semisterile, as are carriers of balanced translocation in general due to production of genetically imbalanced gametes (Generoso, 1978). However, reduced numbers of liveborn pups from heterozygote matings occurred at rates greater than can be attributed to translocation-associated effects alone, which suggested that the mutation may also be lethal in at least some fraction of the homozygous mice. In accordance with this observation, anecdotal evidence obtained during breeding experiments at ORNL suggested that a relatively low number of homozygous mutants were being born live in 12Gso carrier crosses (on a BLH [C3H/Rl x C57BL/10a] background; K.T. Cain and W.M. Generoso, unpublished data). The number of liveborn offspring exhibiting the skeletal phenotype in 12Gso carrier crosses was reduced significantly after the stock was moved to LLNL and transferred from the BLH strains to an inbred C3Hf/Rl background, and later, to a pathogen-free B6C3 [(C3H/HeJ X C57BL/6J) hybrid; The Jackson Laboratory, Bar Harbor, ME] genetic background. In later experiments, the hypothesis was tested by karyotyping all liveborn pups from carrier crosses during a year-long period of breeding at Lawrence Livermore National Laboratory (LLNL). During this period, experimental results indicated that homozygous offspring were dying pre- or perinatally (discussed in Chapter 3), but were generated *in utero* in expected ratios (1/6). Later experiments transferred the stock onto two very different genetic backgrounds, C57BL/10J and 129x1/SvJ (The Jackson Laboratory), both of which were more supportive of mutant production than either C3Hf/Rl or B6C3 and provided a strong suggestion that both the survival of 12Gso homozygotes and the severity of the skeletal defects expressed by the 12Gso/12Gso mice was profoundly influenced by genetic background.

Two explanations could be offered for the variations in numbers of animals expressing the skeletal disorder after transferal to these different backgrounds: (1) some of the homozygotes were born without physical manifestations of the mutation, that is, the 12Gso phenotype could be characterized by incomplete penetrance, or, (2) homozygotes were affected with varying degrees of severity on the different genetic backgrounds and the most severely affected may have been dying prematurely. Even in the mice that exhibited an obvious skeletal malformation, the 12Gso skeletal phenotype was expressed in a highly variable manner; for example, animals have been born with slightly shortened spines and kinked tails, severely affected spinal columns but normal tails, and many intermediate variations. This has also been observed in other skeletal mouse mutations; mutations such as *achondroplasia*, *snub-nose*, *meander tail*, and *luxoid* (Table 1) all exhibit variable penetrance. The mutation *luxoid* (Table 1) is particularly notable, as the *luxoid* phenotype is almost completely absent on a C3H background (Green, 1955). The phenotypic variation in these classical mouse mutations suggest that the 12Gso homozygous skeletal phenotype is likely to be expressed with variable penetrance and influenced by background genetic modifiers.

Normal skeletal development

The skeletal phenotype exhibited by 12Gso mice suggested a defect in the early stages of skeletogenesis, and lead to the focus on potential candidate genes that are known or implicated in the classical pathways involved in this complex developmental process. Since the foundations of the skeletal system occur during the very early stages of development, and bone development is essentially completed before birth, molecular and genetic access to human conditions involving congenital skeletal abnormalities has been extraordinarily difficult. As a result, only a small number of genes had been implicated as root causes in the etiology of skeletal anomalies until recent years. Because of the accessibility of most developmental stages in mouse embryos, mouse models have provided critical insights into the nature of human developmental syndromes. By examining the expression patterns of genes at different stages of development, as well as interpreting the fully developed adult mouse skeleton in the

Table 1. Mouse skeletal mutations mapping to Mmu4 and Mmu9

Mmu	Mutation	Symbol	Map position	Phenotype	Ref.
4	meander tail	<i>mea</i>	44.8 cM	ankylosis or fusion in the tail vertebrae; cerebellar disruption; incomplete penetrance	Hollander and Waggie, 1977; Fletcher <i>et al.</i> , 1991
	skeletal fusions with sterility	<i>sks</i>	54.6 cM	skeletal fusions occur in the ribs and one or more vertebrae; mutants are also sterile	Handel <i>et al.</i> , 1988
	Achondroplasia	<i>cn</i>	55.4 cM	limited viability; a square body shape, bulging a short tail and domed skull; limited chondrocytes vulnerable to regressive change	Konyukhov <i>et al.</i> , 1970; DiLeone <i>et al.</i> , 1997
9	luxoid	<i>lu</i>	23 cM	polydactyly, kinked tails, an increased number of vertebrae and ribs; at E10.5 the number of somites is increased; penetrance is almost null in C3H strains, and is close to 90% in C57Bl/10	Green <i>et al.</i> , 1955; Burda <i>et al.</i> , 1969
	Abnormal feet and tail	<i>Aft</i>	32 cM	heterozygotes exhibit syndactyly and kinked tails; penetrance of the mutation is very low in the 129 strain	Lane, 1987
	tail-kinks	<i>tk</i>	48 cM	short, very kinked tails, and minor aberrations in the vertebral column; genetic background can influence viability	Gruneberg, 1954; Imai <i>et al.</i> , 1993
	kyphoscoliosis	<i>Ky</i>	56 cM	progressive kyphoscoliosis in the thoracic and lumbar vertebrae, preceded by musculature aberrations; mutation in transglutaminase-like muscle-specific protein	Blanco <i>et al.</i> , 1998; Blanco <i>et al.</i> , 2001

context of the presence or absence of these genes, researchers have begun to elucidate the mechanisms of normal and abnormal skeletal development in mammals.

Vertebrae and rib formation essentially begins at gastrulation with the production of the primitive streak in the posterior of the developing mouse embryo. At approximately 6.5 days post coitus (dpc; Theiler stage [TS] 9), the primitive streak arises from the cells of the epiblast, which are laid down in a posterior to anterior direction. From the anterior end of the primitive streak the mesoderm cells differentiate into the notochord (Psychoyos and Stern, 1996). Fully developed, the notochord blocks the progression of other mesodermal cells, also derived from the primitive streak, into the midsagittal area of the embryo. This results in two sheets of mesoderm, which accumulate on either side of the centrally located neural tube and notochord (Hall, 1977). The sheet adjacent to the neural tube, the paraxial mesoderm, will give rise to the somitomes (presomitic mesoderm) at 7.5 dpc (TS 11), which will undergo initial segmentation to form the somites by 8 dpc (TS12). Somites are paired, segmental structures, the first of which arises from the anterior end of the primitive streak, near the head region. Each somite becomes epithelialized soon after formation. This epithelial somite is subject to the dorsoventral patterning influence of the surrounding tissues (Kaufman and Bard, 1999), and subsequently partitions into three zones: dermatome, myotome, and sclerotome. The ventrally located sclerotome reverts to mesenchymal tissue and separates from the dermomyotome. The rostrocaudal compartmentalization of the sclerotome occurs in the presomitic mesoderm, prior to the initial segmentation of this mesodermal sheet, and is separate from the epithelialization that is responsible for dorsoventral specification of dermomyotome and sclerotome (Stern and Keynes, 1987; Bronner-Fraser and Stern, 1991; Saga and Takeda, 2001). This compartmentalization can be identified in the sclerotome by an area of dense mesoderm and an area of looser mesoderm, demarcated by a cleft. Each vertebra derives from a rearrangement, or resegmentation, of these mesodermal regions in the sclerotome of the somites. The areas of loose and dense mesoderm in the sclerotome separate, and the loose rostral portion rejoins with the dense caudal portion of the next posterior sclerotome. This results in the prevertebrae being slightly offset from the dermomyotome and the segmental units of

the neural tube, which remain in the original position of the somite. The dense rostral portions give rise to the intervertebral discs, while the lower density caudal regions give rise to the centra and processes of the vertebrae (Goldstein and Kalcheim, 1992; Kaufman and Bard, 1999).

As the notochord disappears at approximately 11 dpc (TS 18), the centers of chondrification appear and begin the formation of cartilage. Cartilage, the precursor of ossified bone, is derived from the mesenchymal cells (embryonic connective tissue cells) of the mesoderm. These cells differentiate from their branched formation to a more spherical one and begin to secrete matrix, at which point they are termed chondrocytes (Feduccia and McCrady, 1991). Eventually, the chondrocytes become trapped within the lacunae (spaces) in the same matrix they have secreted (Romer, 1970). By 13 dpc (TS 21/22), the cartilaginous axial skeleton of the embryo is almost completely developed.

Vertebrae are considered endochondral bone; that is, they arise from the replacement of cartilage by bone. While cartilage begins to degenerate centrally through the action of chondroclasts, calcification occurs by deposition of a complex form of calcium phosphate called apatite. Blood vessels break through into the degenerated areas of cartilage, bringing with them the osteoblasts, which secrete the bone material and aid in the calcification process (Romer, 1970). Ossification proceeds outward toward either end as well as from the epiphyses, which are ossification centers located at the ends of bones. In vertebrae, several primary ossification centers occur, causing the irregular shape of the bone. During the first postnatal week in the mouse -- when bone formation has replaced the cartilage formation -- the waves of ossification from the shaft and the epiphyses unite to complete the bony skeleton (Kaufman and Bard, 1999).

Anatomically, vertebrae and ribs are considered to be members of the somitic axial skeleton. The vertebrae have two principal parts: the centrum, which is the replacement of the notochord, and the neural arch, which serves to protect the spinal cord on either side. Each vertebra is connected to those in front and behind by the zygapophyses, which extend from the neural arch. Above the spinal cord two arches fuse to form the neural spine, at the base of which is the transverse process

(diapophysis), where rib attachment occurs. Rib formation occurs at approximately the same time as sclerotome amasses around the notochord. The ribs, also derived from the sclerotome, are attached dorsally to thoracic vertebrae and ventrally to a sternum, with the exception of a few shorter, posterior “floating ribs”, which are only attached to the vertebral column. These are the components of “typical” vertebrae, although vertebrae vary regionally in functional morphology along the column (Weichert and Presch, 1975). The vertebral column is fundamentally divided into five categories (anteriorly to posteriorly): the cervical (7 vertebrae); the thoracic (13 vertebrae); the lumbar (6 vertebrae); the sacral (4 vertebrae); and the caudal (30 to 31 vertebrae).

The molecular basis of skeletal defects

Human developmental abnormalities involving the skeleton have been documented extensively, ranging from complex heritable bone disorders to the simple absence of one tiny phalanx. The genes responsible for many of these disorders have been cloned, and encode such products as collagen matrix components, cellular signaling molecules and receptors, and transcription factors. These defects can be grouped into three categories: defects in collagen metabolism, aberrant differentiation or proliferation of chondrocytes and osteoblasts, and abnormal mesenchymal condensation and its associated incorrect cellular differentiation (Mundlos and Olsen, 1997a, b).

An example of a disease resulting from a defect in collagenous metabolism is osteogenesis imperfecta. Osteogenesis imperfecta is more commonly known as brittle bone disease, and is characterized by skeletal fragility. This fragility derives from mutations within the genes for *COL1A1* and *COL1A2*, which encode the α_1 and α_2 chains of collagen, respectively (Byers and Steiner, 1992). The mutations can range from mild anomalies caused by decreased collagen formation to lethal effects caused by the inability to form the collagen triple helix (Cotran *et al.*, 1999).

Achondroplasia results from an abnormal proliferation of chondrocytes within the growth plate. The root cause of the disease is a point mutation in the cellular signaling molecule *FGFR3*. The mutation hyperstimulates the receptor, leading to constant inhibition of cartilage production and the resulting dwarfism (Mundlos and

Olsen, 1997a). The homologous mouse mutation achondroplasia (*cn*) maps to 55.4 cM on mouse chromosome 4 near *Bmp8a* and *Bmp8b*, members of the bone morphogenic protein family (DiLeone *et al.*, 1997). Mutants are clearly recognizable by the square body shape and bulging abdomen, accompanied by a short tail and domed skull. The major effect is seen in the chondrocytes, which are limited in number (Konyukhov and Paschin, 1970) and are vulnerable to regressive change (Silberberg *et al.*, 1976).

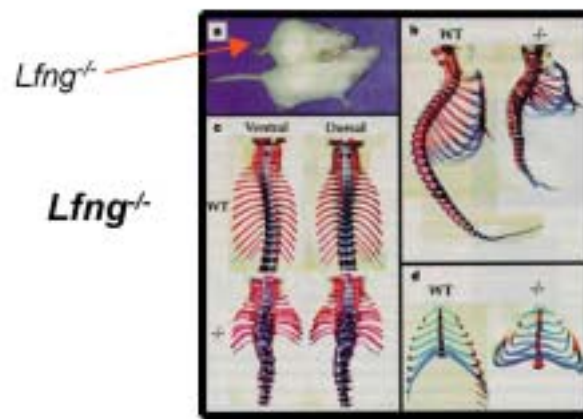
The majority of skeletal mutations manifest before cartilage is fully developed. Since cartilage provides the template from which the bone of the axial skeleton derives, the effects of mutations in this tissue persist in the fully developed skeleton. Chondrification centers of the developing cartilage are not typically direct targets of gene action, therefore effects of these mutations are generally displayed before cartilage formation. Mutations in somites, mesenchyme or sclerotome, the notochord, or the primitive streak affect the cartilaginous skeleton, since these tissues are precursors to cartilage development (Johnson, 1986). Defects in mesenchymal condensation, as well as aberrant cellular differentiation, usually lead to congenital malformations. These malformations are exceedingly difficult to study at a molecular level in humans, as access to the developmental pathways is limited, and many of the affected genes from which the defect derives are no longer expressed in adult tissues. It is also to this category of aberrations that 12Gso bears the most resemblance, and will thus be the category for which 12Gso represents the strongest model.

One of the most common vertebral malformations is scoliosis, particularly in the form of kyphosis, in which the vertebral column arches dorsally. Scoliosis is generally analyzed when present as a secondary condition of other skeletal syndromes, such as Basal Cell Nevus Syndrome (BCNS; also called Gorlin Syndrome). As the name implies, the most identifiable characteristic of this syndrome is the numerous basal cell nevi. However, this syndrome also manifests severe skeletal deformities, including kyphoscoliosis, bifid, fused, or missing ribs, and vertebral anomalies deriving from improper segmentation (reviewed by Gorlin, 1987). This syndrome derives from mutations in *PTCH* and *PTCH2*, vertebrate homologs of the *Drosophila* gene *patched* (Hahn *et al.*, 1996).

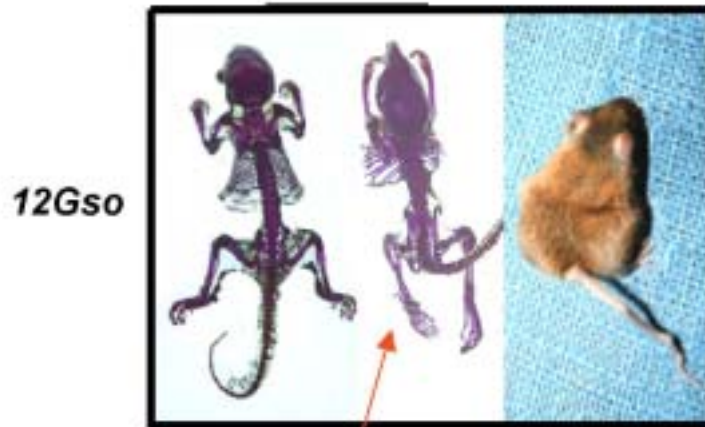
Within the classification of congenital malformations is included a broad, generic category termed spondylocostal dysostosis. This disease is characterized by shortened stature; fused, block, and hemivertebrae; nonprogressive kyphosis; fused, missing, and bifid ribs; shortened neck; apparent normal appearance of appendicular structures; seemingly normal neurological function; and numerous cases of neonatal death associated with respiratory infection (Jarcho and Levin, 1938; Lavy *et al.*, 1966). Mutations in *delta-like 3 (DLL3)*, a mammalian homologue of the *Drosophila* gene *Delta*, are associated with the autosomal recessive form of this disease (Bulman *et al.*, 2000). Mutations in the mouse homologue of this gene result in the classical mutation *pudgy (pu)*. *pu* arose from a spontaneous point mutation (Greenfield and Brown, 1987) in *Dll3*, which maps to chromosome 7 in mouse (Kusumi *et al.*, 1998). *Dll3* is a member of the *Notch* signaling pathway, which includes a cascade of pair-rule genes that control positional effects during development.

The 12Gso mouse mutation became the central focus of this thesis work because of its obvious effects on skeletal development. The mutation manifests as a profound skeletal deformity, visually identifiable at one day postnatal (Figure 2). Before it was mapped and characterized, the mutant was nicknamed “pudgy” by members of Dr. Generoso’s group because of its outward morphological similarity to the previously mentioned mutation of the same name. The gene associated with the *pu* phenotype has been cloned and identified as the mouse delta-like 3 (*Dll3*) locus (Kusumi *et al.*, 1998). Phenotypically, 12Gso also strongly resembles targeted null mutations in the lunatic fringe (*Lfng*) gene, which, like *Dll3*, participates in the *Notch-Delta* developmental pathway. Because of the phenotypic similarities, a relationship between the 12Gso mutation and genes encoding members of this pathway was hypothesized. *Dll3* and *Lfng* mutant mice have severe vertebral and tail anomalies, including irregularly shaped vertebrae and hemivertebrae that have been fused and jumbled extensively, rib and sternum anomalies, and a kinked or missing tail (Figure 3). Abnormalities seem to be restricted to the axial skeleton, since the appendicular structures appear normal. The abnormalities in the tail have been attributed to lack of segmentation in the sclerotome and/or somitic tissue; this could also be the source of the malformation in the vertebral

Figure 3. The 12Gso homozygote skeletal phenotype strongly resembles that of mutations in the genes from the *Notch-Delta* pathway. Orange arrows indicate homozygotes from all three genotypes. Note the wedge-like shape, abnormal skeleton, and shortened body length peculiar to all three mutations. Mutations: *Lfng*^{-/-}, lunatic fringe deficiency; *Dll3*^{pu}, delta-like 3 deficiency. The *Lfng*^{-/-} knockout mutation was generated by Zhang and Gridley, and the *Dll3*^{pu} mutation was characterized by Dunwoodie *et al.*



Nature 1998 **394**:374-377



12Gso/12Gso



Nat Genet 1998 **19**:274-278

column. In addition to the deficiency in segmentation, the somites present in *pu* embryos do not extend as far caudally as the somites in a normal developing embryo, thereby causing the shortening of the vertebral column. Like 12Gso homozygotes, *pu* embryos have limited viability after the weaning stages (Gruneberg, 1961). These studies suggest that the skeletal malformations and limited viability of 12Gso might also represent manifestations of a loss-of-function mutation in a single gene.

Aspects of hematopoiesis relevant to bone marrow components

As phenotypic studies have revealed, the 12Gso skeletal phenotype is accompanied by aberrations in hematopoiesis within the bone marrow. To understand the genetic factors that might contribute to this aspect of the phenotype, the basic stages of normal hematopoietic development must first be considered. Hematopoiesis is the generation of the cells of the hematopoietic system: blood cells, blood precursors in the bone marrow, and cells of specific lineage in the spleen, lymph nodes, and other lymphatic tissues. In normal progression, the first, or mesoblastic, phase occurs as early as 7 dpc, and involves the production of primitive erythroblasts from the mesoderm of the blood islands in the yolk sac and the splanchnopleura (TS 10; Kaufman, 1991; Godin *et al.*, 1993). The second, or hepatic, phase begins at early- to mid-gestation, peaks at approximately mid-gestation, and subsides at birth. This phase takes place principally in the fetal liver, but is also active in the spleen and lymph nodes. The third phase begins at mid- to late-gestation and continues past birth, taking place in the bone marrow. The rate of hematopoiesis in the bone marrow gradually decreases until puberty, at which time approximately half of the marrow space is hematopoietically active, as indicated by the presence of red marrow only in the skull, pelvis, vertebrae, ribs, sternum, and the proximal epiphyseal regions of the humerus and femur (Cotran *et al.*, 1999).

The precursors to the cells involved in hematopoiesis are undifferentiated hematopoietic stem cells (HSCs). The HSCs are first found in the fetal liver and spleen, and gradually move exclusively into bone marrow during the third phase of hematopoiesis. From these HSCs derive two cell lineages: the lymphoid stem cells and the trilineage myeloid stem cells. From the trilineage myeloid stem cells derive the

erythrocytes, megakaryocytes, granulocytes, eosinophils, monocytes, and macrophages, and from the lymphocytic line derive the B-cells, T-cells, and natural killer cells (Cotran *et al.*, 1999). In B-cell development, the HSC first differentiates into a lymphoid stem cell, the precursor to both T- and B- cells, localized in the fetal liver or bone marrow. The progeny of the lymphoid stem cell either migrate to the thymus to differentiate into T-cells, or remain in the marrow to differentiate into B-cells. Differentiation of the lymphoid stem cell into B-cells begins at approximately E14 in the mouse.

The morphology of the bone marrow structure can be broken down into bone trabeculae and the medullary space. The medullary space contains adipose tissue, the previously mentioned hematopoietic cells, and the connective and vascular tissue of the stroma. The vascular tissue of the stroma is intricate; it consists of an artery that branches into arterioles, which further branch into the capillaries that connect to a system of sinusoids. While in earlier stages the marrow cellularity is approximately 80%, in the human adult, normal bone marrow composition is a one to one ratio of fat cells to hematopoietic elements, of which approximately 60% are granulocytic and 10% - 15% are lymphocytic in origin. Extreme alterations in these percentages are indicative of disorder, such as the hyperproliferation that is associated with erythroid hyperplasia or leukemia, or the hypocellularity associated with some forms of anemia (Litz *et al.*, 1996; Cotran *et al.*, 1999).

Maturation of B-cells in the bone marrow occurs as the cells migrate towards the central venous sinus of the marrow. That is, earlier progenitor cells would be located near the endosteum, while the immature and mature B-cells surviving apoptosis and phagocytosis would be closest to the central sinus and released into circulation. The maturation of B-cells is dependent on a number of cytokines and growth factors, and is thought to be maintained, at least in part, by the stromal and adventitial reticular cells that line the interior of the cavity between the endosteum and central sinus. It is from the adventitial cells that the fat cells of the marrow derive.

Because of the morphological similarity between stages in B-cell development, classification of maturation stage is typically done using terminal deoxynucleotidyltransferase (Tdt), cell surface antigens, and immunoglobulin (Ig)

expression (Loken *et al.*, 1987). Lymphoid stem cells, which express Tdt but no cell surface antigens, proliferate and differentiate, and those that are destined to be in the B-cell lineage undergo Ig heavy chain gene rearrangement. Ig gene rearrangement begins with D-J rearrangement, and concludes with V-D rearrangement (Felix and Poplack, 1991). While undergoing recombination, the cells are termed pro-B-cells, and besides Tdt, also express CD10, CD72, CD34, and progressively express CD19, CD20, and CD21. Expression of CD10 ends when the cells emerge as pre-B-cells, which by this time have assembled heavy chains of the μ isoform. The μ heavy chains are at first expressed in the cytoplasm, but as the stage progresses, are expressed at the surface, associated with surrogate γ light chains, and accompanied by Ig α and Ig β , the remaining components of the B-cell antigen receptor (Litz *et al.*, 1996). At this time, the Ig light chain genes rearrange to produce a κ or λ light chain, and cell surface expression of the intact B-cell receptor, which could be either IgM- κ or λ + Ig α /Ig β , commences. It is at this time that the cell ceases to express Tdt, is classified as an immature B-cell, and is committed to making a specific antibody based on the expressed antigen receptor (Felix and Poplack, 1991; Litz *et al.*, 1996). As the cell progresses into a mature B-cell, both IgM and IgD are expressed as surface Ig. Expression of CD23 is present in immature and mature B-cells, but is mostly found on activated B-cells. After activation by antigen, the B-cells either differentiate into antibody-forming cells (which terminally differentiate into plasma cells), or into memory cells.

Common forms and genetic causes of inherited bone marrow hyperplasia

Most neoplasms, or foci of excessive and aberrant cellular growth, are somatically derived and associated with extensive DNA damage. This damage typically involves genes that are classified as either oncogenes or tumor suppressor genes, although other classes, such as those that regulate DNA repair and apoptosis, could also contribute to abnormal proliferation (Cotran *et al.*, 1999). In normal modes of action, oncogenes should regulate cellular growth and differentiation, whereas tumor suppressor genes usually encode products that have a negative influence on cellular growth. Due to the roles of these genes in the induction of proliferation and/or differentiation, activation

of only one copy of an oncogene can lead to neoplastic growth (Lebovitz, 1986; Muller, 1986). These oncogenes may encode growth factors, growth factor receptors, cell cycle regulators, nuclear regulatory proteins, or proteins of a signal transduction pathway (Cotran *et al.*, 1999). However, as tumor suppressor genes inhibit cellular growth, two “hits” are typically necessary to inactivate both copies of the gene, and thus, make that locus ineffectual for growth inhibition (Levine, 1993). While the pathways through which tumor suppressor genes contribute to neoplasms is less understood than those pertaining to oncogenes, it is likely that the inhibitory signals are connected to cell cycle or transcription regulation (Cotran *et al.*, 1999). Oncogenes, in particular, have been well studied in relation to immunological neoplasms, and it appears that different oncogenes affect specific cell types with respect to growth stimulation, as exemplified by the *c-myc* oncogene in B-cell lymphomas (Taub *et al.*, 1982; Della-Favera *et al.*, 1982). The genes may be activated either by changes in structure or regulation, which may occur by mechanisms such as gene amplification, point mutations, or chromosomal rearrangements such as translocations (Cotran *et al.*, 1999). All lymphoid and myelocytic neoplasms are traditionally classified as malignant, but a distinct difference lies between the hematopoietic neoplasms termed leukemia and lymphoma. While the two may present overlapping clinical findings, they are distinct with respect to origin and tissue distribution. Leukemia can be defined as neoplasms of lymphoid or myelocytic origin with focused involvement of the bone marrow, and is usually accompanied by infiltration of the peripheral blood by a large volume of tumor cells. Lymphomas, on the other hand, are characterized by proliferations that have arisen in separate tissue masses, and may later deposit tumor cells into the blood at leukemic levels. However, all lymphoid neoplasms are monoclonal, that is, they derive from a single cell, and are transformed after antigen receptor gene rearrangement. For this reason, the diagnosis and classification of the type of lymphoid neoplasm is often based on the cell type of origin (e.g. B lymphocyte, myelocyte) and differentiation stage that appears to be amplified (Cotran *et al.*, 1999).

Another term for bone marrow hyperplasia is leukemia, as it is defined as the abnormal proliferation of hematopoietic cells whose initial locales are the blood and

bone marrow. Briefly, leukemias can be either lymphoproliferative or myeloproliferative, and acute or chronic. Acute leukemias are marked by a predominance of blast cells, and often there is evidence of marrow failure. Chronic leukemias, on the other hand, are marked by leukocytosis and predominantly mature cells. Early marrow failure is rare (Litz *et al.*, 1996). Occurrences of all forms of leukemia in the United States averages approximately 9.5 cases per 100,000 individuals, per year (Ries *et al.*, 1991), and is usually more prevalent in males than females.

The four major forms of leukemia are acute myeloid leukemia, acute lymphoid leukemia, chronic myeloid leukemia, and chronic lymphocytic leukemia. Acute myeloid leukemias (AMLs) occur in the United States at a proportion of 2.2 individuals per 100,000, annually, and can be characterized by hyperproliferation of a precursor or immature myeloid cell, principally located in the marrow. The proliferating cells can be monoblasts, promonocytes, megakaryoblasts, promyelocyte, myeloblasts, or erythroid precursors. The extensive proliferation of the immature cell type and the resulting lack of normal myeloid cells is the principal cause of bone marrow failure in these patients. Depending on which form of AML is present, different cellular antigens may be present in the proliferative population; these markers include CD13, CD14, CD15, CD33, and CD36. Common cytogenetic diagnostics for AML are also available, such as the presence of t(15;17)(q11; q11-12) in over 90% of cases of promyelocytic leukemia (Litz, *et al.*, 1996).

Chronic myeloid leukemia (CML) occurs in the United States at a rate of 1.3 per 100,000, and is commonly diagnosed by use of the Philadelphia chromosome, t(9;22)(q34;q11). The bone marrow in CML patients is almost 100% cellular, mostly composed of granulocytic precursors (Cotran *et al.*, 1999). In addition to neutrophilic hyperplasia in the bone marrow, CML patients also present marked leukocytosis and splenomegaly. The Philadelphia chromosome (Ph), present in 95% of cases, results in the fusion of the major breakpoint cluster region gene (*BCR*) on chromosome 22 to the *c-abl* oncogene (*ABL*) on chromosome 9 (Kutrzrock *et al.*, 1988; Litz *et al.*, 1996). However, in the juvenile form of the disease, Ph is not present, but presenting findings do include hepatosplenomegaly, lymphadenopathy, anemia, and skin rashes. Prognoses

in these cases are typically poor; median survival is less than one year (Freedman *et al.*, 1988; Litz *et al.*, 1996).

The precursor B-cell neoplasm acute lymphoblastic leukemia (ALL) is manifested in the United States at a rate of 1.5 per 100,000, and is characterized by a proliferation of lymphoblasts. The majority of cases are manifested during childhood. Depending on the blast morphology, ALL may be classified as L1, L2, or the less frequent L3, and can be either populous in B-cell precursor cells, T-cells, or B-cells (Foon and Todd, 1986). A common marker for the B-cell form is CD10, also known as CALLA (Cotran *et al.*, 1999), although infantile forms of the disease typically lack this antigen and are typed as “null”. ALL may also be identified cytogenetically. Almost all cases present a translocation that disrupts the *c-myc* oncogene on HSA8 and an immunoglobulin chain locus. The second disrupted locus may be a gene on HSA14 encoding the heavy chain, on HSA2 encoding the kappa light chain, or on HSA22 encoding the lambda light chain. The transposition of these loci facilitates the aberrant transcription of *c-myc*. A small percentage of cases also present the Philadelphia chromosome (Vogler *et al.*, 1981; Ribeiro *et al.*, 1987). Clinically, it is very similar to AML. As in other leukemias, bone marrow failure is linked to the replacement of normal marrow by the hyperproliferative lymphocytes, and the disease is often accompanied by lymphadenopathy, splenomegaly, and hepatomegaly derived from neoplastic infiltration of these particular organs (Litz *et al.*, 1996).

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in the United States, occurring at an incidence of 2.6 per 100,000 individuals. The disease is a peripheral B-cell neoplasm that is characterized predominantly by hyperproliferation of light chain-restricted mature B-cells (Bennett *et al.*, 1989), and accordingly, most neoplasms are found in patients over 50 years old (Cotran *et al.*, 1999). It is always observed in the bone marrow, and lymphadenopathy and/or hepatosplenomegaly is present in 50-60% of cases. Very often the disease is accompanied by hypogammaglobulinemia, and occasionally, autoimmune hemolytic anemia derived from neoplastic, self-reactive B-cells (Cotran *et al.*, 1999). The bone marrow varies, and diffuse patterns are associated with a poor prognosis. Besides the reduction in light

chain-restricted surface immunoglobulin, the disease also presents CD5, a pan-T-cell antigen that is normally expressed only on a small subset of circulating B-cells (Litz *et al.*, 1996).

Few spontaneous mouse models of leukemia exist, but there has been a considerable amount of work done with respect to transgenics and gene knockout models that target genes known to be involved in human leukemias. A recent example is the mouse model of CLL generated by the insertion of a *TCL1* transgene (Bichi *et al.* 2002). *TCL1*, an oncogene overexpressed in T prolymphocytic leukemia, exhibits normal expression in immature T-cells, and a range of early B-cells. The mice carrying a human transgene for *TCL1* exhibited an expanded population of CD5+ B-cells, indicative of B-CLL, and upon aging, developed all related symptoms of CLL. Another of these mouse models was a targeted mutation of the *Mll* gene, which lies near the *luxoid* mutation on mouse chromosome 9 and is homologous to the *Drosophila* gene *trithorax*. The product of this gene exists in multiply spliced forms and has at least partial similarity to DNA methyltransferases (Ma *et al.*, 1993). The mutation is a homozygous lethal, and heterozygotes exhibit not only hematopoietic abnormalities, but also skeletal malformations. These malformations are associated with aberrant expression of the homeotic genes *Hoxa7* and *Hoxc9*, which are regulated through a conserved SET domain in the *Mll* sequence (Yu *et al.*, 1995).

The role of genes for somitic development in both skeletal development and hematopoiesis

Two major signaling pathways, one centered on the Notch-Delta receptor-ligand pair and one involving the Sonic Hedgehog secreted morphogen, have been well documented with regard to somitogenesis, and have implications in hematopoietic development. The components of the *Notch-Delta* pathway are conserved from *Drosophila* to human, and maintain a similarity in basic function: to participate in the specification of cellular fates by use of a signaling mechanism between adjacent cells. However, the genes expressed in *Drosophila* are neurogenic, and probably not involved in segmentation (Artavanis-Tsakonas *et al.*, 1999), supporting the morphological data

that indicate different mechanisms for segmentation are present in flies. In mammals, however, this pathway is critical for the establishment of rostrocaudal polarity and periodicity in the presomitic mesoderm (Saga and Takeda, 2001, and refs. therein).

Specific members of the *Notch-Delta* pathway are present in the presomitic mesoderm, initially expressed in a cyclical fashion. In mouse, some of these include *Lfng* and the *hairy* and *enhancer of split*-related genes *Hes1*, *Hes5*, *Hes7*, and *Hey1* (Aulehla and Johnson, 1999; Leimeister *et al.*, 1999; Bessho *et al.*, 2000; Jouve *et al.*, 2000; Dunwoodie *et al.*, 2002). The suggested role of *Notch* signaling has been to maintain the oscillating expression patterns of these genes by cellular communication through its own wave-like activity which traverses the presomitic mesoderm for each somite formed (Jiang *et al.*, 2000).

Expression of *Notch1* and some of its ligands have also been identified in hematopoietic precursors, developing B-cells, T-cells, and myeloblasts (Jones *et al.*, 1998; Varnum-Finney *et al.*, 1998; Osborne and Miele, 1999; Walker *et al.*, 2001; Kawamata *et al.*, 2002). Indeed, it has been speculated that although *Notch1* appears to be particularly responsible for early T-cell development, as indicated by expression in fluorescence-activated cell sorter analysis (e.g., Kawamata *et al.*, 2002) and genetic overexpression that resulted in T cell lymphoma/leukemia (Pear *et al.*, 1996; Pui *et al.*, 1999; Wilson *et al.*, 2001). However, it has been indicated that this T-cell development occurs at the expense of B-cell development, because of suppression of B-cell development in *Notch1* overexpression and the proliferation of B-cells upon inhibition of *Notch1*, presumably through ligands such as *Deltex* and *Jagged1* (Pui *et al.*, 1999; Bertrand *et al.*, 2000; Izon *et al.*, 2002). This role of *Notch1* in hematopoiesis seems to be in accordance with its role in segmentation: regulation of cell fate through a complex series of interactions. Two *Delta*-like genes, *Dll1* and *Dll4*, and a *Serrate*-like gene, *Jagged1*, are expressed in the bone marrow stroma and endothelium and have also been implicated in the control of *Notch* signaling within the bone marrow. As in somitic tissue, these two ligands appear to differentially regulate *Notch1*, in a manner similar to lateral inhibition (Bertrand *et al.*, 2000).

The *Hedgehog* pathway, particularly the members that interact with *Sonic hedgehog* (*Shh*), is also evolutionarily conserved and has dual roles in both somitogenesis and hematopoiesis. The expression of *Shh* and its progenitors in mesodermal tissue induces dorsoventral polarity. *Shh* is expressed at sites of epithelial-mesenchymal interaction (Bitgood and McMahon, 1995). Its relevance to somitogenesis lies in its secretion as a morphogen gradient extending from the notochord which induces the ventral somite to revert to mesenchymal tissue, thus becoming the sclerotome from which the somites derive (Pourquie *et al.*, 1993; Fan and Tessier-Lavigne, 1994). *Shh* functions through the activation of the transcription of *patched* (*Ptc/Ptch*), *wingless* (*wg*) or its homologs (e.g., *Wnt3*), and *decapentaplegic* (*dpp*) or its homologs (e.g. *Bmp4*), often by the release of repression of associated complexes (Hidalgo and Ingham, 1990; Ingham *et al.*, 1991; Capdevila *et al.*, 1994). The binding of *Shh* to the *Ptch-smoothened* (*Smo*) receptor complex relieves the repression of *Smo*, which in turn activates the transcription of *cubitus interruptus* (*ci*) homologs, the *Gli* genes (Hui *et al.*, 1994; Nusse *et al.*, 1999; Stone *et al.*, 1996; Kalderon *et al.*, 1997). *Shh* is also thought to be involved in epithelial proliferation, as overexpression contributes to the expression of both *Ptch* and *Gli1* in mesenchyme, leading to rapid proliferation of both tissues (Grindley *et al.*, 1997).

A role for *Shh* in hematopoiesis has also been implied. *Shh*, which induces the proliferation of HSCs, acts through *Ptch*, *Smo*, and the downstream *Gli* genes to activate a *Bmp4*-inhibitor, *Noggin*. *Bmp4* has been previously documented in its role of hematopoietic cell maintenance and survival (Maeno *et al.*, 1996; Dale and Jones, 1999), but this role for *Shh* has only recently been elucidated (Bhardwaj *et al.*, 2001).

As embryonic hematopoietic cells derive from mesodermal tissue, the finding that both pathways involve the same signaling members is not a novel idea and has been studied in nonmammalian systems (Zon, 1995; Dosch *et al.*, 1997). However, in light of the fact that the associated skeletal phenotype closely resembles mutations within the somitogenesis pathway, the finding of the lymphocyte hyperproliferation only further solidifies the relationship between the genetic mutation(s) of 12Gso and those within these developmental pathways.

The genetic relationship between translocation breakpoints and gene(s) associated with the mutant phenotypes

Many of the experiments described in this study proceed according to a basic hypothesis, that is, that the 4;9 translocation inherited by 12Gso homozygous mice disrupts either the structure or the regulation of transcription unit(s) that are directly associated with the skeletal abnormalities, bone marrow defects, and low survival rates of the mutant mice. This hypothesis is based on several lines of evidence, the strongest of which has been provided by genotype-phenotype correlations that have been documented before and during the course of this study. First and most importantly, strict concordance has been observed between expression of the skeletal/early death phenotype and homozygosity for the 12Gso translocation. These data, which will be described in further detail in chapter 3, suggest that the chromosome break has disturbed the structure. These data suggest that the chromosome break has disturbed the structure or expression regulation of a gene or genes that are directly associated with the skeletal defects and early lethality. The breeding methods employed for maintenance of the 12Gso stock have been based primarily on selection for the translocation, and not for presence/absence of the phenotype (L. Stubbs, K.T. Cain, and W. Generoso, pers. comm.). Because of this fact, we would expect that after many generations of selection for the translocation, segregation between the translocation and the mutation associated with the phenotype would have occurred if the two were not coinciding or very closely linked. If the translocation were not associated with the phenotype, the aberrations seen in the mutants would have disappeared after generations of stock maintenance. At the very least, we would expect to document the expression of the skeletal and/or leukemic phenotype among animals that did not inherit the translocation in homozygous form. This has never been documented despite more than 10 years of maintenance breeding of this mutant stock.

The phenotype could, however, be explained by a second mutation closely linked to the translocation site. The *a priori* argument against involvement of a linked mutation is based on two pieces of evidence: (1) the mutagenesis load expected from the

treatment that produced the 12Gso line, which though relatively unknown, is unlikely to be highly mutagenic, and (2) the amount of founder DNA [JH stock; (C3Hf/Rl X 101/Rl) F1] remaining in current mouse stocks after years of selective outbreeding. Although 12Gso was maintained at Oak Ridge on a BLH [(C3Hf/Rl x C57BL/10a) F1] hybrid background, we know from molecular evidence that the lesion occurred on the C3Hf/Rl chromosome 4. It is therefore possible to estimate how much founder DNA, in centimorgans (cM), remains after each generation based on calculations used for the generation of congenic strains (Silver, 1995), that is, $[200 (1-2^{-N})/N]$, where N represents the generation number. To simplify this further, after the fifth generation, the calculation $[200/N]$ may be used. Because the mutation/allele of interest, in this case the translocation breakpoint, is the selection reference for each generation, the genetic region surrounding this mutation/allele decreases the amount of donor DNA much more slowly. That said, it must be noted that 12Gso is already at F20 for several mice, and the regions surrounding the chromosomal breakpoints in these mice can be calculated to have retained an average 10 cM, or 20 Mb, of founder DNA. While this number is an estimate, and would certainly be subject to genetic variation in the region, it is still useful in determining the likelihood of a second mutation linked to the site where the lesion generating the translocation occurred. While a true estimate of the presence of a second mutation is not possible with the data currently available, it is known that the nature of MBA is to generate large lesions which lead to chromosomal rearrangements. The presence of a second lesion in the form of another translocation may be ruled out; gametes with this cytology would not be viable. Large deletions and inversions can also be ruled out using mapping data generated for 12Gso in this 20 Mb region. The elimination of these categories of lesions further reduces the chances of a mutation event within the calculated 20 Mb. Since the likelihood of a linked mutation, though reduced, cannot be ignored, experiments were designed to address the link between inheritance of the translocations and the 12Gso mutant phenotype. Tests included genetic mapping, breeding, and the use of microsatellite markers surrounding the breakpoints to determine both the strain on which the mutagenic lesion occurred, and how much of this original DNA is maintained in current stocks. A more in-depth analysis of the likelihood of non-

concordance will be addressed in later chapters. This analysis will be centered on an estimate of the maximum possible distance existing between the map locations of the translocation and the mutation causing the phenotype, without making the assumption that the two are coextensive. While these calculations can not prove or disprove the role of the translocation in the mutant phenotype, the data can be used to strengthen the argument for concordance.

Chromosome rearrangements as a tool for identifying genes associated with human disease

Localization of genes associated with inherited disorders can be an extremely lengthy process, involving years of pedigree analysis or extensive animal breeding and genotyping. Although localization of candidate genes has been streamlined significantly with the recent advent of human and mouse genome sequence, making a link between genes in a candidate interval and mutant phenotypes can still be an overwhelming task. The use of a genetic approach for gene mapping in humans is difficult, therefore large chromosomal rearrangements such as deletions, inversions, duplications and insertions have assumed a prominent role in the discovery of genes associated with human disease. A classic example that illustrates the usefulness of chromosomal rearrangements in linking candidate genes to phenotypes is the story behind the cloning of the autosomal dominant polycystic kidney disease gene, *PKD1*. The 1 megabase (Mb) critical region to which the *PKD1* gene had been localized was searched exhaustively for many years (Reeders *et al.*, 1985; Germano *et al.*, 1992), and although many different genes were discovered and analyzed, none could be clearly associated with the disease. The critical breakthrough finally came when a second group of researchers identified a *PKD1* patient carrying a balanced translocation that disrupted the candidate region. Disrupted in the breakpoint interval was a 14-kb transcript, which was confirmed as the causative agent of ADPKD when deletions within this gene were also discovered in *PKD1* patients (European Polycystic Kidney Consortium, 1994).

Another prominent example is the *DMD* gene. The locus for Duchenne muscular dystrophy had been localized to Xp21 by a linkage analysis (Davies *et al.*,

1983), deletions in male subjects (e.g. Clarke *et al.*, 1986; Greenburg *et al.*, 1987) and by numerous translocations (e.g. Lindenbaum *et al.*, 1979; Jacobs *et al.*, 1981; Boyd *et al.*, 1988). The *DMD* locus was finally cloned and confirmed through the use of an array of deletions, most of which were contained within a 2 kb region of the cloned transcript (Koenig *et al.*, 1987). The use of translocations in isolation of the *NF1* gene for neurofibromatosis type I narrowed the region to under 1 Mb (Fountain *et al.*, 1989; O'Connell *et al.*, 1989) before the gene was cloned by three separate groups, again with the aid of deletions and translocations (Cawthon *et al.*, 1990; Viskochil *et al.*, 1990; Wallace *et al.*, 1990). Numerous other examples of genes associated with human disease identified as a result of their disruption by chromosomal rearrangements can be found in recent literature. Prominent examples include the *FMRI* for Fragile X mental retardation (Verkerk *et al.*, 1991); the genes *BRCA1* and *BRCA2* involved in familial breast cancer (Miki *et al.*, 1994; Tavtigian *et al.*, 1996); *WT1* for Wilm's tumor/aniridia (Call *et al.*, 1990); the *TBX5* gene in Holt-Oram syndrome (Li *et al.*, 1997); the *SOX9* gene for autosomal sex reversal/campomelic dysplasia (Wagner *et al.*, 1994); and the *RIEG* gene for Rieger syndrome (Semina *et al.*, 1996).

In addition to these inherited disease phenotypes, the use of somatic rearrangements -- especially translocations -- in the cloning of genes associated with leukemic phenotypes is widespread, and has been well reviewed by many sources (Rowley, 1990; Rabbitts, 1994; Heim and Mitelman, 1986; Look, 1997; Rowley, 1998; Rabbitts, 2001). Some of the more notable examples of leukemia-associated genes cloned by use of translocations are *MLL/ALL/HRX*, which is disrupted in cases of acute myeloid leukemia and acute lymphocytic leukemia (Zieman-van der Poel *et al.*, 1991; Djabali *et al.*, 1992; Gu *et al.*, 1992; Tkachuk *et al.*, 1992), the *AML1/CBFA2* gene, which is disrupted by translocations in acute myeloid leukemia and acute lymphoblastic leukemia (Myoshi *et al.*, 1991), and the familiar Philadelphia chromosome, which fuses the *BCR* and *ABL* genes in approximately 95% of chronic myeloid leukemia cases and 5% - 30% of acute lymphoblastic leukemia (de Klein *et al.*, 1982). The discovery of these genes would have been much delayed without the aid of a cytological marker such as a translocation.

Human geneticists have therefore learned to exploit the information given by disease-associated balanced chromosome rearrangements, as a study done by a consortium in Sweden and Denmark has indicated (Bugge *et al.*, 2000). Even excluding the most frequently seen cytogenetic aberrations, Robertsonian translocations and chromosome 9 inversions, these laboratories found *de novo* rearrangements at a frequency of six times the normal population in their cytogenetic archives, the data of which was collected during diagnosis of an aberrant phenotype in an observed patient. Of the rearrangements the group found, several disorders were either cloned or localized within a finite interval as a direct result of cytogenetic anomalies. These include Prader-Willi Syndrome (*SNRPN*; Schulze *et al.*, 1996; Sun *et al.*, 1996; Conroy *et al.*, 1997; Kuslich *et al.*, 1999), Nail-patella syndrome (*LMX1B*; Duba *et al.*, 1988; Silahtaroglu *et al.*, 1999), Beckwith-Wiedemann Syndrome (Tommerup *et al.*, 1993), Grieg cephalopolysyndactyly syndrome (Tommerup and Nielsen, 1983), Diamond-Blackfan anemia (Gustavsson *et al.*, 1997), Duchenne muscular dystrophy (Nielsen and Nielsen, 1984), and Trichorhinophalangeal syndrome type I (*TRPS*; Brandt *et al.*, 1997). Other disorders, e.g., Smidt syndrome, Noonan syndrome, ulna aplasia, polydactyly, atypical ataxia, and various forms of mental retardation, were localized to candidate regions using the chromosomal abnormalities (e.g., Skovby and Niebuhr, 1974; Kristoffersen *et al.*, 1974; Rasmussen *et al.*, 1982; Friedrich *et al.*, 1982). Eleven of the 14 well-characterized disorders that were documented in this analysis presented evidence that linked the chromosomal breakage event and the phenotype by independent verification of involvement of the disrupted locus in the disease. The translocations in the remaining 3 diseases did disrupt genes, but not the genes later proven to be associated with the disease phenotype. While results of this study are preliminary, they contribute to an overwhelming body of solid evidence that exists to support the usefulness of cytogenetic abnormalities, such as translocations, as tools for cloning the genes responsible for aberrant phenotypes.

Chromosome rearrangements and inherited disorders in mice

Because of anatomical, physiological and developmental similarities between mice and humans, and because of the facility of maintenance and breeding, mice have long been used for analysis of human development and disease. With regard to gross chromosomal abnormalities, mice have been an ideal source of study. While the study of human inversions, deletions, and translocations usually relies initially on a subject or descendant of a subject with a spontaneous rearrangement presenting an obvious phenotype, this is not the case for mice. By subjecting mice to specific mutagenesis schemes, chromosomal rearrangements can be easily induced and studied. A few studies have used chromosome rearrangements to identify genes associated with mutant phenotypes in mice.

One of the most notable studies directly related to the 12Gso project is that of cloning the gene associated with the non-agouti coat color phenotype. Although the *Agouti* (*A*) locus had been a research subject for a number of years, it was not until 1990, when an γ -radiation induced mutation caused a deletion, an insertion and an inversion, placing the *A* gene adjacent to the previously characterized *limb deformity* (*ld*) locus, which maps approximately 20 cM away in normal mice (Woychik *et al.*, 1990). This inversion (Bultman *et al.*, 1991) enabled initial characterization of the region, ultimately leading to the cloning of the *A* locus (Bultman *et al.*, 1992).

Deletions and deletion complexes have been used extensively as tools for cloning mouse genes. A large collection of deletion mutations exist for loci associated with markers used in the specific locus test (Russell, 1951; Russell *et al.*, 1982; Lyon *et al.*, 1992; Metallinos *et al.*, 1994; Rinchik, 1994; Rinchik *et al.*, 1994; Bell *et al.*, 1995; Johnson *et al.*, 1995; Russell *et al.*, 1995; O'Brien *et al.*, 1996 reviewed by Rinchik, 2000). These deletion complexes permitted the use of hemizygosity as a method to identify the functions of numerous loci linked to the region of mutation (e.g., Johnson *et al.*, 1995; Rinchik *et al.*, 1999; Rinchik *et al.*, 2002). The deletion complexes have also led to the cloning of several genes in these regions, including the *cp1/Gabrb3* gene for cleft palate (Culiat *et al.*, 1993; Culiat *et al.*, 1995); juvenile fitness, growth, and sterility

(*jdf2*; Walkowicz *et al.*, 1999); fumarylacetoacetate hydrolase (*Fah*; Klebig *et al.*, 1992); extra embryonic mesoderm (*eed*; Sharan *et al.*, 1991); and *Emv-23* (Rinchik *et al.*, 1989).

Since a relatively small number of mouse translocation stocks are available, there have been fewer mouse genes localized and/or cloned through the use of these rearrangements. But examples do exist, including translocations that permitted analysis of the Ts65Dn trisomy in a Down Syndrome model (Akeson *et al.*, 2001) and the identification of imprinted domains in mice (Cattanach and Kirk, 1985). More recently, the use of a t(2;10) translocation has aided in the localization and cloning of the *bicc* gene for autosomal recessive polycystic kidney disease in the mouse models 67Gso, jcpk, and bpk (Chittenden *et al.*, 2002; Price *et al.*, 2002; L. Flaherty, pers. comm.). 67Gso, like 12Gso, is a stock from a large collection of translocation mutant mice, the previously mentioned Gso stocks generated in mutagenesis studies at Oak Ridge (reviewed by Culiati *et al.*, 1997; Stubbs *et al.*, 1997). Even with these examples of mouse models, documentation of the use of chromosomal rearrangements as tools for cloning mouse genes is not as prevalent as publications reporting the role of cytogenetic abnormalities in the genetic characterization of human disease.

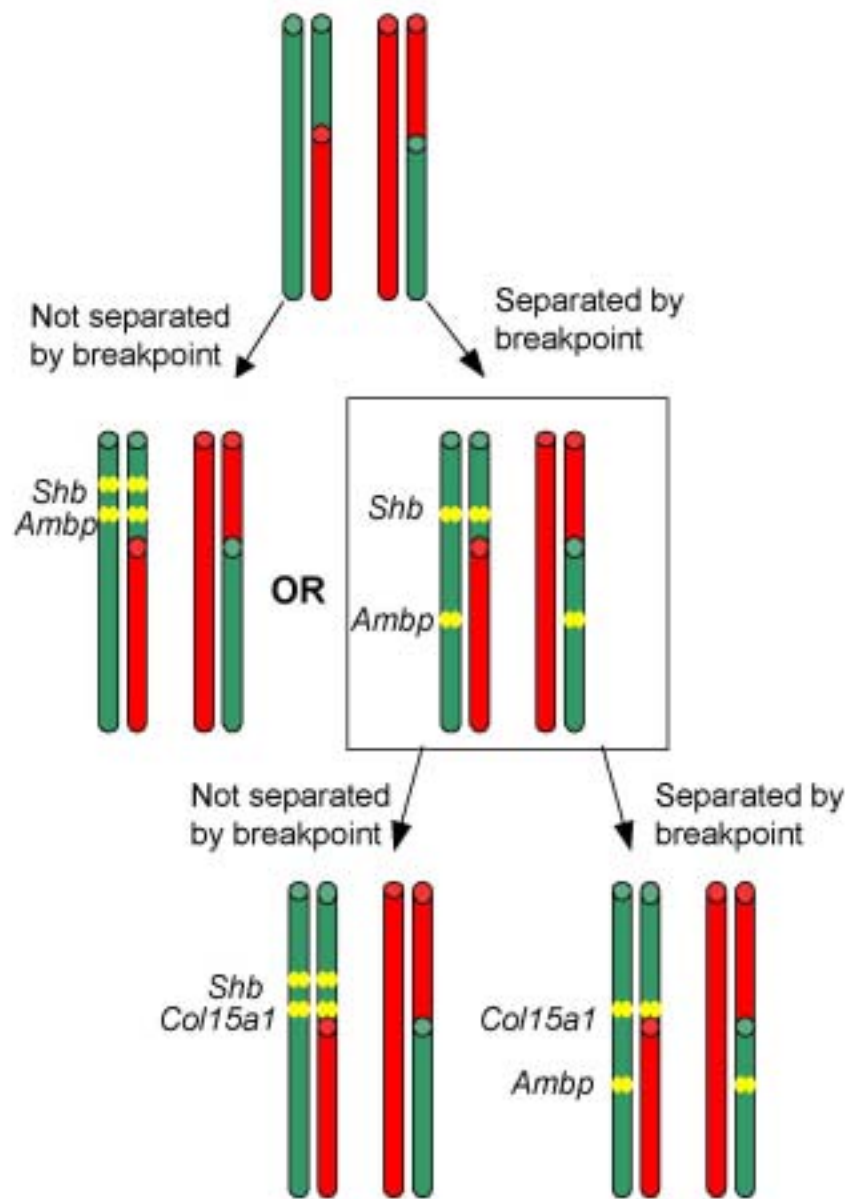
Using translocations as a tool to map and clone mutant genes

When searching for candidate genes, one of the most common ways to examine the probability of a gene as being responsible for the phenotypic expression of a mutation is to localize it within a region to which the mutation has been mapped. The converse may also be used: establish finely tuned mapping of the mutation to a region in which there is a gene whose misexpression, underexpression, or overexpression could prove to be responsible for the phenotypic variations. Using a translocation as a guide and fluorescent *in situ* hybridization (FISH)-based strategies (Mercer *et al.*, 1993; Culiati *et al.*, 1997; Stubbs *et al.*, 1997) to narrow the region surrounding the estimated breakpoint interval of the 12Gso translocation, the search for likely candidate genes is considerably facilitated. This FISH-based approach has been in use in the Stubbs laboratory to localize the Mmu2 breakpoint of the 1Gso mouse (Stubbs, 1997; Carver

and Stubbs, pers. comm.), the Mmu7 breakpoint of the 14Gso mouse (Culiat *et al.*, 1997), and the Mmu10 breakpoint of the 67Gso mouse (Chittenden *et al.*, 2002; Price *et al.*, 2002); FISH has also been used in the cloning of human disease genes as well (e.g., Menkes disease; Mercer *et al.*, 1993).

Because translocations create a visible disturbance in chromosome structure, the mutation can be mapped through cytogenetic techniques to a well-defined chromosomal region (Rutledge *et al.*, 1986; Cacheiro *et al.*, 1994). Therefore, the chromosomal position can be estimated very early in the study of these mutations without the need for the requisite large crosses that are generally used to map mutations in mice. Further delineation of the breakpoint regions can be accomplished through the use of fluorescent *in situ* hybridization (FISH) methods (Mercer *et al.*, 1993; Culiat *et al.*, 1997; Stubbs *et al.*, 1997). Gene markers in the outermost borders of the chromosomal band containing the breakpoint are first used to isolate a series of bacterial artificial chromosome (BAC) clones to be labeled with fluorescence or a hapten, and hybridized to metaphase spreads generated from mice either homozygous -- or preferably, heterozygous -- for the translocation. Because the map order of these genes is known, we expect the breakpoint to lie between these two markers because they frame the band containing the breakpoint. If breakpoint-flanking markers are indeed selected, FISH signals generated by these two BACs will be separated onto two distinct translocation chromosomes. By contrast, the same two markers will be linked on a normal mouse chromosome. However, if the markers both mapped either above or below the translocation breakpoint, the signals produced by the labeled BACs would remain paired together on the mutant chromosome, just as on the normal chromosome. For example, if the markers *Shb* and *Ambp* are used to isolate BACs, which are then hybridized to heterozygote chromosome spreads (Figure 4), we would expect one of two outcomes. In the first outcome, two pairs of signals will appear on two chromosomes, indicating that the breakpoint does not lie between the two markers (Figure 4). In the second possible outcome, a linked pair of signals will be detected on the normal chromosome in heterozygous spreads, and one pair of signals will be seen on each of the translocated chromosomes. This second outcome indicates that the two markers flank the breakpoint site. Once the breakpoint

Figure 4. FISH-based strategy for mapping the chromosomal breakpoint. Solid cylinders represent normal chromatids at mitosis, multicolor cylinders represent translocated chromatids at mitosis, and yellow circles represent fluorescent-labeled BAC clones. When hybridizing a pair of BAC clones with known map position to a heterozygote metaphase chromosome spread, two scenarios are possible. The two clones could both map above or below the chromosomal breakpoint (chromosome pairs on the left side show clones mapping above breakpoint), or the clones can be separated by the chromosomal breakpoint (chromosome pairs to the right). Once a pair of markers is separated by the translocation, a clone mapping internal to this pair is used in combination with the proximal and distal markers individually to further subdivide the region (see text for more details).



has been “anchored” by two markers, a marker internal to these is chosen for BAC isolation and co-hybridization with each of the anchoring probes to divide the breakpoint region (Figure 4). This process is repeated sequentially to narrow the breakpoint region from a distance of 10+ cM to that of a few hundred kilobases, spanned by a single BAC clone. Within that region, the candidate gene search can begin in earnest.

Knowledge of gene marker location within a given genetic interval is crucial to this project for several reasons. First, gene markers that have been localized within the designated breakpoint interval are the starting points of BAC isolation. Once the gene order has been determined, it is possible to use BACs that have screened positive for these genes as accurately placed probes for FISH. Second, these gene markers can give some indication as to the remaining distance surrounding the breakpoint. Third, these gene markers are the sources for candidate genes causing the phenotypes in the mutant. Any gene(s) localized within the breakpoint region are fair game for disruption, and any of these with any indication that they could play a role in the mutant phenotype are worth more scrutiny. For this reason, the 12Gso project began with the mapping of numerous gene markers to chromosomes 4 and 9. Before mapping began, the sizes of the breakpoint regions measured approximately 15 centimorgans (cM) on Mmu 4 (homologous to portions of human 9) and 10 cM on Mmu 9 (homologous to portions of human 3q, 6q, 11q, and 15q). Once these breakpoint regions were determined, genetic mapping of the breakpoint region markers was done using interspecific backcross (IB) mapping panels (Bonhomme, 1979). Although the possibility of a disrupted gene was equivalent for either chromosome, more probes for Mmu4 were available because of a genetic mapping experiment (Chakrabarti *et al.*, 1996) that was completed prior to the beginning of this project. Consequently, mapping experiments were begun on chromosome 4.

After these probes were mapped, clones suitable for FISH analysis were isolated as cosmids and bacterial artificial chromosomes (BACs), selected by screening libraries with markers that mapped to the regions of interest, and then rescreened to verify positive clones. These isolated clones were used as probes for FISH, initially on Mmu 4, to pinpoint the exact location of the breakpoint.

12Gso carries a cytologically obvious reciprocal translocation, and as the breakpoints were mapped to specific chromosomal bands, the next step in the characterization of this mutation was the most tedious: pinpointing the exact location of the breakpoint within this specific region. By determining the location of the breakpoints, the gene or genes associated with the phenotype could be more easily found and analyzed. Though this process is the most difficult in cloning any mutation, the cytogenetic landmarks available in the 12Gso mutation provide two advantages to this end: the aforementioned assignment to a specific chromosomal band by cytogenetic analysis, and the ability to use FISH as a means to efficiently span that chromosomal band and cross the physical breakpoint boundary in a manner that is visually evident. This FISH-based approach was used to narrow the region containing the breakpoint to a single BAC clone, with the ultimate goal of finding a candidate gene or genes implicated in producing the mutant phenotype. These genes would then be analyzed for verification of candidacy and to explore developmental, hematopoietic, and other expression patterns relevant to the gene(s).

The search for candidate genes

When examining the breakpoint regions, all genes that are located near the sites of translocation, regardless of whether or not those genes are physically disrupted, need to be analyzed because the possible effects on gene expression caused by chromosomal rearrangements could occur from some distance away. This is exemplified by a translocation breakpoint analyzed in patients with autosomal sex reversal/campomelic dysplasia, where although not disrupted by the translocation, the nearby *SOX9* was determined to be responsible for the phenotype (Wagner *et al.*, 1994). Other examples also exist, such as *TWIST* for Saethre-Chotzen syndrome (Krebs *et al.*, 1997), the *HOXD* cluster for mesomelic dysplasia (Spitz *et al.*, 2002), and *TBX5* for Holt-Oram syndrome (Li *et al.*, 1997). For this reason, the experiments to examine the expression of a neighboring gene with characteristics that might also be consistent with some aspects of the 12Gso phenotype will also be described.

Sequence analysis of the BAC spanning the Mmu 4 breakpoint region indicated the presence of the gene *Abca1*, whose position was already known, two *Nipsnap*-related transcripts, and a short transcript annotated as an olfactory gene. The *Abca1* gene is of particular interest as a possible candidate for the early lethality exhibited by 12Gso homozygotes. The 1 megabase (Mb) region surrounding Mmu9 breakpoint appears to be relatively gene-poor, but contains two potential gene candidates: *Tbx18*, which is expressed in somatic tissue, and *Rock1*, a *ras*-related kinase. Other genes in this region include *Snap91*, a neuronally expressed gene, a hemoflavin-related EST, and an uncharacterized EST expressed in brain. All of these genes will be discussed further in Chapter 3.

Methods of locating and testing candidate genes

There are several ways in which a particular gene can be tested for candidacy. Obviously, since we are working under the hypothesis that translocation-associated gene disruption is the root cause of the phenotypes expressed by 12Gso mice, map position is the first criterion. Although, as previously mentioned, the translocation may not physically disrupt the gene, one would still expect the responsible gene to be located near the break. Secondly, analysis of the gene product could aid in determining the likelihood of candidacy by the kind of protein encoded by this gene, and if unknown, the relationship of the protein encoded by the putative candidate gene to other proteins whose functions might be applicable to the phenotype. Thirdly, relative levels and locations of gene expression would also indicate candidacy. Even without the comparison of mutant and wild type tissues, expression pattern could be a strong indicator; however, it is a much stronger positive indicator than a negative one. Although expression information can be used to encourage further analysis of genes that exhibit the expected patterns of tissue expression, it can be used to rule out candidate genes only when fully documented and the phenotype in question is very well understood. One method of testing expression, useful in the case of small samples from which little RNA can be isolated, is that of reverse transcriptase polymerase chain reaction (RT-PCR). Using available sequencing data, primers specific for various exons

can be used to amplify specific regions of mutant RNA. RT-PCR screening could detect secondary alterations that could affect gene regulatory regions, and could also determine which portion of the gene was truncated in the generation of the breakpoint (Dirks *et al.*, 1996; Pai *et al.*, 1994; Sato *et al.*, 1996). It is more common, however, to test the gene on a comparative Northern containing RNA from wild type and mutant tissues. Northern blots are useful not only for determining differences in gene expression/ expression levels, as in RT-PCR, but can also determine if an aberrant transcript is produced in mutant RNA. Finally, microarrays, which allow the quantitative and qualitative measurement of gene expression through hybridization to an array of ESTs, can be used as a means to determine which genes out of a large subset are expressed in a particular tissue, and also to compare expression levels between various tissue samples (e.g. McNeish *et al.*, 2000).

In order to elucidate possible candidacy of the various genes within the 12Gso breakpoint regions, analysis of the gene expression was carried out using a variety of these methods.

The aims of the 12Gso project

The discovery of the 12Gso mutation provides a unique opportunity to map and identify genes associated with skeletal development and leukemia, and to study their molecular mechanisms in an animal model. Because the 12Gso translocation breakpoints do not contain genes known to be involved in the *Notch-Delta*, *Shh*, or classic leukemia gene pathways, the mutant phenotypes exhibited by 12Gso homozygous mice are very likely to be associated with novel genes, or known genes interacting in previously unknown pathways involved in somitogenesis, segmentation, or hematopoietic development. This dissertation work has been devoted to the characterization and development of this unique mouse model, including both phenotypic and genetic studies as well as physical mapping and molecular analysis of the translocation breakpoint sites. The long-range goals of this work are to identify the genes that are disrupted in 12Gso mice, and to use these genes as the foundations for understanding the molecular bases of inherited skeletal defects, reduced fitness, and leukemia in mammals.

CHAPTER 2: MATERIALS AND METHODS

Alizarin red staining of the skeleton

To study skeletal structure, animals were euthanized with an approved inhalant (chloroform or isoflurane) in order to not damage bone structure. Mice were skinned and all internal organs, including the palate and tongue, were removed. Using a 20-gauge needle, the brain was removed through the small hole at the base of the skull. For mice with a higher fat content, the entire skeleton was placed in acetone overnight, rinsed with distilled water, and placed in 2% potassium hydroxide (KOH) for the second night. If skeletons were not fat-laden, they were placed immediately into 2% KOH the first night; skeletons of young mice were treated in 1% KOH. The following day, the skeleton was removed from the KOH and incubated 16-48 hours in 1:100 dilution of 0.4% Alizarin Red in 2% KOH (or 1% for younger skeletons). After incubation, the skeleton was removed from the staining solution, rinsed with ethanol, and placed in a clearing solution of 20% benzyl alcohol, 40% glycerol, and 40% ethanol for two days or until sufficiently clear. Stained skeletons were stored indefinitely in 100% glycerol.

Combined staining of the skeleton and cartilage

After euthanasia, all skin and soft tissues were removed from the animal and the carcass was soaked overnight in 95% ethanol. The following day, the skeleton was incubated in a mixture of one volume glacial acetic acid, four volumes 95% ethanol, and 15 mg Alcian Blue (Sigma) per 50 mL final volume. The skeleton was left in this solution for 16 to 48 hours for cartilage staining. Once the cartilage staining solution was removed, the skeleton was rinsed in 95% ethanol for one hour, and then placed in 2% KOH for 6 to 24 hours. After incubation in 2% KOH, the skeleton was placed in 1% KOH supplemented with 75 µg/mL Alizarin Red S (Sigma) for 12 to 24 hours to stain the skeleton. Once the skeleton was sufficiently stained, it was cleared in a solution of

20% glycerol/1% KOH for one week, with the solution changed daily. After clearing, the stained skeleton was stored in a solution of 50% glycerol/ 50% ethanol for analysis.

Perfusion of the mouse for sectioning

Mice were euthanized as described for embryo isolation. The carcass was sterilized with 70% and was dissected to expose the heart. A small cut was made in the right atrium to vent the heart. A syringe attached to a 22 gauge needle was injected into the left ventricle to flush the animal with 1x PBS containing 0.5% sodium nitrate and 10 units/mL heparin, followed by fixation with 4% paraformaldehyde (freshly made, in 1xPBS). Complete fixation was indicated by stiffness in the extremities of the carcass. Tissues were examined for gross morphological abnormalities, then placed in 4% paraformaldehyde solution overnight for the final fixation step, followed by storage in a diethylpyrocarbonate (depc)-treated 70% ethanol solution.

Immunohistochemistry

All immunohistochemistry for this project was performed by Dr. Xiaochen Lu and Edward Wehri. Slides for immunohistochemistry (IHC) were dewaxed by first warming to approximately 60°C for 30 minutes, followed by three 5-minute xylene washes. The slides were then rehydrated with a series of ethanol washes to prepare for IHC. IHC was performed with the Histomouse-Plus kit (Zymed; South San Francisco, CA). Manufacturer instructions were followed, with two exceptions. Prior to blocking, the slides were incubated for 20 minutes in Target Retrieval Solution (DAKO; Carpinteria, CA) at 96°C. The slides were then removed from the heat and cooled to room temperature for 20 minutes. Following this step, the protocol proceeded with blocking reagents as indicated until the hybridization step, which was carried out at 4°C overnight.

Determination of carrier status by breeding tests

The genotype of translocation carriers was first determined by semi-sterility testing. According to the traditional method, males were bred to two females with a normal karyotype that were dissected late in gestation and the number of dead implants relative to normal, live fetuses was recorded. Carriers exhibited semi-sterility, or sired litters with +/+ females that resulted in at least 50% dead implants and/or resorption moles. Female carriers were tested by tabulation of resulting litter size when mated to +/+ males. Females from the translocation stock consistently producing litters of less than 5 offspring (compared to a normal average litter size of 10) were considered carriers. Males in LLNL were tested in a manner similar to that of females. Karyotypic analysis or FISH was used to confirm mouse genotypes.

Determination of heterozygosity by whole chromosome paints

Chromosomal painting was performed using FITC-conjugated mouse whole chromosome paints (Cambio, Cambridge, UK). Manufacturer protocol was followed through hybridization of the paints to the chromosomes. After incubation overnight at 42°C, the slides are washed twice for 5 minutes each in 50% formamide/2xSSC at 45°C, followed by two washes of 5 minutes each in 0.1xSSC at 45°C. Biotinylated anti-fluorescein (Vector) was diluted 1:200 and FITC-conjugated Avidin (Vector) was diluted at 1:100 in 4xSSC/1% Tween-20/1%BSA. Slides were incubated with the diluted anti-fluorescein for 15 – 20 minutes at 42°C. After incubation, slides were washed three times in 4xSSC/1% Tween-20 at 42°C for 5 minutes per wash. The slides were then incubated with diluted Avidin for 15 – 20 minutes at 42°C, and washed in 4xSSC/1% Tween-20 as previously described. Chromosomes were counterstained with 0.2 ng/mL DAPI and examined using DAPI/FITC excitation filters (Applied Imaging, Santa Clara, CA) on a Zeiss Axiophot microscope.

Isolation of embryos for expression studies and prenatal analysis

To generate embryos for usage in the expression studies, mating pairs of either C57BL/6J x C3H/HeJ (B6C3) hybrid mice (Jackson Laboratory) or pairs consisting of

12Gso heterozygous males and females (abbreviated 12Gso/+ X 12Gso/+) were caged together and examined daily for vaginal plugs. The genetic background of the 12Gso/+ mice was B6C3, C57BL/10J, or 129x1/SvJ. When a vaginal plug was found, the female was separated from the male. The developmental time was designated at 0.5 days post coitum (dpc) on this date. Embryos were isolated at 9.5, 10.5, 11.5, 12.5, 15.5, and 18.5 dpc. On the date designated for isolation, the pregnant female was euthanized and her abdominal cavity was opened for uterine access. The uterus was removed and placed in cold phosphate buffered saline (PBS), where individual embryos were separated. For extraction of the embryo from the embryo sac, the sacs were transferred individually to a fresh petri dish containing cold PBS. All embryos were dissected from the deciduum and yolk sac and transferred into freshly prepared cold 4% paraformaldehyde in PBS; each 12Gso/+ X 12Gso/+ offspring was housed separately until genotyped. The yolk sacs of 12Gso/+ X 12Gso/+ embryos were placed into a cell lysis solution [100 mM Tris, pH 8.5, 5 mM EDTA, 200 mM sodium chloride (NaCl), 0.2% sodium dodecyl sulfate (SDS), 100 mg/mL Proteinase K] overnight at 37°C in preparation for genotypic analysis. Tail biopsies of older embryos were taken for karyotype analysis (see below), and the musculature of the back was opened for complete fixation. Fixation occurred by rocking at 4°C; length of fixation was dependent on embryo age: 1-2 hours for E9.5, 2-4 hours for E10.5, 4-6 hours for E11.5, 6-8 hours for E12.5, and overnight for the remaining embryos. After fixation, embryos were dehydrated through a series of PBS and methanol washes at 4°C: PBS, PBS, 25% methanol, 50% methanol, 75% methanol, 100% methanol, 100% methanol, and then stored at -20°C in a fresh change of 100% methanol. Genomic DNA was isolated from the yolk sacs by isopropanol precipitation. The supernatant was removed, and the pellets were air-dried and resuspended in dH₂O. Embryos for sectioning were embedded in paraffin, sectioned to a thickness of 5 - 10 microns using a Leica motorized microtome and attached to slides (embedding and sectioning done by Dr. Xiaochen Lu).

Outcrosses to test effects of genetic background

To test the effects of different genetic backgrounds on expression of the skeletal phenotype in liveborn pups, 12Gso was crossed onto the mouse strains 129x1/SvJ and C57BL/10J (The Jackson Laboratory). These two strains were chosen because of documented evidence of higher penetrance of similar skeletal mutations, like *Dll3^{pu}* (Dunwoodie *et al.*, 1997), when maintained on these two backgrounds. The 12Gso translocation was transferred from B6C3 onto genetic backgrounds containing 129x1/SvJ and C57BL/10J alleles by mating females from these strains with male 12Gso heterozygotes, followed by backcrossing of the male offspring to the inbred mother for 4 generations. The new lines were then maintained by heterozygote intercrosses.

Complementation testing

To determine whether or not 12Gso was allelic to the known recessive mutation, *tk*, complementation crosses were carried out between 12Gso male heterozygotes and female *tk* homozygotes (TKDU/Dn; Jackson Laboratories). Litters were observed and recorded. Offspring were examined and genotyped for 12Gso carrier status by whole chromosome paints. Male offspring heterozygous for 12Gso were backcrossed to *tk/tk* females for two more generations, and litters were observed and recorded.

Amplification of microsatellite markers

Polymerase chain reaction (PCR) amplification of microsatellite markers was carried out in an MJ Research thermocycler using a 55°C annealing temperature. Standard PCR conditions were used (1x Taq polymerase buffer, 1 mM dNTP mix, 2.5 mM MgCl₂, 200 ng each forward and reverse primers, and 2 units Taq polymerase). Sequences for the microsatellite markers were found on the Whitehead Institute/Massachusetts Institute of Technology website (WI/MIT; <http://www-genome.wi.mit.edu/>).

Acrylamide gel electrophoresis to resolve amplified products

Precast 15% TBE acrylamide gels (Biorad, Hercules, CA) were used in a small vertical electrophoresis chamber (Biorad). Running buffer was 1xTBE. Samples were mixed with TAE dye to monitor the run. After the dye had migrated past the end of the gel, the gel was removed from its cast and stained for 45 minutes in 100 mg/mL ethidium bromide and photographed.

Estimation of the maximum map distance between the translocation and the mutation in 12Gso

To determine the expected frequency of a distribution of karyotypes at a given recombination fraction, r , the class of 12Gso/12Gso was calculated with $[(1-r)^2] M$, where M equals the number of observed 12Gso/12Gso. The class of 12Gso/+ was calculated with $2r(1-r) M$, and +/+ was calculated with $[(r)^2] M$. Because the values in the +/+ class were very small, an approximation is made and the two non-mutant classes (12Gso/+ and +/+) were combined to a single class (reducing degree of freedom to one).

The data from each class was then subjected to chi-squared analysis using the formula $(\text{observed} - \text{expected})^2 / \text{expected}$, and the corresponding values for each recombination fraction were combined and either rejected or not at the .01 and .05 significance levels (6.3 and 3.84, respectively).

Because of the small numbers of generated 12Gso/12Gso animals, estimations were made from all homozygotes produced, and not by individual background strains.

Probes used for genetic mapping

The probes selected for mapping were initially selected based on research publications and marker mapping data publicly available on the Mouse Genome Database (MGD; <http://www.informatics.jax.org/>) or the Human Genome Database (GDB; <http://www.gdb.org>). Clones for these markers were acquired as gifts or ordered from either American Type Culture Collection or the IMAGE Consortium. Plasmid inserts were generated either by restriction digest or by PCR from the vector promoter sequences. Later probes were generated by PCR from either genomic or cDNA

templates, and primer sequence was determined from publicly available sequences in the above-mentioned databases or in Genbank (www.ncbi.nlm.nih.org/). Besides the initial locations, the various publicly available radiation hybrid (RH) maps at The Jackson Laboratory (<http://www.informatics.jax.org/>) and the Whitehead Institute (<http://www.wi.mit.edu/>), Ensembl database (<http://www.ensembl.org/>), and Unigene and BLAST searches (<http://www.ncbi.nlm.nih.gov/>) were also used to find gene or microsatellite markers for placing on the genetic map. Probes were originally chosen to be spaced one to four centimorgans (cM) apart in the breakpoint region. Probes mapping within smaller intervals were later obtained as the breakpoint region narrowed and as the publicly available maps included more markers positioned within the determined breakpoint intervals.

Interspecific backcross mapping

The interspecific backcross (IB) mapping panel used for genetic mapping was generated by E.M. Rinchik and colleagues at ORNL [(C3Hf/RI-*Mgf*^{SL-ENURg} / + X *Mus spretus*) X C3Hf/RI], and was typed for markers specific to related projects (Doyle *et al.*, 1996; Stubbs *et al.*, 1996). The IB map currently contains more than 500 mapped markers, extending over all autosomes and the X chromosome. The initial interspecific cross used as a basis for these panels is a *Mus musculus* female and a *Mus spretus* male. Since F1 males are sterile, the F1 females of this outcross are backcrossed to a male of either of the parental genotypes. The panel in use in our laboratory uses the DNA from 160 of the F2 progeny from the crossing of the F1 female to a *Mus musculus* male; thus, all F2 would carry at least one *Mus musculus* allele and either *Mus musculus* or *Mus spretus* for the other allele at each locus. Genomic DNA was isolated from each of these F2 progeny, and aliquots from each sample were subjected to a series of enzymatic digests, one enzyme at a time, electrophoresed, and transferred to Southern blots to generate a series of panels specific to each enzyme (Silver, 1985; Avner *et al.*, 1988; Copeland and Jenkins, 1991). Probes were hybridized to Southern blots (Sambrook *et al.*, 1989) containing the F2 digests, and the *spretus* restriction fragment length variants (RFLVs) were recorded, as previously described (Avner *et al.*, 1988; Stubbs *et al.*,

1990). Gene linkage, order, and intergenic distances with associated standard errors were calculated according to standard methods (Silver, 1985) using Map Manager data analysis software (Manley, 1993).

Isolation of plasmid DNA

Plasmid DNA was isolated using either a standard alkaline lysis preparation (Sambrook *et al.*, 1989), or by using commercial kits and following the manufacturer's protocol. Commercial kits used for plasmid isolation were Wizard kits (Promega, Madison, WI), Mini-prep Spin kits (Qiagen, Valencia, CA), or Midi Prep kit (Qiagen).

Isolation of DNA inserts

Inserts were generated in one of four ways: enzymatic digestion of the plasmid, PCR of the plasmid, PCR with gene-specific primers from genomic DNA template, or PCR with gene-specific primers from a cDNA template.

Enzymatic digestion was dependent on the vector into which the insert had been ligated. Inserts were isolated from the vector using 1-2 units enzyme/ μ g DNA and the associated buffers and conditions as indicated by the enzyme manufacturer.

Most PCRs were carried out using DNA template (100 ng plasmid, 50 ng cDNA, 500 ng genomic DNA), manufacturer-recommended buffer dilution, 2 mM MgCl₂, 1 mM dNTPs, 200 ng primers, and 0.2 units Taq polymerase. PCRs were carried out on either a Perkin-Elmer 9600 thermocycler or an MJ Research thermocycler. Cycling times were usually 5 minutes at 94°C, 25-30 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1.5 minutes at 72°C, 5 minutes at 72°C, then maintained at 4°C. Adjustments were made to component concentrations and cycling conditions as necessary.

Once the digestion or PCR was completed, the sample was electrophoresed on a standard 1% agarose TPE gel, and the band containing the insert DNA was excised. Purification of the DNA from the agarose was performed using either overnight β -agarose digestion followed by phenol/chloroform extraction and ethanol precipitation, or by using commercially available kits (QiaQuick Gel Extraction Kit, Qiagen; Spin-X

columns). Concentration of the isolated insert was determined by electrophoresis with standard markers or by spectrophotometric analysis.

Isolation of bacterial artificial chromosome (BAC) clones

Initial BAC clones were isolated from the mBAC-6x library, generated from females of a 129 strain. Later clones were isolated from the RPCI-23 library, derived from C57BL/6 females.

Library screening was performed using EST clones or overgo oligonucleotides. Labeling and hybridization conditions for EST probes were carried out as previously described (Stubbs *et al.*, 1990). Stringency of the filter washes progressed to 0.5xSSC, 0.1% SDS, at 65°C for 30 minutes. Filters were then wrapped in plastic wrap for exposure on phosphorimager screens or X-OMAT autoradiographic film (Kodak).

Pairs of 24-mer oligonucleotides overlapping by 8 base pairs (overgos) were generated using a web-based program (OvergoMaker, <http://genome.wustl.edu/gsc/overgo/>). Prior to ^{32}P incorporation, 0.5 – 2.0 pmol of each overgo in the pair were denatured at 80°C and annealed at 37°C. Labeling of the denatured overgos was performed in 2% bovine serum albumin (BSA), 2 μL of buffer, 10 μCi ^{32}P -dATP, 10 μCi ^{32}P -dCTP, and 2 units Klenow polymerase. The BAC library filters were prehybridized at 60°C in hybridization solution (1 mM EDTA, 7% SDS, 0.5 M NaPi, pH 7.2, 1% BSA) for 1 – 4 hours. One-half volume of each TE^{-3} and sodium phosphate buffer was added to the labeled probe and the entire mixture was denatured at 80°C. Two-thirds of this mixture was added to the hybridization solution and applied to the BAC library filters for 1-3 days at 60°C. After hybridization, the filters were first washed in a solution of 1% sodium dodecyl sulfate (SDS) and 40 mM sodium phosphate buffer, followed by two washes in a solution of 2xSSC buffer and 1% SDS. Each wash proceeded for 20 minutes at 60°C. If necessary, the filters were washed in a fourth wash solution (0.5xSSC, 0.1% SDS) for 5 minutes at 60°C. After washing, the filters were rinsed quickly in hybridization solution and wrapped in plastic wrap. The BAC library filters were exposed to phosphorimager screens and scanned using a STORM 860 phosphorimager and associated software. Images were analyzed for positive clones using

a program (SCIL) developed by Laura Mascio-Kegelmeyer (LLNL) to run on a UNIX platform.

Secondary screening of the positive clones isolated from the BAC library was done mostly by robotics (LLNL mapping group) for large sample sets and by manual methods for small sample sets. Samples were rearranged for robotics by inoculation of positive clones into individual wells of a 96-well plate containing L-Broth supplemented with chloramphenicol and 10% glycerol (rearranges done by M. Groza, LLNL). The 96-well plates were incubated at 37°C overnight, then stamped onto an equivalent-sized L-Broth agar plate supplemented with chloramphenicol. The agar plates were grown at 37°C overnight and then transferred onto nitrocellulose. The nitrocellulose was incubated overnight at 37°C and nicked with ultraviolet light (stamping done by H. Badri, LLNL). Manual rescreening involved the restreaking of the positive BAC clones onto L-Broth agar plates and overnight incubation at 37°C. Single colonies from these plates were inoculated and grown in small-scale plasmid preparations. These plasmids were digested with *EcoRI* or *BamHI*, electrophoresed, and subjected to Southern blotting. The filters generated by either protocol were subjected to hybridization under conditions equivalent to that of the initial library screening.

Culture of mouse splenocytes and generation metaphase chromosome spreads

After isolation of a portion of the spleen from the selected mice, splenocytes were cultured in RPMI 1640 media supplemented with 20% heat-inactivated fetal bovine serum (JHR Biosciences, Woodland, CA), 730 mg/mL L-glutamine (Gibco BRL, Rockville, MD; 200mM/100x), 125 units/mL penicillin-G (Gibco BRL; 5000 iu/mL), 125 units/mL streptomycin sulfate (Gibco BRL; 5mg/mL), 90 mg/mL Concavalin-A (Sigma, 1mg/mL), and 10 mg/mL β -mercaptoethanol (Sigma, St. Louis, MO; 5 mg/mL). Cells were harvested after 48 hours and metaphase spreads were prepared. One hour before the harvest, cultures were treated with ethidium bromide (Sigma; 10 mg/mL). Thirty minutes later, 0.25 mg/mL colcemid (CIBA Pharmaceuticals, Suffern, NY) was added. Cultures were centrifuged at 900 rpm for 5 minutes, and the pellet was resuspended in a hypotonic solution of 75mM potassium chloride and incubated at 37°C

for 8 minutes. The cell pellet was again collected by centrifugation and fixed in Carnoy's (1 part glacial acetic acid to 3 parts methanol). Following four washes with the fixative, one to two drops of the cell suspension were dispensed on moist slides. The slides were air-dried and subsequently baked overnight at 62°C - 65°C, and stored under nitrogen at -20°C.

Direct fluorescent labeling of probes by enzymatic methods

Enzymatic labeling of probes for FISH was done using the Prime-It Fluor kit (Stratagene, La Jolla, CA), with slight modifications to manufacturer protocol. Approximately 50 – 200 ng of each DNA probe to be analyzed was labeled. After incorporation of fluorescein-conjugated dUTP, probes were precipitated with the addition of 10 µg sonicated salmon sperm DNA, 5 µg Mouse Cot-1 DNA, 5 µL 3 M Sodium Acetate pH 5.2 and 150 µL absolute ethanol. Slide denaturation, probe hybridization, and slide washing steps proceeded as per manufacturer protocol. Slides were counterstained with the propidium iodide supplied with the kit, or with Vectasheid plus propidium iodide (Vector Laboratories) and protected with cover glass. Slides were examined using a Zeiss Axiophot microscope equipped with a Cohu CCD camera. Image capturing and analysis was done with hardware/software from Perceptive Scientific Instruments (now Applied Imaging, Santa Clara, CA).

FISH using biotinylated probes generated by degenerate oligonucleotide-primed PCR

Probes were generated by degenerate oligonucleotide-primed PCR (DOP-PCR; Telenius *et al.*, 1992a, 1992b), using biotin-16-dUTP for the labeled nucleotide. The procedure includes two rounds of PCR, the first of which uses the degenerate primers to reduce the size of the DNA to be labeled to between 300 and 600 base pairs. The second PCR incorporates the labeled dUTP into these small fragments. Mouse Cot-1 and a hybridization solution of 50% formamide, 10% dextran sulfate, and 2xSSC were added to the probes, which were subsequently denatured for 10 minutes at 84°C and preannealed for 60 - 90 minutes at 37°C. Slides were treated with RNase, dehydrated in a series of ethanol washes, denatured at 71°C in 70% formamide, 2xSSC buffer, and

dehydrated again. Hybridizations were carried out overnight at 42°C. Following hybridization, stringent washes were performed at 45°C: two washes for 10 minutes each containing 50% formamide and 2xSSC buffer, one wash for 5 minutes in 2xSSC buffer, and one wash for 5 minutes in PN buffer (0.1 M Na₂HPO₄, 0.05% NP40, pH 8.0). Slides were then preblocked with PNM for 5 minutes. All secondary detection reagents (avidin-fluorescein, biotinylated anti-avidin) were diluted in PNM (PN, 5% dry milk, 1 mg/mL sodium azide). Diluted avidin-fluorescein (Vector, Burlingame, CA) was applied for 30 minutes at room temperature. Slides were then washed three times for 3 minutes each in PN buffer, then again preblocked with PNM for 5 minutes. Biotinylated anti-avidin (Vector) was then applied for 30 minutes at room temperature. Slides were again washed and preblocked, and the avidin-fluorescein step was repeated. After the final PN wash, slides were counterstained with DAPI and examined using a Zeiss Axiophot microscope. Imaging was done with hardware/software from Perceptive Scientific Instruments (now Applied Imaging, Santa Clara, CA).

PCR analysis of the 12Gso breakpoint region

The sequence of the *Abca1* BAC contig and the ordered and oriented sequence for RPCI-23-353G1 were submitted to the Repeat Masker database (<http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker>) to block repetitive sections in the sequence. Primer sets of 28-mer to 30-mer oligonucleotides specific to each the interspersed non-repetitive regions were generated and used to amplify the non-repetitive regions, followed by a second round of PCR to span repetitive regions (see Appendix). Presence, absence, or size alteration of a 12Gso fragment was noted for each PCR. Touchdown PCRs were carried out at a 60°C annealing temperature, with an extended 5 min extension at 68°C for 30 cycles. Samples were electrophoresed on a 1.5% agarose TAE gel by standard protocol.

Generation and screening of comparative genomic Southern hybridizations

The amplified regions of non-repetitive DNA generated in the PCR analysis were pooled according to 10 kb spans, either directly from the PCR or after gel-isolation. The

pools were ^{32}P -labeled by standard protocol for hybridization onto genomic Southern blots. Genomic Southern blots were generated by the digestion of 12Gso homozygote genomic DNA and the genomic DNA of various background strains (C3Hf/R1, 101/R1, C57BL/10Ei, SEC/R1) with a variety of enzymes. The digests were electrophoresed on a 0.8% agarose TPE gel, and subjected to Southern blotting under standard conditions.

Reverse transcriptase PCR (RT-PCR)

The cDNA for use in RT-PCR was generated from total RNA isolated from mouse liver. All reagents used to generate the cDNA were from the Amersham cDNA Synthesis kit (Amersham), according to manufacturer instructions for first-strand cDNA synthesis. All PCR was carried out under standard conditions, using exon-crossing gene-specific primer sets. RT-PCRs of +/+ and 12Gso/12Gso tissues were done in parallel.

Isolation of mouse tissues and extraction of total RNA from mouse tissues

Mice were euthanized by cervical dislocation and sterilized with ethanol. The tissues were removed and snap frozen in liquid nitrogen for storage. For extraction of total RNA, the Trizol (Invitrogen) method was used. Manufacturer protocol was followed, including the spin to remove excess cellular debris and fat. Prior to precipitation, however, a second phenol extraction was performed to further purify. After the precipitation, the RNA was treated with DNaseI for 15 minutes at 37°C. The reaction was stopped with 0.5 M EDTA, and a final phenol extraction and precipitation were used to isolate the purified RNA species.

Preparation of Poly-A enriched mRNA

Poly-A enriched mRNA was generated using the Message Maker Kit (Invitrogen). Manufacturer protocol was followed.

Expression analysis using Northern blotting methods

RNA samples to be analyzed were denatured in a solution of 0.5x MOPS buffer (0.2M MOPS, 50 mM sodium acetate, 5 mM EDTA), 17.5% formaldehyde, and 50% formamide and labeled with ethidium bromide. Samples were electrophoresed on a denaturing 1% agarose gel (1xMOPS buffer, 20% formaldehyde) in 1xMOPS buffer. After electrophoresis, the gel was subjected to standard Northern blotting methods (Sambrook *et al.*, 1989).

Microarray analysis

Bone total RNA samples from a 12Gso/12Gso female and an age-matched B6C3 female were submitted to Gene Expression Resource (Department of Medical Pathology, University of California, Davis) for comparative microarray analysis on Affymetrix gene chip MG-U74Av2. The 12Gso mouse was the offspring of a 12Gso/+ male on a B6C3 background and a F4 C57BL/10J 12Gso/+ female.

Culture of mouse fibroblasts and corresponding metaphase chromosome spreads

Biopsies of the mouse tails were dissected and digested overnight in 2000 units/mL collagenase II diluted in prepared fibroblast culture medium (DMEM/F-12 (Gibco BRL, Rockville, MD) supplemented with 25% heat-inactivated fetal bovine serum (JHR Biosciences, Woodland, CA), 730 mg/mL L-glutamine (Gibco BRL; 200mM/100x), 125 units/mL penicillin-G (Gibco BRL; 5000 iu/mL), 125 units/mL streptomycin sulfate (Gibco BRL; 5mg/mL) in a 37°C humidified CO₂ incubator. The remaining fibroblasts were pelleted by centrifugation and resuspended in prepared fibroblast culture medium. The resuspended fibroblasts were transferred into to a 75 cm² flask that was coated with gelatin (Sigma). Cells were grown in a 37°C humidified CO₂ incubator, and media was replenished every 48 hours. When the cells grew to approximately 70% confluency, 0.5 mg/mL colcemid was added and the flask was incubated an additional 4 – 6 hours. The cellular layer was then washed twice with sterile PBS (Gibco BRL) and attached cells were released with trypsin/EDTA solution (Gibco BRL). The cells were centrifuged at 1100 rpm for 5 minutes, and the pellet was

resuspended in a hypotonic solution of 75mM potassium chloride and incubated at 37°C for 20 minutes. The cell pellet was again collected by centrifugation, then fixed in Carnoy's (1 part glacial acetic acid to 3 parts methanol). After 4 washes of fixative, 2 – 3 drops of the final cell suspension were dispensed onto moist slides. Slides were allowed to air-dry overnight and were stored at -20°C in vacuum-sealed bags.

Breakpoint fragment isolation and sequencing

Generation of breakpoint-containing fragments was done using the Universal Genome Walker kit (Clontech); reactions of 12Gso/12Gso and C3Hf/RI were run in parallel according to manufacturer instructions. Final products were PCR-amplified under standard conditions using the primer set generating the fragments [*Abca1*d-R (see Appendix) and the AP1 primer from the kit]. Fragments were prepared for sequencing by Dye Terminator reaction (Applied Biosystems) with 30 ng of each sequencing primer [*Abca1*dnested-R (see Appendix) and the AP2 primer from the Universal Genome Walker kit]. Sequencing reactions were run on an ABI 377 (Applied Biosystems) and analyzed by BLAST analysis (NCBI; <http://www.ncbi.nlm.nih.gov/>).

CHAPTER 3: RESULTS

Characterization of defects in the skeleton and cartilage of 12Gso mutant mice

The homozygous 12Gso mutant mice can be visually identified at one day of postnatal life by their shortened body length, kyphoscoliosis, hip dysplasia, and often, a reduced or kinked tail (Figure 2). Examinations of skeletal preparations of surviving adult mutants indicate the presence of bifid ribs, fused, jumbled, and hemivertebrae, as well as block vertebrae in the tail (Figure 5). Malformations in the tarsals, cranium, and sternum were also present in those mice on a BLH background, but these types of bone malformations were not seen after the mutation was transferred onto different genetic backgrounds in the move to a clean facility at LLNL (e.g., B6C3, C57BL/10J, 129x1/SvJ; The Jackson Laboratory). Despite these skeletal malformations, no apparent effect is seen in the majority of the appendicular skeleton, for the remainder of the limbs appear normal.

Skeletal staining was later done using a combination of Alizarin red and Alcian blue to determine if cartilage irregularities were also present. The skeleton of an affected 12Gso homozygote was stained at one-day postnatal, showing not only the bifid ribs and bone fusions, but also an altered morphology along the vertebral column. The vertebrae appeared to have developed in a slightly more regular fashion than those of the skeletons stained by Alizarin red alone, but there was also incomplete closure along the backbone, indicating multiple ossification centers, and improper attachment of the ribs in the thoracic vertebral region (Figure 6).

Histopathology

As part of the phenotypic characterization, pathologic examinations of all major organ systems were conducted for adult and weanling animals. All organs examined from the homozygous mutant animals, including heart, lungs, liver, kidney, stomach and intestines, appeared normal on gross examination (X. Lu, pers. comm.). Sections taken of E17.5 homozygous mice and normal littermates were examined by standard hematoxylin and eosin (H&E) stains. Although staining revealed the relative persistence

Figure 5. Skeletal anomalies of the adult 12Gso homozygous mouse. Panels **A.**, **C.**, **E.**, and **G.** are 12Gso/12Gso; panels **B.**, **D.**, **F.**, and **H.** are +/+. Aberrations are seen in the axial skeleton, but the appendicular skeleton (e.g., the tarsals) appears normal. **A.** 12Gso/12Gso ribs. Note the presence of a bifid rib (arrow) and the crowded attachment of the ribs to the vertebral column (top of image). **B.** +/+ mouse ribs. **C.** 12Gso/12Gso thoracic vertebrae. The vertebrae are crowded and block-like, and incomplete closure of the vertebral column is evident (demarcated by open arrows). **D.** +/+ thoracic vertebrae. **E.** 12Gso/12Gso lumbar and sacral vertebrae. Arrows indicate the extreme fusion of the vertebrae in this region, also evident by the lack of space between the vertebral structures. **F.** +/+ lumbar and sacral vertebrae. **G.** 12Gso/12Gso tarsals. No apparent defect is evident. **H.** Normal tarsals. **I.** 12Gso/12Gso (top) and +/+ (bottom) skeletons. The 12Gso/12Gso skeleton is shorter than the +/+ skeleton, and the 12Gso/12Gso tail is extended at an odd angle.

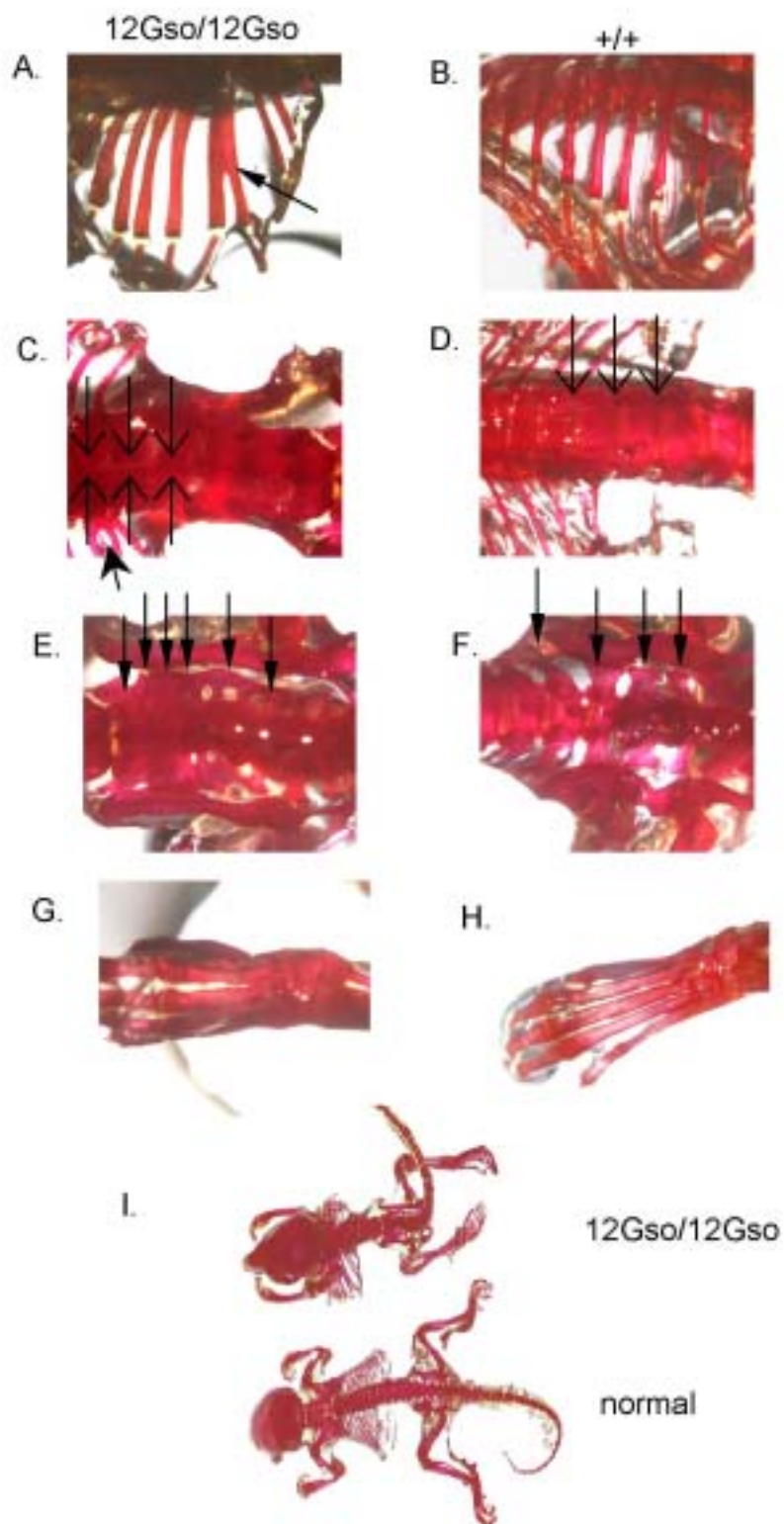
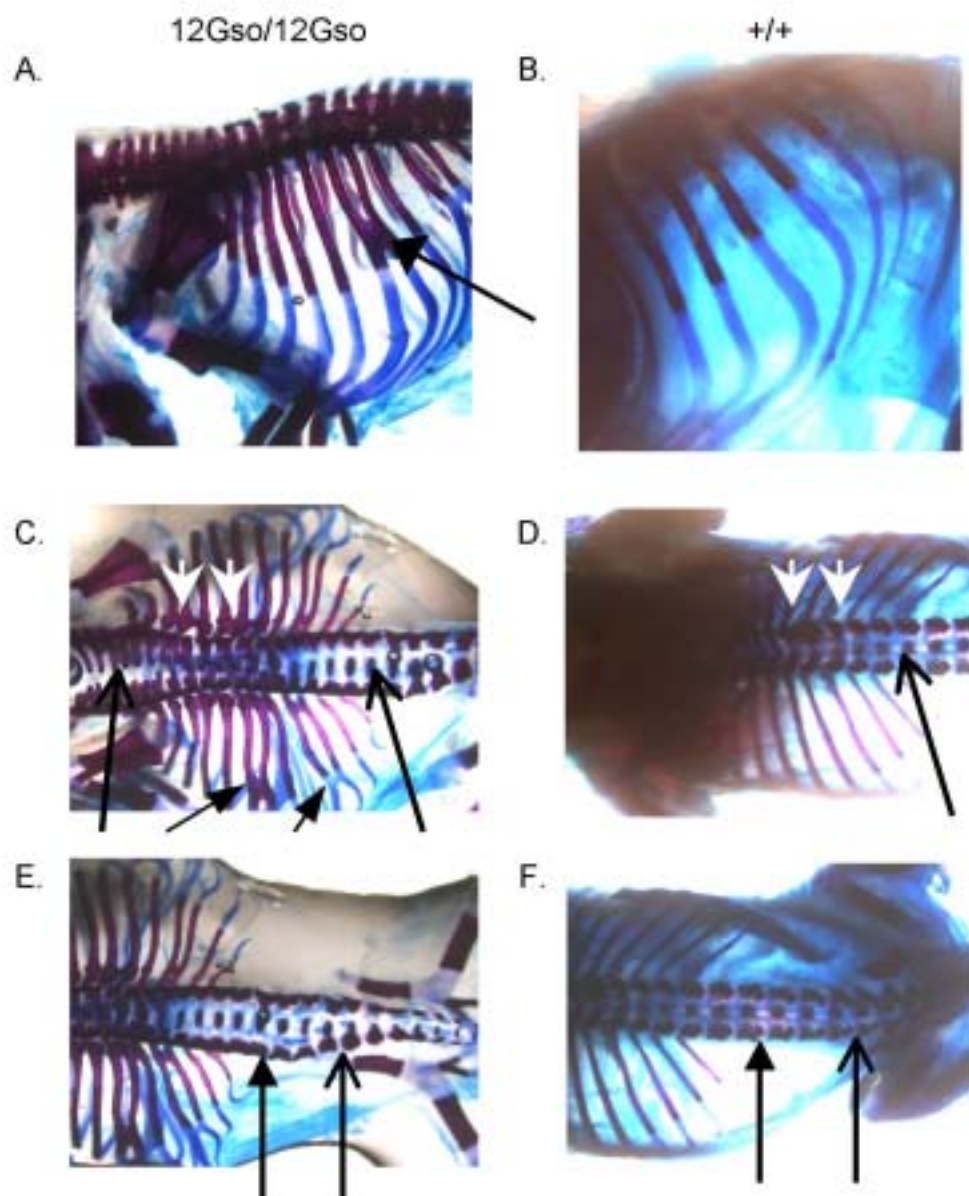


Figure 6. Skeletal anomalies are present in E18.5 12Gso/12Gso mice. 5x magnification. (A.) The 12Gso/12Gso embryo has bifid ribs (arrow), and the remaining ribs are flimsy, crowded, and malformed, as compared to the straight and evenly spaced ribs of the +/+ sibling (B.). The vertebral column of the 12Gso/12Gso embryo (C.) has delayed closure in the cervical and lumbar regions (open arrows). Rib fusions are evident near the sites of attachment (e.g., white arrows) and as bifid fusions (closed arrows). (D.) The +/+ sibling maintains an even closure of the vertebral column (open arrow) and consistent amounts of ossification at the sites of rib attachment (white arrows). (E.) Again, highlighting the improper closure of the vertebral column in the 12Gso/12Gso lumbar region (solid arrow), and the presence of thickened prevertebrae in the narrowed sacral region (open arrow). (F.) The +/+ embryo, however, maintains an even closure along the vertebral column (closed arrow) and consistent thickness in the prevertebrae (open arrow).



of a metameric pattern in the prevertebrae of 12Gso homozygotes, this tissue was developmentally delayed and presented as abnormally shaped and poorly spaced segments. These segments were adjacent to the spinal cord, which not only appeared thickened in the 12Gso homozygote, but also lacked the characteristic arch presented in the normal embryo. The chest cavity in the 12Gso/12Gso embryo was also slightly larger in comparison to the normal mouse, possibly because of the decreased thickness of the sternum in the mutant (Figure 7). Magnified regions of the cervical and thoracic vertebrae verified the fusion of prevertebral segments and the misalignment of the vertebrae (Figure 7). Bone sections from a 6-week old 12Gso/12Gso mouse also exhibited a dramatic decrease in the intervertebral space, resulting in a compression of the nucleus pulposus (Figure 8). The bone is also being eroded internally by excessive osteoclast activity in various parts of the skeleton, possibly through the same mechanism causing unusual proliferations of nucleated cells within the marrow (Figure 8).

Examination of bone sections from several 12Gso/12Gso mice on BLH, B6C3, and C57BL/10J background strains revealed a proliferation of nucleated cells in the bone marrow of homozygotes, indicative of the type of hyperplasia that occurs in leukemia (Figure 9). The magnitude of this affected growth and the clustering of the cells into diffuse patches within the marrow suggested that this disorder may contribute to the early death of 12Gso homozygous mice (X. Lu, pers. comm.). The +/+ bone sections show many other cell types, such as granulocytes, neutrophils, and megakaryocytes, whereas the 12Gso/12Gso sections do not present these cell types. It may be, however, that the massive expansion of the lymphocytic cell type is preventing visual observation of the other cell types. Immunophenotyping of the bone marrow was attempted as a means to determine the form of leukemia present. Antigens against B-cells and T cells bound to cell populations in both the 12Gso/12Gso and +/+ bone marrow sections (X. Lu, pers. comm.; data not shown), but accurate quantitative analysis from these slides is not possible without flow sorting the cell types. For this reason, all that can be conclusively said about the leukemia present in 12Gso homozygotes is that it is

Figure 7. Aberrations are present in the skeleton of E17.5 12Gso/12Gso embryos.

Panels **A.** and **C.** are 12Gso/12Gso, and panels **B.** and **D.** are +/+. All sectioned embryos pictured are stained with hematoxylin and eosin. **A.** and **B.** 10x magnification. The 12Gso/12Gso embryo (**A.**) does not have the characteristic arch in either the spinal cord (open arrow) or the prevertebrae (closed arrow) that is present in the normal embryo (**B.**). **C.** and **D.** 40x magnification, second cervical vertebra to the fourth thoracic vertebra. The 12Gso/12Gso embryo (**C.**) has fusions in the prevertebrae (closed arrows) that contribute to the misalignment of the vertebrae (indicated by a dashed line, connecting the dorsal portion of the vertebrae to the ventral portion). The vertebrae retain a metameric pattern, but the size and shape of the structures are not consistent (e.g., c4 and c5) as in the wild type mouse (**D.**). These alterations in alignment, size, and shape in the 12Gso/12Gso embryo have also constricted the spinal column in the thoracic region (open arrow). Abbreviations: sc, spinal column; c2, second cervical vertebra; c4, fourth cervical vertebra, c5, fifth cervical vertebra; t4, fourth thoracic vertebra.

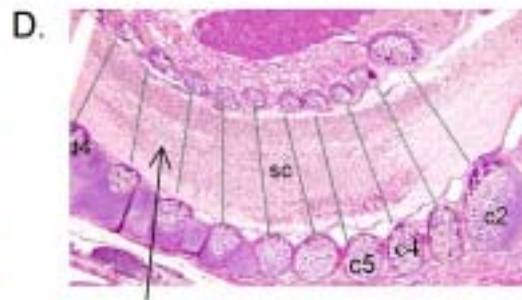
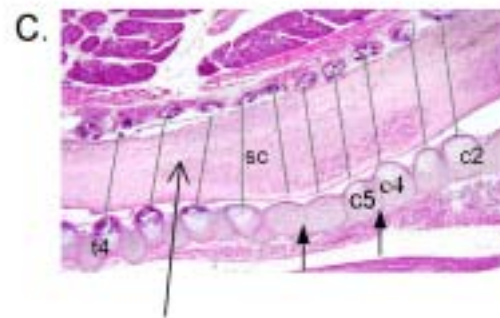
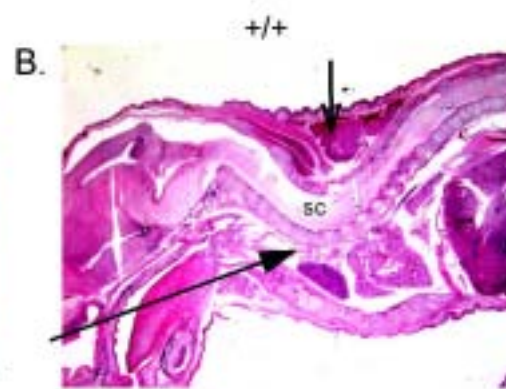
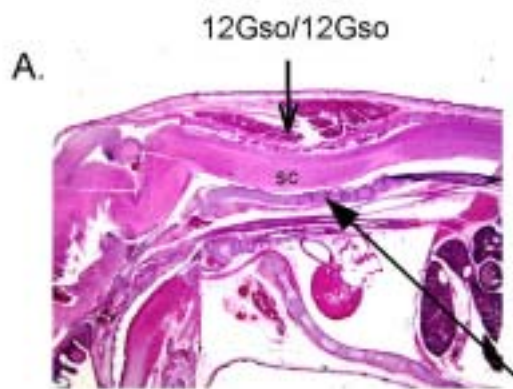


Figure 8. Sections of 6-week old 12Gso/12Gso mice indicate abnormal bone and marrow structure. Abbreviations: L4, 4th lumbar vertebra; L5, 5th lumbar vertebra, ivs, intervertebral space. 40x magnification of sections stained with hematoxylin and eosin. **(A.)** Sections through the 4th and 5th lumbar vertebrae of the 12Gso/12Gso mouse indicate a drastic decrease in the intervertebral space, causing a constriction of the nucleus pulposus (closed arrow). The cellular distribution of the bone marrow is also abnormal (open arrow); nucleated cells (purple) are proliferating in dense dispersed patches, with reduced interspersed levels of blood cells (pink). In the +/+ mouse **(B.)**, however, the intervertebral space and nucleus pulposus are much larger than in the 12Gso/12Gso (closed arrow), and a normal distribution of cells is seen in the bone marrow (open arrow). A section through the end of the tibia in 12Gso/12Gso **(C.)** shows the thinned walls of the bone (closed arrows), being eroded by the marrow components (arrowheads). The soft bone at the knee joint is extensively eroded (tissue flap; teal arrow), and an increase in proliferating cells from the marrow at the joint can be seen (green arrow). Again, the marrow is proliferating in dense patches, leaving large empty areas in the marrow (open arrow). The +/+ mouse **(D.)** has much thicker bone walls (closed arrows), and no erosion of the inner surfaces (arrowheads). The marrow is distributed normally (open arrow), and only a small concentration of marrow is present at the knee joint (teal arrow, green arrow).

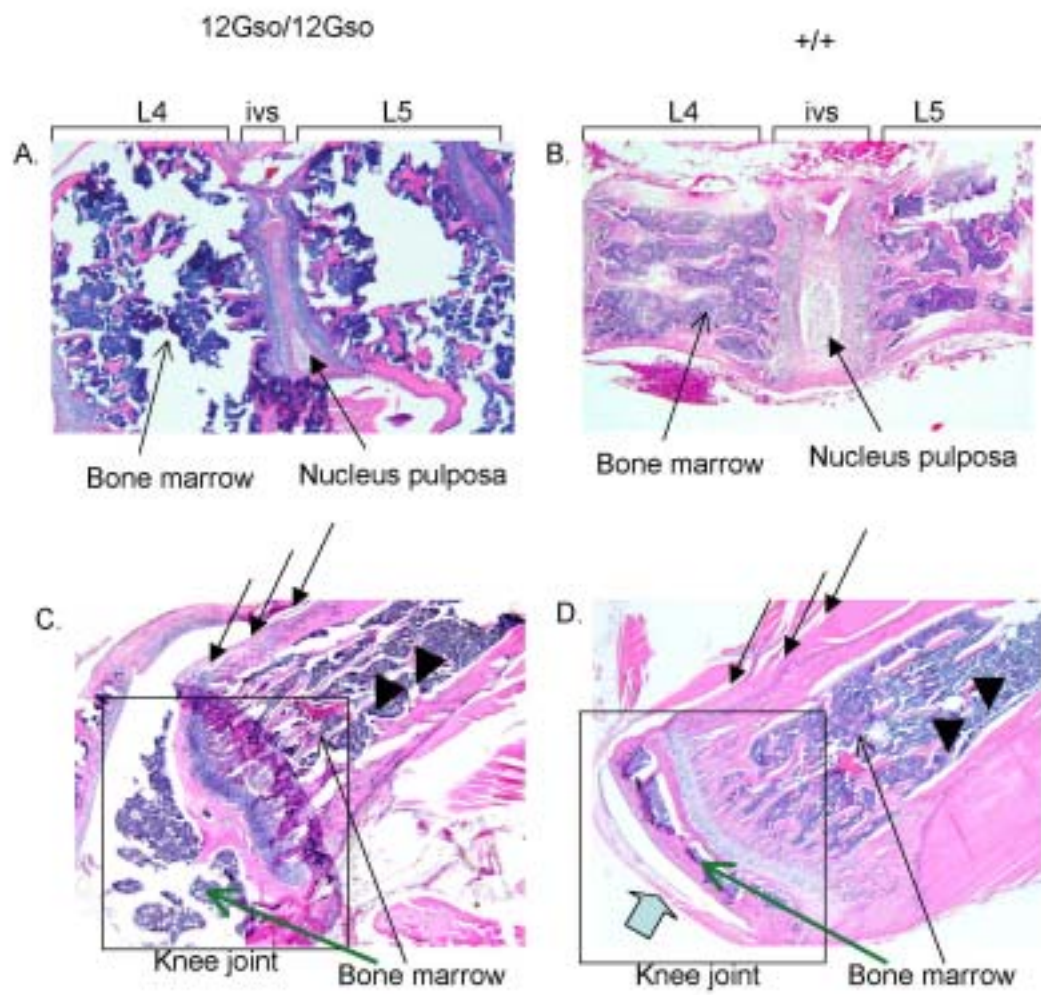
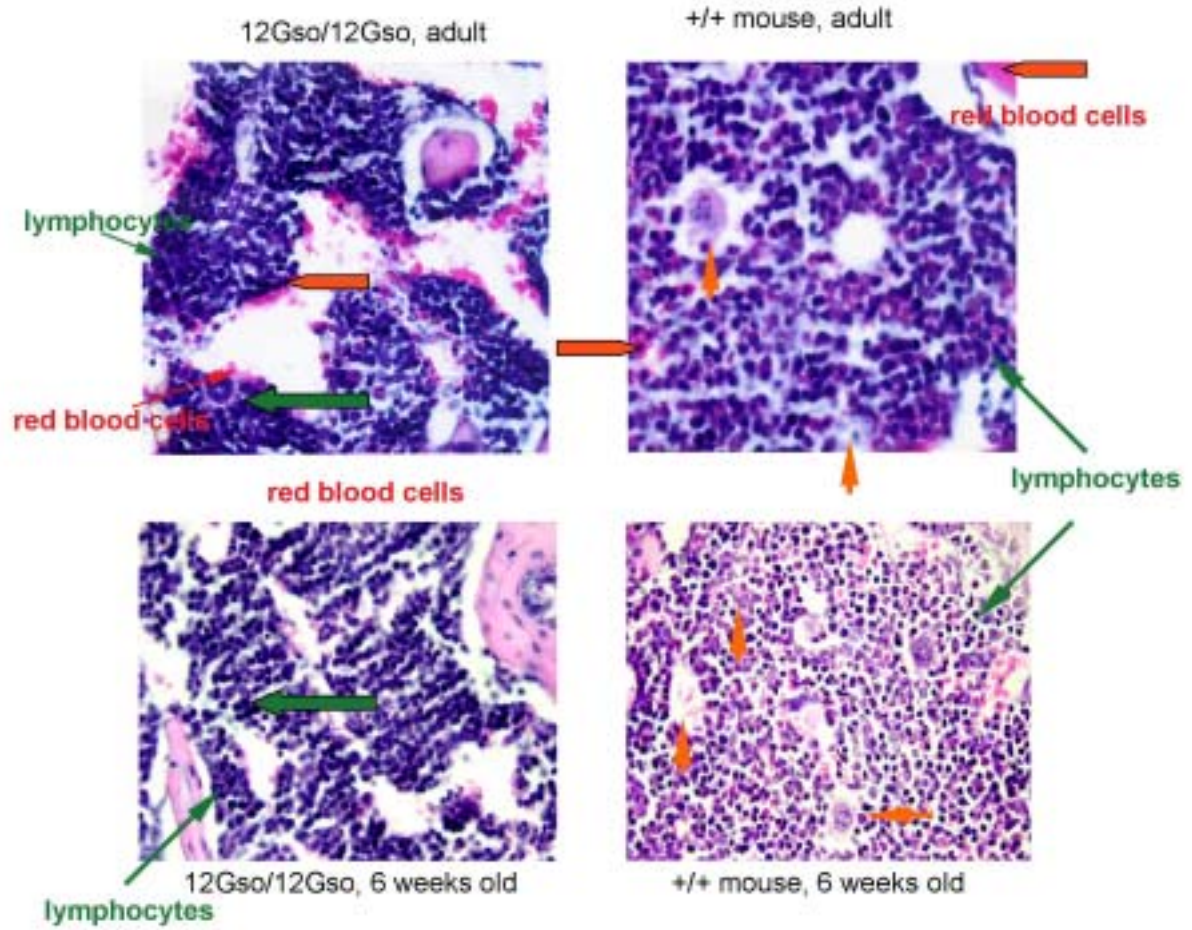


Figure 9. The bone marrow of 12Gso homozygous mice shows a diffuse pattern of lymphocyte proliferation. 400x magnification of bone marrow sections stained with hematoxylin and eosin. The bone marrow in the 12Gso/12Gso adult mouse **(A.)** shows dense, patchy proliferation of nucleated cells (purple, indicated by green arrow) of lymphocytic origin, and a decrease in the blood cell population (pink cells, indicated by red arrow), leading to large open spaces in the bone marrow. The +/+ adult mouse **(B.)** shows a normal distribution of lymphocytes (purple cells, green arrow), other hematopoietic cell types (orange arrows), and an adequate supply of blood cells (pink cells, red arrow). **(C)** The bone marrow in the 12Gso/12Gso 6 week old mouse also shows hyperproliferation of lymphocytes (green arrow), and the bone marrow of the +/+ 6 week old mouse **(D)** displays a wide range of hematopoietic cell types (light purple and pink stains; orange arrows).



lymphocytic, and the proliferation patterns of these lymphocytes in the bone marrow imply a chronic form of B-cell leukemia.

Preliminary studies of the effects of genetic background on survival and expression of the skeletal phenotype

To determine whether or not homozygous mutants were being born live but without discernable phenotype, the karyotypes of liveborn pups from heterozygote matings on several backgrounds were tracked. The results of the genotyping experiments contradicted the hypothesis that homozygous mice were being born live without skeletal malformations. 177 liveborn animals from 12Gso heterozygote crosses on various genetic backgrounds were karyotyped; of these, 14 were 12Gso/12Gso (Table 2; 36 animals from ORNL not included). The first background strains to be tested were BLH, C3Hf/Rl, and B6C3, on which 12Gso/12Gso production was suppressed (Table 2). Crosses between translocation carriers produce many embryos with an unbalanced complement. The number of either mutants or wild types from these crosses should equal 1/6 of liveborn progeny, much more than the observed numbers for 12Gso on BLH, C3Hf/Rl, or B6C3.

To further study the reasons for the lack of surviving mutants on the B6C3 genetic background, a series of embryos in developmental stages from E9.5 to E18.5 (inclusive) were isolated from heterozygote crosses. Though none of the embryos could be conclusively identified by gross observation as a mutant, a few appeared developmentally delayed in the earlier stages or runted at later stages (8 embryos of 46 embryos isolated). Preliminary karyotyping of embryos at later stages of E17.5 and E18.5 indicated that an expected number of mutants were developing to this stage (4 embryos karyotyped as 12Gso/12Gso out of 14 total karyotyped offspring), suggesting that the homozygous animals die during the final stages of development or perhaps just after birth.

To test the possibility of genetic background effects, and for use in possibly increasing the number of liveborn pups expressing the mutant phenotype, 12Gso was crossed onto two very different inbred strains, C57BL/10J and 129x1/SvJ (The Jackson

Table 2. Karyotype analysis of liveborn offspring from 12Gso/+ X 12Gso/+ crosses

	12Gso/12Gso	12Gso/+	+/+	Total
C3Hf/R1	0	2	3	5
B6C3	4	60	14	78
129x1/SvJ	1	11	2	14
C57BL/10J	5	27	12	44
Totals	10	100	31	141

Laboratory). After only a few generations, the number of liveborn animals with skeletal malformations (and a 12Gso/12Gso karyotype) increased, most notably on the C57BL/10J background, on which the production of homozygotes is approximate to expected ratios (Table 2). These data are very preliminary, but suggest that alleles shared by C3Hf/Rl and [C3H/HeJ X C57BL/6J] hybrids are associated with increased perinatal mortality of 12Gso mutant mice, whereas alleles carried by C57BL/10J inbred line increase viability of the mutant animals.

Concordance between skeletal phenotype and inheritance of the 12Gso translocation

While collecting data for large breeding experiments at ORNL, all offspring from 12Gso translocation carrier crosses that displayed aberrant phenotypes were subjected to cytological analysis. All 4 liveborn animals (from 36 liveborn offspring) clearly displaying skeletal defects, ranging from obviously shortened spine to kinky tails, were genotyped as homozygotes, while littermates or the animals expressing other potential phenotypes were genotyped as heterozygotes or wild types (K.T. Cain, W.M. Generoso, N.L.A. Cachiero, pers. comm.). Additional breeding studies conducted at LLNL have also identified 10 liveborn 12Gso mice with the skeletal phenotype (10 mutants/141 total karyotyped from carrier crosses), and all of these were found to be homozygous in FISH or chromosome painting experiments. Analysis of breeding records from 12Gso/+ X 12Gso/+ matings indicated that the birth rate of live homozygotes on the C3H, B6C3, and 129x1/SvJ genetic backgrounds was lower than predicted, though production of heterozygote and wild type offspring was normal (Table 2).

Estimation of the maximum map distance between the translocation and the mutation in 12Gso

To estimate maximum map distance, an iterative chi-squared analysis was done on data from records of karyotyped offspring from 12Gso heterozygote crosses. 14 karyotyped 12Gso/12Gso mice have been generated (M). Because of the small number of homozygotes produced, relatively large regions of map distance were expected. Data indicated that the hypothesis of the maximum distance between the mutation and the

translocation could be rejected for distances of 11 cM and 17 cM, but not rejected at 10 cM and 16 cM, at the .05 and .01 levels, respectively. It can then be said with 95% certainty that the maximum possible distance between the translocation and the mutation is less than 11 cM, and with 99% certainty that the distance is less than 17 cM.

Testing genetic background of the Mmu4 breakpoint region and surrounding DNA.

As suspected by analysis of results generated from genomic Southern blots hybridized with probes within the 12Gso critical region (data not shown), microsatellite markers mapping closest to the chromosome 4 breakpoint in 12Gso indicated that the DNA surrounding the translocation breakpoint was of the C3Hf/R1 origin. This would indicate that the mutagenic lesion initially occurred on a C3Hf/R1 chromosome 4 (Table 3). Because most of the generations of 12Gso were maintained on stocks that contained C3Hf/R1 genetic material, it was necessary to extend the microsatellite analysis further from the breakpoint to determine if there was any 101/R1 DNA present at more distant loci (Table 3). Because DNA contributed by the mutated founder male and animals from subsequent crosses could not be distinguished, the size of the interval remaining from the originally mutagenized chromosome could not be measured in recent representatives of the 12Gso stock. Loci surrounding the chromosome 9 breakpoint were not analyzed because of the lack of specific breakpoint location on that chromosome at the time of these studies.

Genetic mapping: locating genes on Mmu4 and Mmu9

The original sizes of the breakpoint regions, based on chromosomal band size, were approximately 10 cM for either chromosome. The original Mmu 4 region was homologous to portions of human 9 and the original Mmu 9 region was homologous to portions of human 3q, 6q, 11q, and 15q. Once these breakpoint regions were determined, genetic mapping of the breakpoint region markers was done using IB mapping panels. Mapping was begun on chromosome 4, establishing the placement of genetic markers at a depth of approximately 1 marker every 1-2 cM within the region of

Table 3. Microsatellite and gene markers used to characterize the 12Gso Mmu4 breakpoint region.

Marker	JAX position (cM from centromere)	MIT position (cM from centromere)	Ensemble position (Mb from centromere)	12Gso DNA at this locus?
D4Mit99	5.0	8.7	11.408	i
D4Mit18	5.2	8.7	-	C3H
D4Mit107	12.1	16.4	41.680	C3H
D4Mit238	17.9	21.9	-	C3H
D4Mit229	20.8	23.0	-	C3H
D4Mit112	20.8	24.0	55.309	C3H
D4Mit271	20.8	24.0	-	C3H
D4Mit88	20.8	24.0	58.671	C3H
D4Mit110	21.9	24.0	59.545	C3H
Nipsnap4	23.1	-	59.686-59.697	C3H
Nipsnap3	23.1	-	59.709-59.719	C3H
<i>Abca1</i>	23.1	-	59.731-59.901	C3H
D4Mit272	21.9	25.1	60.091	i
*D4Mit184	21.9	25.1	60.533	C3H
*D4Mit163	21.9	25.1	-	C3H
*D4Mit240	21.9	25.1	-	C3H
D4Mit151	28.6	29.5	66.983	C3H
D4Mit82	32.5	31.7	72.150	i
D4Mit178	35.5	30.6	75.527	C3H
D4Mit348	40.0	33.9	-	C3H
D4Mit116	40.0	36.1	95.928	C3H

i = impossible to determine; C3Hf/Rl and 101/Rl alleles are the same size

- = marker not in database

* = map order unclear

interest (Figure 10). Additional mapping on Mmu 9 also verified the genetic interval of various markers within the breakpoint region on this chromosome as well (Figure 11).

Mapping the Mmu 4 translocation breakpoint using FISH and the available public databases

Early rounds of FISH isolated the breakpoint between the markers Shb and Ambp (Figure 12). FISH experiments with genetic markers gradually narrowed the breakpoint region to one in which we had no probes available at the time, between flanking markers Baat and Lv. Further FISH experiments were greatly facilitated by the plethora of information that has recently become publicly available. Not only have sequences been submitted, but also maps that contain probe contigs and sequence annotation. These rapidly developing tools can only accelerate the discovery of new mutations within our colony. Prescreened BACs from the BAC/PAC database (<http://www.chori.org/bacpac/home.htm>) were then used to narrow the breakpoint interval to a region flanked by Baat and the microsatellite marker D4Mit272.

With the advent of databases such as the FPC database and the Jackson Laboratory and the Whitehead Institute/Massachusetts Institute for Technology radiation RH maps, estimation of probes lying between the markers of Baat and D4Mit272 was facilitated. The size of this region, as estimated from these markers' positions on the publicly available RH maps, and the approximated kilobase (kb) to centiray (cR) distance on Mmu 4 (MIT database), was approximately 4 Mb. Database searches indicated that two of the BACs contained within the FPC contig incorporating the *Baat* marker were positive for D4Mit215, and some distance proximal to this BAC in the same contig, other BACs were positive for D4Mit88. This microsatellite marker was shown by both RH maps to lie between D4Mit215 and D4Mit272, a good indication that the breakpoint could be near (Figure 13). FISH experiments with numerous BACs within this interval narrowed the estimated region to approximately 1 Mb, within the region that lay between two FPC contigs: the proximal contig containing *Baat*,

Figure 10. Genetic map of mouse chromosome 4. Numbers to the left are the distances between the markers in centimorgans; marker placement is not to scale. The chromosomal band that contains the 12Gso breakpoint (Mmu 4B3) is denoted, and extends from *Coll5a1* to *Txn*. Markers listed in green were used to isolate BAC clones.

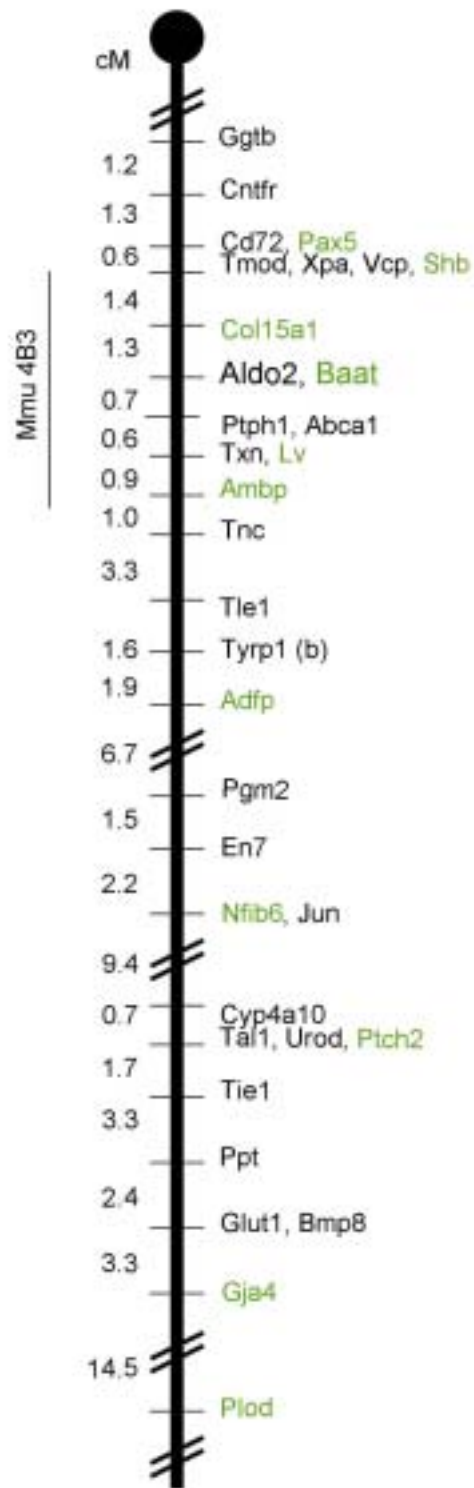


Figure 11. Genetic map of mouse chromosome 9. Numbers to the left are the distances between the markers in centimorgans; marker placement is not to scale. The chromosomal band that contains the 12Gso breakpoint (Mmu 9E3.2) is denoted, and extends from *Htr1b* to *Rbp1*.

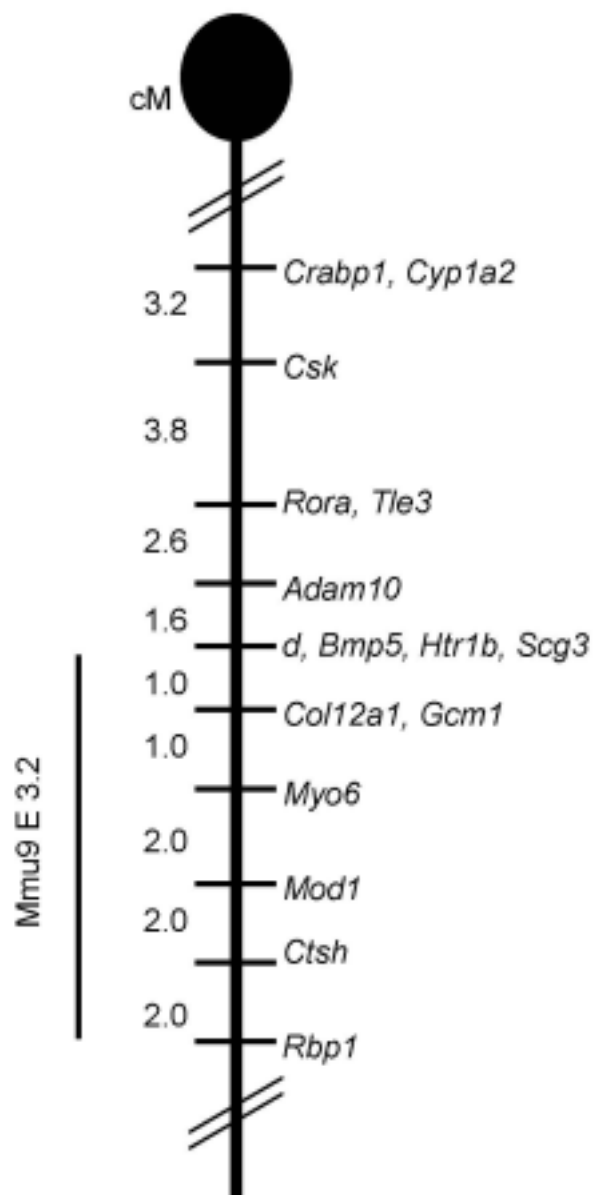


Figure 12. Initial Mmu 4 FISH results. Hybridization of mBAC6x-496F24 (*Shb*) and mBAC6x-202I7 (*Ambp*) to 12Gso homozygote (**A.**) and wild type (**B.**) metaphase chromosome spreads. The pairs of signals on each of the translocated chromosomes in the homozygote indicate the markers are separated by the translocation. **C.** Map of the Mmu 4 breakpoint region, from band B1 to band B3. The current breakpoint region, as determined by FISH, is denoted in red. **D.** Ideotype of the 12Gso translocation, for reference.

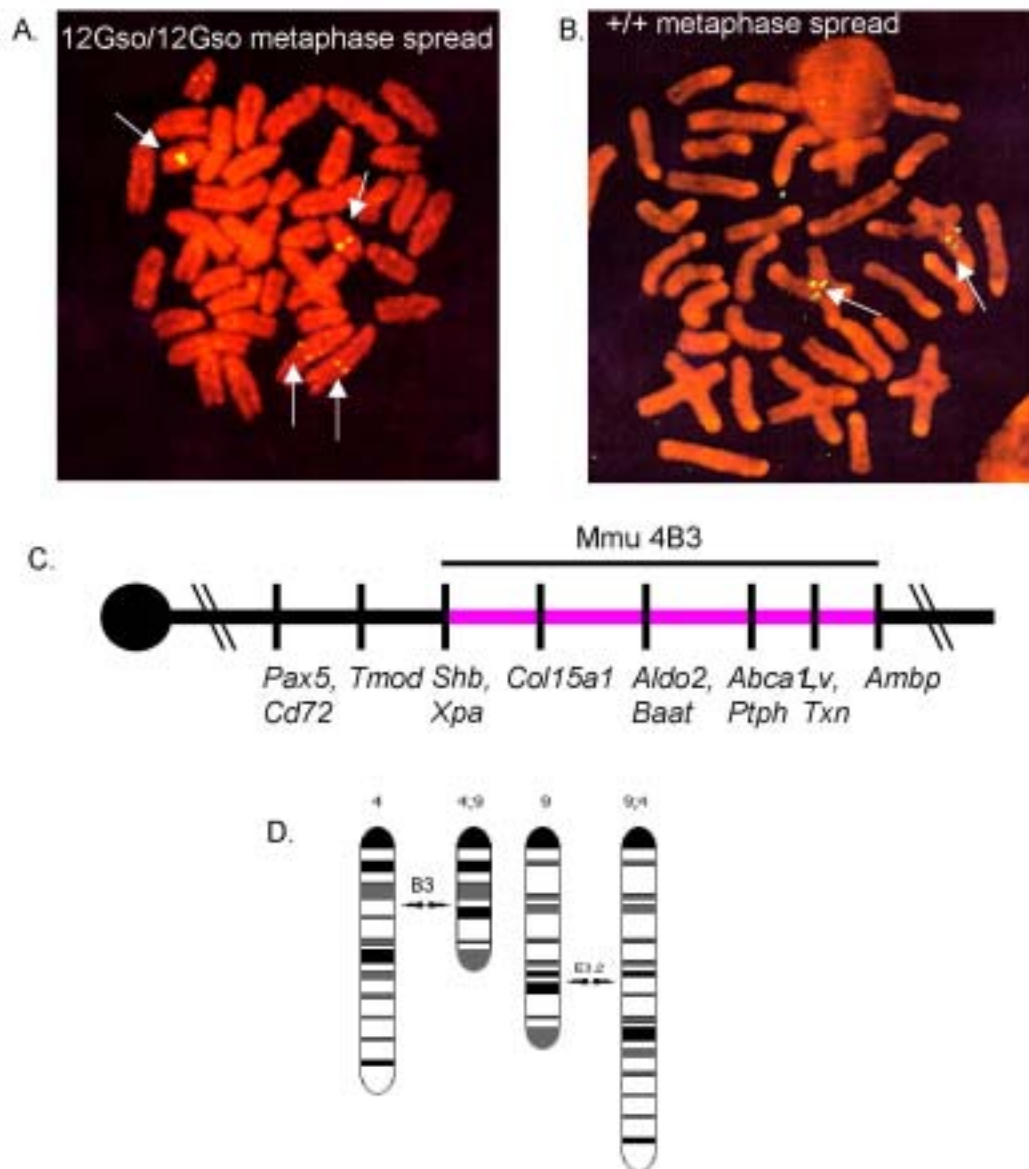
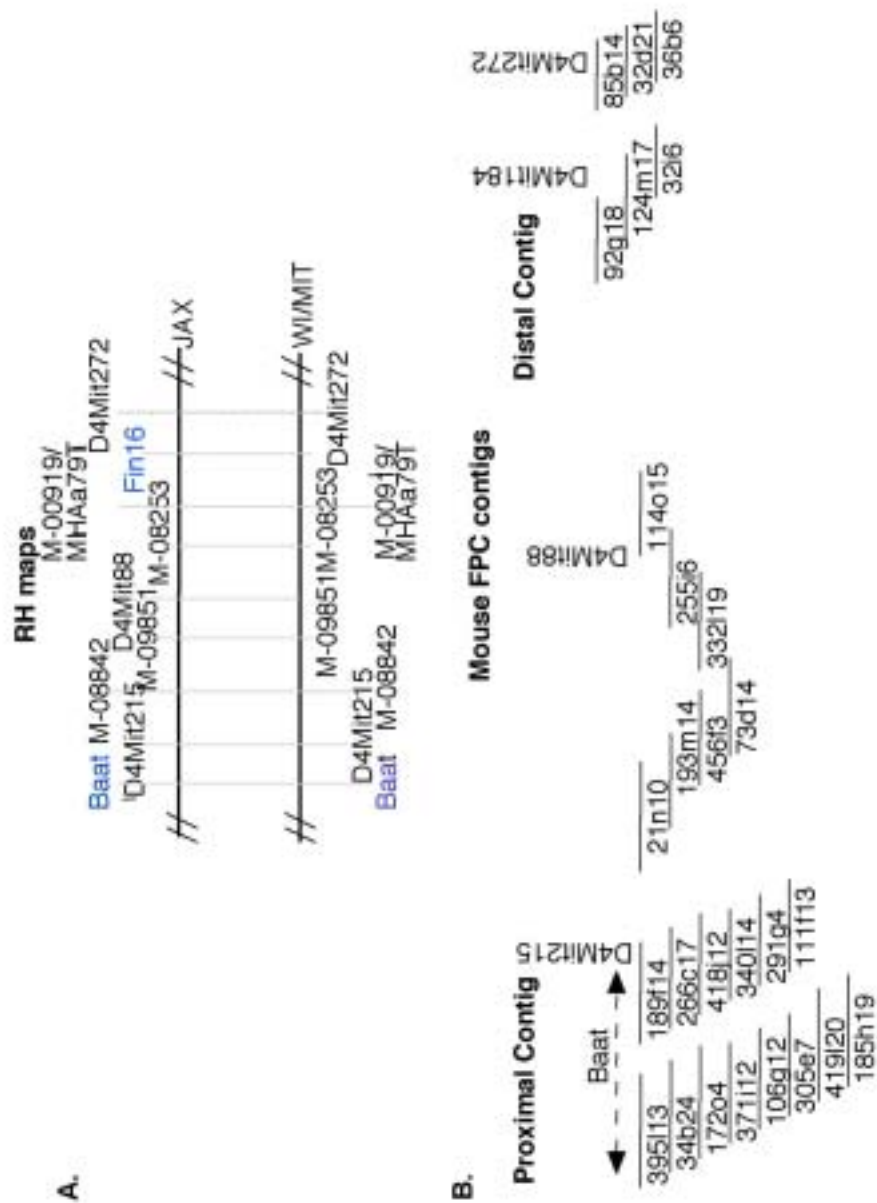


Figure 13. Using the mouse radiation hybrid maps and fingerprinted contigs to narrow the breakpoint region. (A.) Comparison of publicly available radiation hybrid maps. Markers appear to be in accordance, and two genes near the breakpoint region were recognized (blue). **(B.)** A database of fingerprinted BAC contigs was useful in locating BAC clones within the breakpoint region. All BACs listed for the FPC contigs were subject to FISH analysis.



D4Mit215, and *D4Mit88*, and the distal contig containing *D4Mit272* and *D4Mit184* (Figure 13).

To resolve this gap, the corresponding human region, 9q31, was screened for genetic markers that might be used for obtaining BAC clones. Although earlier examination of the region just proximal to *Baat* in human proved to have inconsistent homology, one marker distal to *Baat* on the Ensembl database (*Fin16*) hit not only a BAC sequence in National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>) genomic survey sequence database, but also a new fingerprinted contig in the FPC database. Using the mouse trace sequences indentified from the human contig, more positive clones in the fingerprinted contig containing the *Fin16* positive BAC were found, thus solidifying the indication that this contig might close the gap (Figure 14).

Upon searching the Celera and Ensembl mouse databases, it was determined that these BACs did indeed map into the region between *D4Mit88* and *D4Mit272*, and confirmed the distance between those two markers to be 1 Mb (Figure 15). Using these databases as a guide, BACs were chosen at intervals across this distance for FISH analysis, and the breakpoint region was eventually narrowed to a single BAC clone, RPCI-23-353G1 (Figure 16). To determine more precisely the position of the breakpoint region within this BAC, FISH experiments were continued with BAC clones RPCI-23-302N5 and RPCI-23-426F5, which overlapped RPCI-23-353G1 on the proximal and distal ends, respectively. The estimated interval of overlap of these BACs was determined by NCBI BLAST analysis. The experiments with these BACs narrowed the breakpoint interval to an estimated 7.5 kb (Figure 16).

Examination of the sequence of the breakpoint-spanning BAC, RPCI-23-353G1

The sequence of BAC RPCI-23-353G1 (Genbank accession #AC091466), although in unordered pieces, was submitted to the NCBI Entrez database. Using the automated MyGodzilla comparative sequence analysis display from the VISTA suite of sequence analysis tools (<http://www.vista.lbl.gov/>), it was possible to assemble this mouse sequence based on its best sequence match in the human genome, as well as

Figure 14. Analyzing the human BAC clone contigs for homologous mouse BAC clones. The human region homologous to the 12Gso Mmu4 breakpoint region was searched for links to possible clones that could be used by FISH to close the sequence gap in the breakpoint interval. The BAC clones listed on the top tier are human BAC clones listed on the Ensemble database. The presence of known genes is then indicated, as well as mouse trace sequences that showed some homology to a relevant sequence in the NCBI gss database. Any EST markers within the region present on the RH/FPC databases are indicated. The bottom tier indicates BAC clones for which BLAST hits were generated with the aforementioned sequences, and the subsequent new FPC contigs determined by alignment of these BLAST hits.

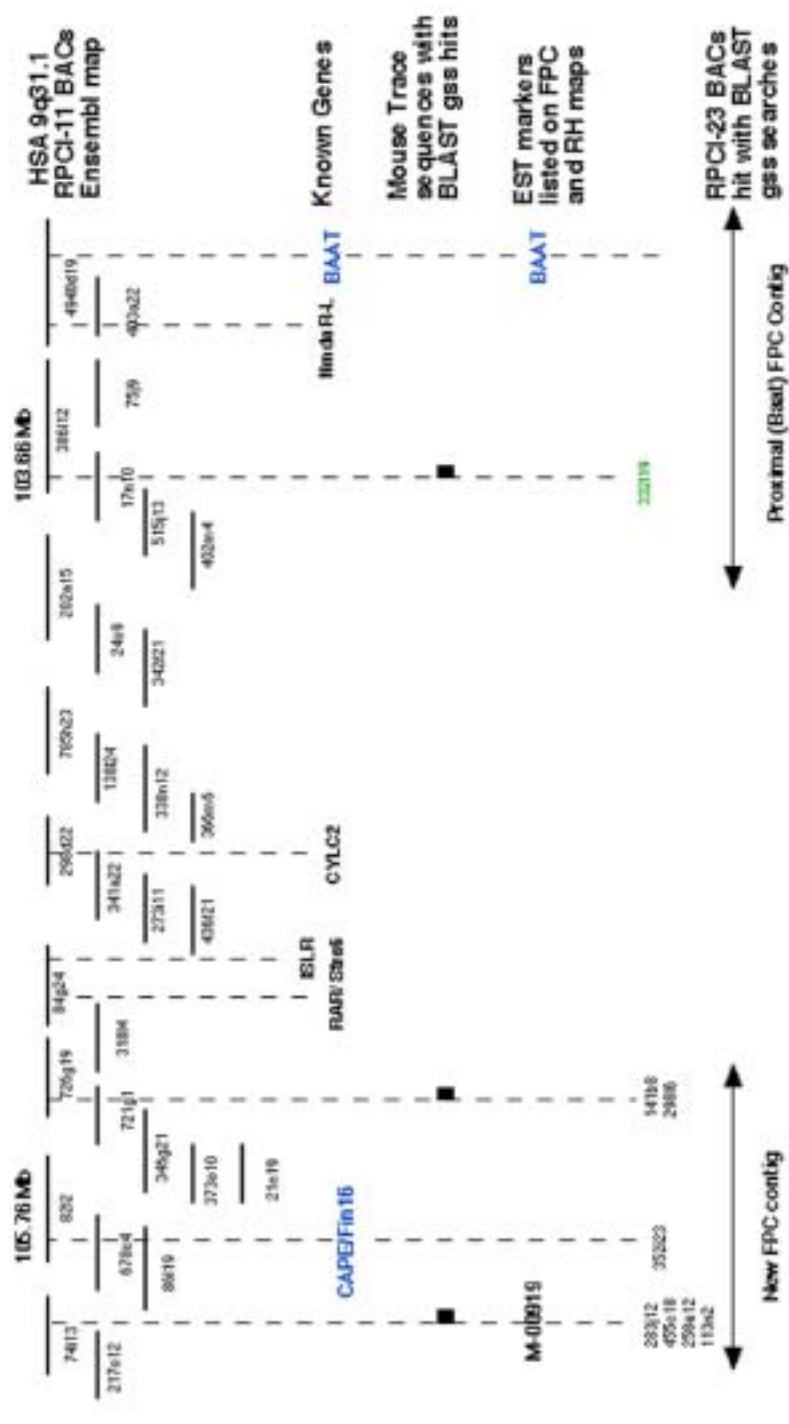


Figure 15. Framing the 1 Mb critical region at the 12Gso chromosomal breakpoint.

(A.) Hybridization of RPCI-23-111F13 (*Baat*) and RPCI-23-32D21 (*D4Mit272*) to 12Gso heterozygote metaphase spread. The three pairs of signals indicate that the markers are separated by the translocation. **(B.)** Hybridization of RPCI-23-114O15 (*D4Mit88*) and RPCI-23-3D21 (*D4Mit272*) to 12Gso heterozygote metaphase spread. Again, these markers are separated by the translocation. Because of the close distance between the markers, multiple signals on the same chromosome appear as a single bright pair. **(C.)** Map of the Mmu 4 breakpoint region, with BAC clones and genetic markers included. The 4 Mb region is the distance represented by the FISH results in panel **A.**, and the 1 Mb critical region (red) is the estimated breakpoint region represented by the FISH results in panel **B.**

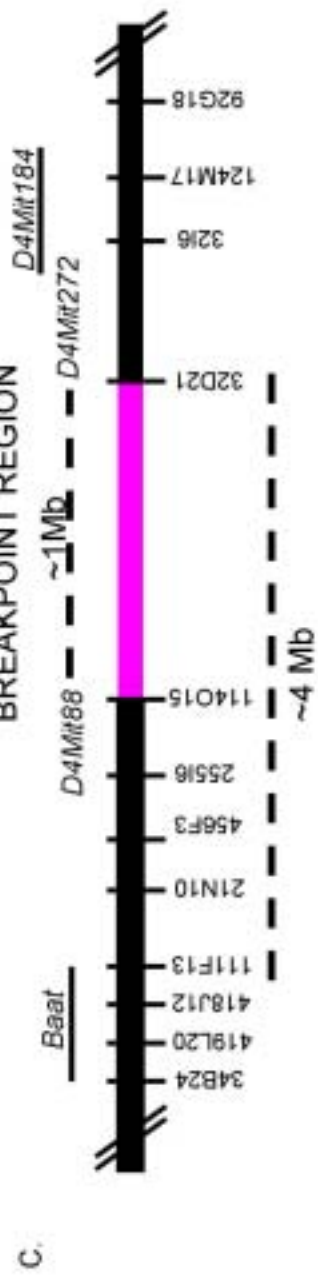
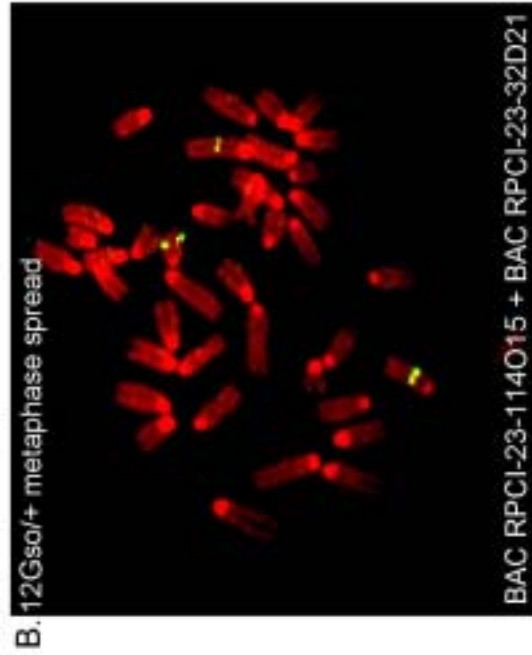
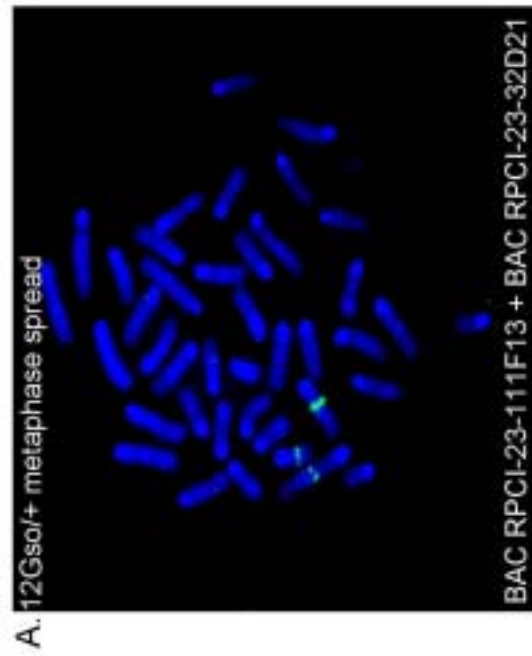
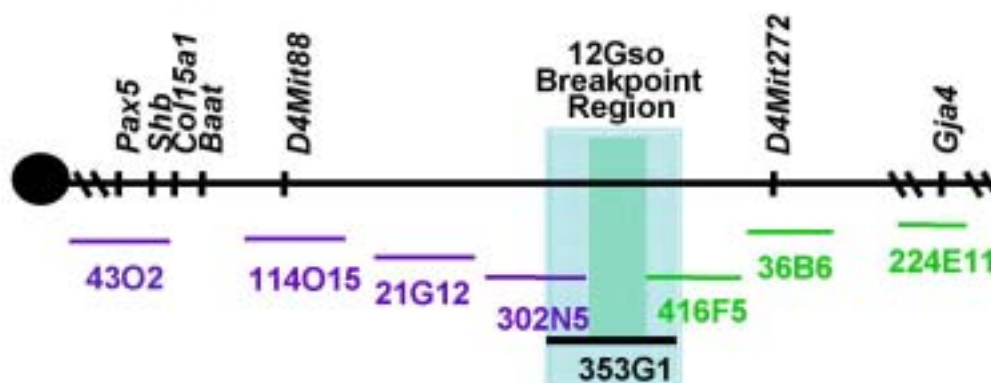
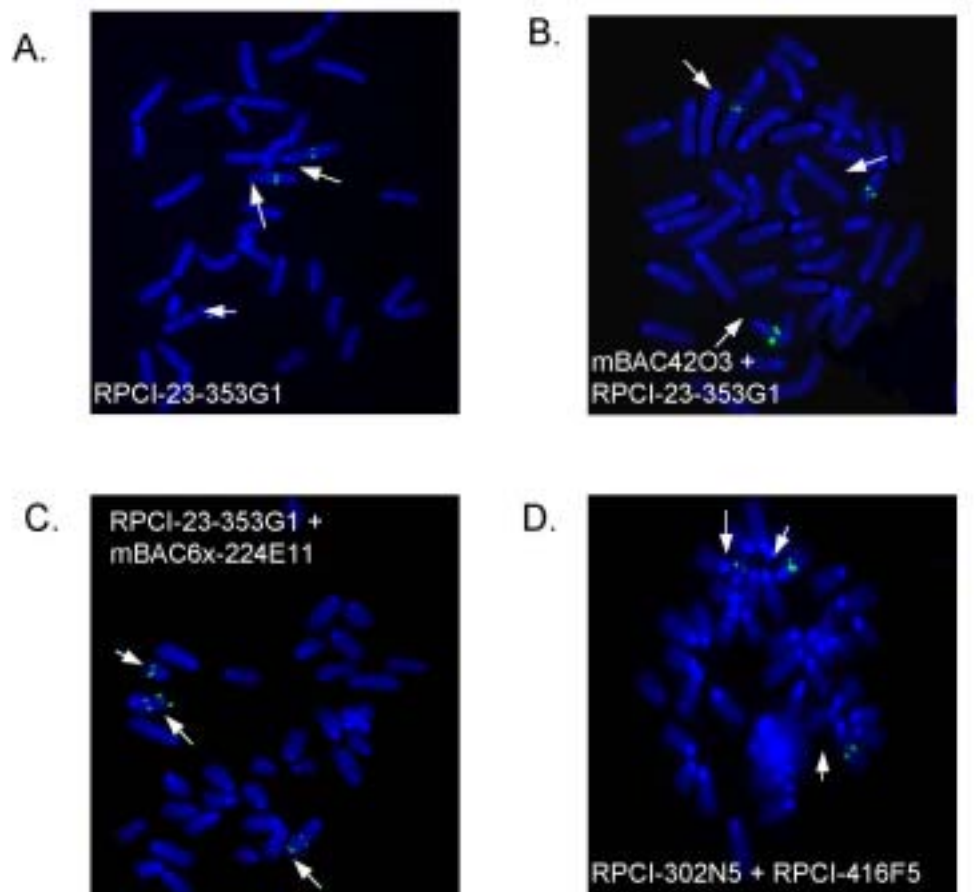


Figure 16. The 12Gso chromosome 4 breakpoint lies in the region spanned by BAC RPCI-23-353G1. FISH analysis of BAC RPCI-23-353G1 with a proximal marker (**A.**), distal marker (**B.**), and alone (**C.**) all present evidence that this BAC clone spans the Mmu 4 breakpoint. (**D.**) BAC clones RPCI-23-302N5 and RPCI-23-416F5 overlap BAC RPCI-23-353G1, and are separated by the breakpoint. The distance between RPCI-23-302N5 and RPCI-23-416F5 endclones is only 7.5 kb. As neither clone presents evidence of spanning the breakpoint when hybridized alone (data not shown), the breakpoint region should lie either between, or near the ends of, these two BACs.



to generate a comparative plot to estimate the positions of possible exons. The plot confirmed the data from the annotation on the Ensembl website: the presence of the gene *Abca1*, whose position was already known, and possibly two other unknown genes. According to our original genetic maps, *Abca1* was outside the breakpoint interval, and thus had not been used to screen the libraries once the region had been narrowed to *Baat* and *Lv*.

In the search for sequences of BAC RPCI-23-353G1, a publication that reported the sequence of the 280 kb surrounding *Abca1* (Qiu *et al.*, 2001) was found. The sequences listed for the contig encompassed all but 20 kb of BAC RPCI-23-353G1. *Abca1* is expressed almost ubiquitously, although highest levels are present in liver. Defects in human *ABCA1* result in Tangier disease, a metabolic disorder characterized by a defect in cellular cholesterol removal. Thus, the most studied role of *Abca1* is with regard to lipid transport, but *Abca1* also has roles in inflammation and macrophage function and recruitment (Schmitz *et al.*, 1999; Harmon *et al.*, 2000). The two Nipsnap-related sequences, *Nipsnap3* and *Nipsnap4*, are immediately downstream of *Abca1*. Each gene spans about 10kb in genomic length, they have 6 and 7 exons, respectively, and they produce ~1.5 kb transcripts that are closely related in sequence. Nipsnaps are related to the *C. elegans* nonneuronal SNAP25 protein (Qui *et al.*, 2001). The functions of these two gene products are unknown. The proteins encoded by these candidate genes have strong identity (70-80%) to the human protein HSPC299, the cDNAs for which are isolated from CD34+ stem cells in blood and from the bone marrow of a patient with chronic myelogenous leukemia (GenBank, NCBI Entrez). CD34 is expressed during the induction of primitive hematopoiesis and vasculogenesis, and although the early molecular and cellular events involved in these processes are not well understood, it is known that transplantation of CD34+ stem cells leads to the production of cells from the lymphoid, myeloid, and erythroid lineages (Sutherland and Keating, 1992). These characteristics implicated the *Nipsnap* genes as potential candidates for the 12Gso bone marrow/lymphoproliferative phenotype. The olfactory receptor sequence located downstream of the *Nipsnap* genes represents an unlikely candidate, as this family of genes encodes receptors expressed exclusively in the olfactory neuroepithelium that

respond to odorants through the regulation of secondary messengers (Restrepo *et al.*, 1996). This sequence, and the ordered and oriented RPCI-23-353G1 BAC sequence, allowed the search for the breakpoint region by PCR analysis.

Strategy using PCR and genomic Southern hybridization to analyze the breakpoint region outside of the Abca1 gene

To identify the Mmu4 breakpoint, PCR primers were generated to span segments of 0.5-5 kb across the entire sequence of RPCI-23-353G1 outside of the *Abca1* gene (Figure 17). PCR was carried out with C3Hf/R1 and 12Gso/12Gso DNA samples as templates to identify fragments that were generated in the normal but not the 12Gso DNA sample, indicating the presence of a rearrangement. No PCRs indicated deletion or chromosomal breakage, as all produced an equivalent product from both C3Hf/R1 and 12Gso/12Gso DNA samples (data not shown).

In addition, pools of normal PCR products from the region of RPCI-23-353G1 outside *Abca1* were hybridized to Southern blots of C3Hf/R1 and 12Gso/12Gso DNA samples, and no alternatively sized or missing fragments detected (data not shown).

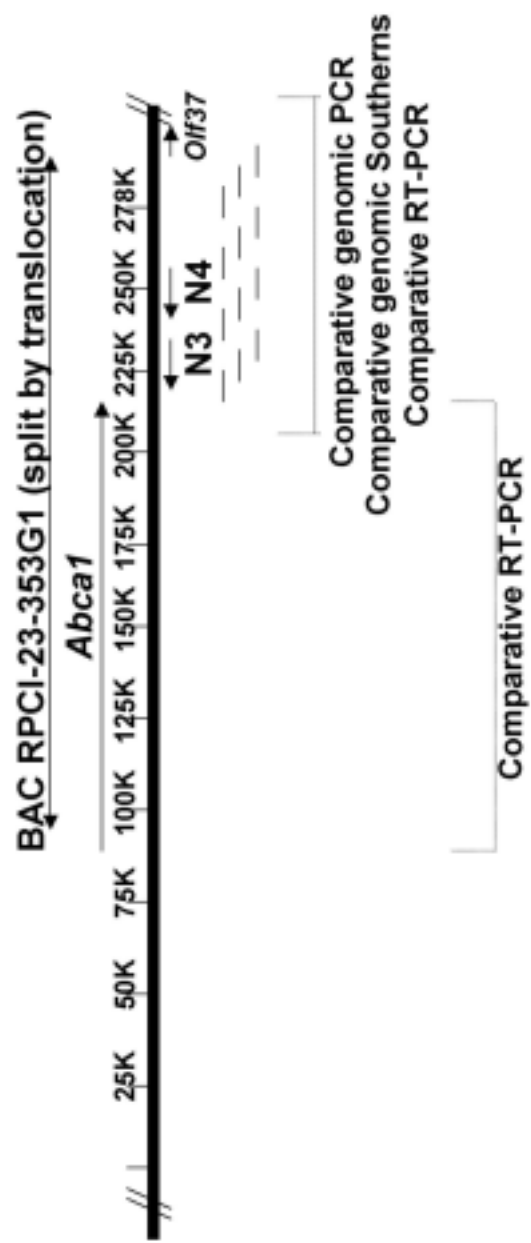
Results of RT-PCR with Abca1

Not until the PCRs and their subsequent hybridization to genomic Southern blots exhausted the region did the search for the breakpoint within the *Abca1* gene proceed. Because of the daunting size of this gene (almost 200 kb of genomic DNA and 8 kb cDNA), RT-PCR was used to cross the region more rapidly.

To traverse the large *Abca1* gene during breakpoint localization, RT-PCR of liver cDNA was performed, using primers sets generated from either end of the gene and gradually walking inward. The rationale for these experiments was that if the breakpoint occurred within the gene, a stable, full-length transcript could not be produced from the gene in mutant tissues, and that either all or part of the transcript would be missing in tissues of 12Gso/12Gso mice.

To span the large *Abca1* gene, we tested the presence of 5'- and 3'-end sequences of the gene in transcripts produced in liver using RT-PCR. RT-PCR on liver

Figure 17. Schematic of the Mmu 4 breakpoint region encompassed by the published *Abca1* contig (Qui *et al.*, 2001) and the methodology used to find the 12Gso breakpoint. The published contig is 278 kb in length, and RPCI-23-353G1 maps from approximately 92 kb from the beginning of the contig sequence, and extends approximately 40 kb past the end of the contig. Arrows above gene names represent the direction of transcription relative to the contig; *Abca1* = *ATP binding cassette 1*; N3, *Nipsnap3*; N4, *Nipsnap4*; *Olf37*, gene from the *olfactory-37* gene cluster. Initially, comparative genomic PCR was performed in the region outside the *Abca1* gene, using primer sets generated from all non-repetitive sequence to span the entire region, and results verified by hybridization to genomic Southern blots. As *Abca1* spans a large portion of both this published contig and the BAC RPCI-23-353G1, RT-PCR analysis was used to traverse the gene.



samples was conducted first with primer sets crossing exons 2 and 3 and crossing exons 49 and 50. Initial results indicated the presence of a stable 5' *Abca1* transcript in 12Gso/12Gso liver, but 3' *Abca1* sequences could not be detected in liver cDNA samples produced from 12Gso homozygotes (Figure 18). Additional primer sets were used to walk inward from the 3' end of the gene; *Abca1* sequences located 3'- of exon 30 were consistently undetected in 12Gso/12Gso cDNA samples whereas primer sets encompassing exons 1 to 30 were consistently detected *Abca1* cDNA in approximately normal abundance in the mutant liver (Figure 18). This indicates the gene must be broken somewhere in the interval contained by the next downstream primer set, between exons 30 and 35. Later RT-PCR experiments established the breakpoint to lie in an approximately 600 bp segment between exons 34 and 35 (Figure 19).

Results of comparative microarray analysis

To identify possible clues to the bone marrow defect exhibited by 12Gso/12Gso mice, bone total RNA samples from a 12Gso homozygote female from a B6C3 background and an age-matched B6C3 female were isolated and sent to the UC Davis Cancer Center microarray facility for analysis on Affymetrix gene-chip arrays. The preliminary data presented from the microarray analysis indicated effects on expression level in numerous genes (Table 4), the vast majority of which were downregulated. The genes have been arranged into the following categories: lipid trafficking and metabolism (n=11), inflammation (n=6), thrombosis and hemorrhage (n=10), steroid metabolism (n=5), hematopoiesis (n=10), liver enzymes (n=8), neurological (n=18), and miscellaneous (n=11).

Wild type mouse Northern blot analysis

Because tissue expression of many of the candidate genes from the breakpoint regions was unknown, probes from these transcripts were hybridized to a series of Northern blots from Clontech (CA). The blots used for this analysis included their mouse RNA Master Blot, which contains a spotted array of normalized mRNA from numerous mouse tissues, the mouse MTN blot, which was an mRNA Northern blot

Figure 18. RT-PCR analysis of the *Abca1* gene. (A.) Initial RT-PCR using 12Gso/12Gso and +/+ liver cDNA generated a product from the 5' end of *Abca1* (exons 2-3; yellow arrow), but no product from the 3' end (exons 48-49; white arrow), indicating the gene produces a stable 5' transcript, but is likely to be truncated. (B.) Primer sets that spanned multiple exons were then used to move upstream from the truncated 3' end of *Abca1* to determine where the gene is truncated. Primer sets A, B, C, and D, which are listed in 3' to 5' order, did not produce a product with RT-PCR (white arrows), but primer sets E, F, and further 5' did produce an RT-PCR product. (C.) Schematic of the *Abca1* gene including localization of the expected products from +/+ RT-PCR relative to exon number. Primer set E, the most 3' primer set to generate an RT-PCR product, spans exons 27-30 of the *Abca1* gene, and primer set D, the most 5' primer set to lack an *Abca1* RT-PCR product, spans exons 30-35. From this data, it can be interpreted that the 12Gso transcript of *Abca1* is stable at the 5' end, but the 3' end is truncated after exon 30.

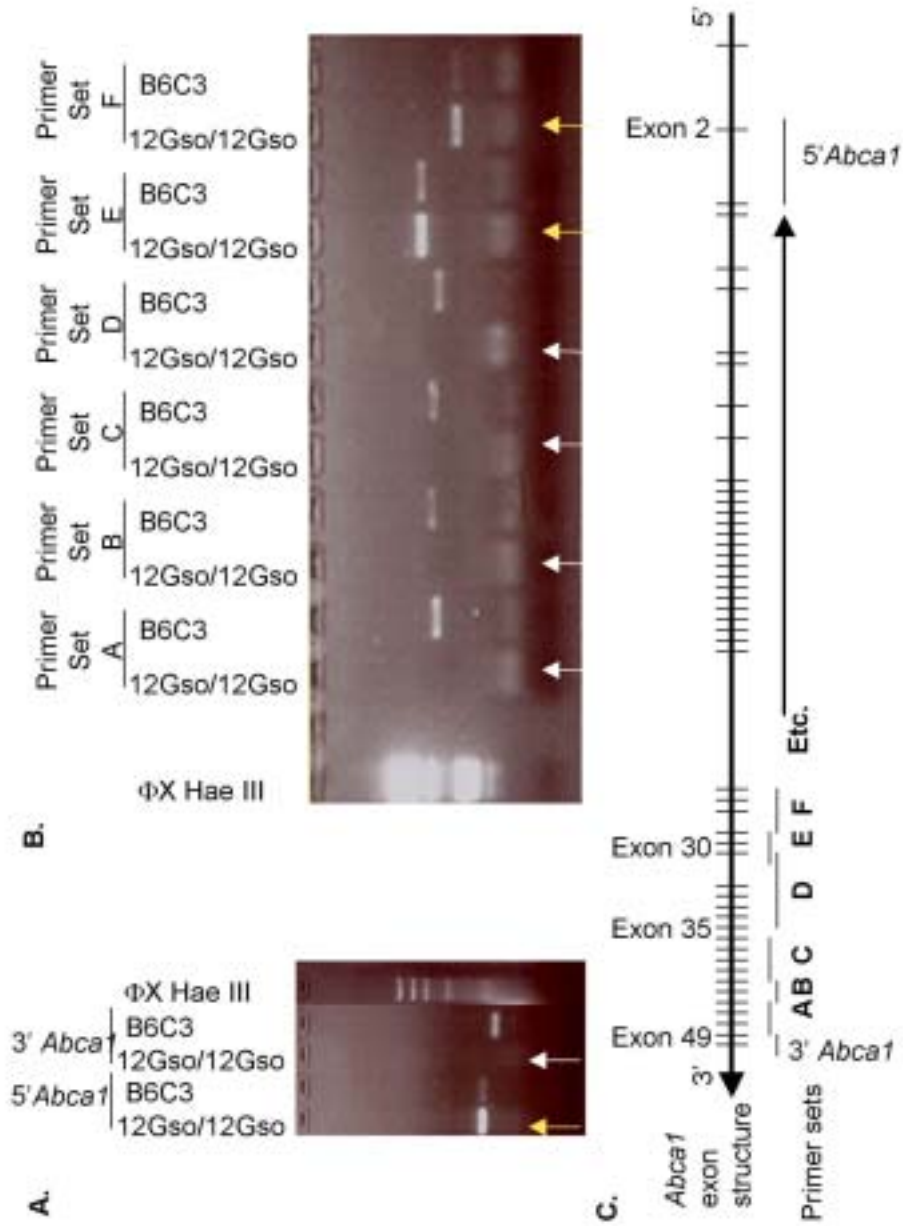


Figure 19. RT-PCR analysis of the *Abca1* region narrows the breakpoint interval to approximately 600 base pairs. The number from the name of each primer represents the exon from which it originated; F, forward primer; R, reverse primer. RT-PCR with liver cDNA in both C3Hf/R1 and 12Gso/12Gso tissues indicated the presence of a transcript in exons 30, 31, 32, and 33, but no product was observed in the amplification of exons 34-35 (arrow), implicating this 600 bp region as the location of the chromosomal breakpoint.

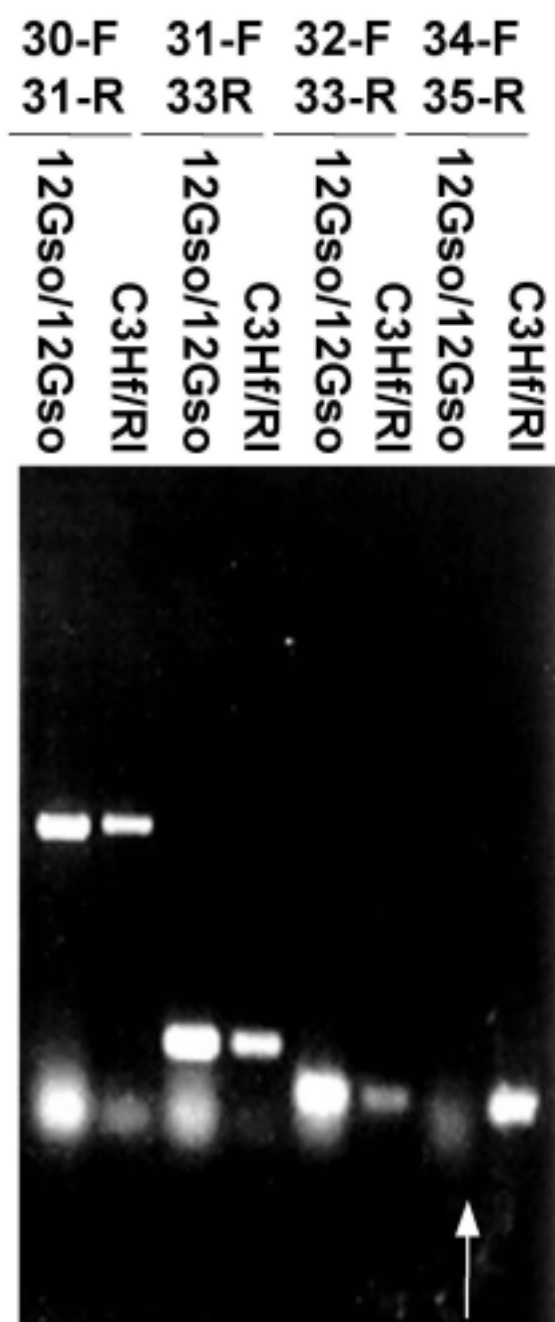


Table 4. Results from the preliminary comparative microarray experiment, using 12Gso/12Gso and B6C3 bone total RNA: genes with 10-fold or greater change in expression between the RNA samples. -denotes a decrease in expression; + denotes an increase in expression.

Functional Category	Gene	Fold Change in Gene Expression	Genbank Accession Number
Lipid pathways	ApoA5	-26	AI785422
	ApoA2	-23	AV038316
	ApoA1	-23	U79573
	L-FABP	-23	Y14660
	ApoH	-18	Y11356
	Adipsin	+10	X04673
	Tap1	+11	U60020
Inflammation	Ambp	-52	X68680
	Urate oxidase	-32	M27695
	α -1 protease inhibitor 5	-26	M75717
	ApoH	-18	Y11356
	Hemopexin	-15	U89889
Thrombosis and hemorrhage	At3	-24	AI226846
	Vitronectin	-13	M77123
	α -2 macroglobulin	-10	M93264
Steroidogenesis	Cyp3a11	-26	X60452
	Cyp3a16	-21	D26137
Hematopoietic	Acid nuclear phosphatase 32	+15	U73478
	Erythroid differentiation factor	+13	AJ007909
	RGS5	+13	U67188
	SCL/Tal1	+10	U01530
	ST2L/IL1RL1/ Lymphocyte antigen 84	+10	D13695
Liver enzymes	Glycine-N-methyltransferase	-32	D89664
	Prohepichidin	-26	AI845514
	Regucalcin	-16	U32170
	Phneylalanine hydroxylase	-13	X51942
Miscellaneous	G substrate mRNA	-26	AF071562
	Gas5	+32	AI8949615
Neurological	Gria1	-35	X57497
	Calbindin	-28	D26352
	Snap25	-16	M22012
	Nsp	-16	AW123115

containing common mouse tissues, and the Immune MTN blot, which contained mRNA from various human immune system sources. *Nipsnap3* was expressed in almost all tissues on the Master Blot, with strongest expression in liver, smooth muscle, epididymus, uterus, submaxillary gland, kidney, and 7-day embryo. Expression on a total RNA multiple tissue Northern blot was limited to liver, stomach, thymus, and weak expression in spleen (Figure 20). The expression level of *Nipsnap4* was weak in all tissues. *Nipsnap4* was expressed only in testes on the Clontech Mouse MTN blot, in the submaxillary gland and epididymus of the Master Blot (weak expression in thymus, ovary, uterus, prostate, skeletal muscle, and 11- and 17-day embryo). Expression was seen in thymus and fetal liver, with weaker expression in the bone marrow and peripheral blood leukocyte on the Clontech Human Immune Blot (Figure 21). *Tbx18* was not expressed in adult tissues, as expected (data not shown).

RT-PCR analysis of nearby Mmu 4 genes

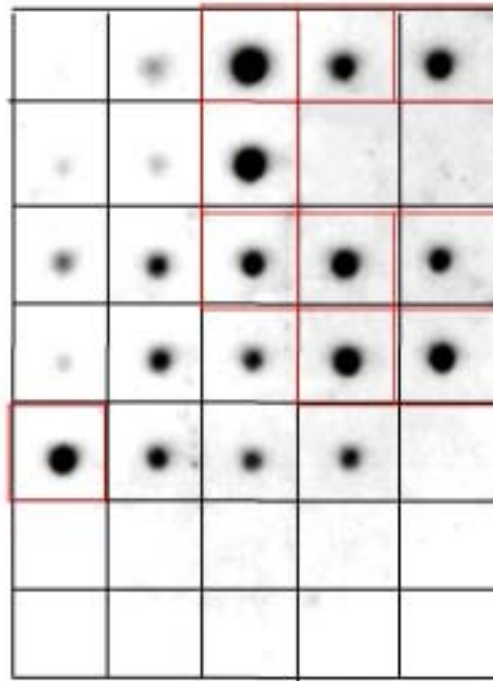
Once the genomic PCR across the region outside the *Abca1* gene was completed, a physical disruption of the *Nipsnap*-related genes was excluded. However, as indicated by the genes cloned in several translocation studies, these genes could not be excluded from our analysis, especially in light of the tissue distribution of *Nipsnap3* transcripts indicated by representation in various EST libraries. RT-PCR of liver using primers generated from the 3' end of *Nipsnap3* indicated that a stable transcript is expressed at similar levels in 12Gso/12Gso and +/+ mice (Figure 22). These results imply that these genes are expressed normally in mutant tissues and are unlikely to be involved in the 12Gso phenotype. The olfactory receptor gene was not tested, as not only is it an unlikely candidate, but it maps further downstream and further from the breakpoint site than even *Nipsnap3* and *Nipsnap4*.

Testing potential allelism between 12Gso and the tk mutation

One of the ways to investigate the relationship between known genes or known mutant alleles and different aspects of the 12Gso phenotype is to perform complementation testing. Complementation testing is a classical genetic method used to

Figure 20. Wild type Nipsnap3 expression. Hybridization of *Nipsnap3* to a Clontech mRNA Master Blot indicated expression of *Nipsnap3* in most tissues (red boxes), with highest expression in liver and smooth muscle (**A.**). (**B.**) Key for interpretation of expression data from the mRNA Master Blot.

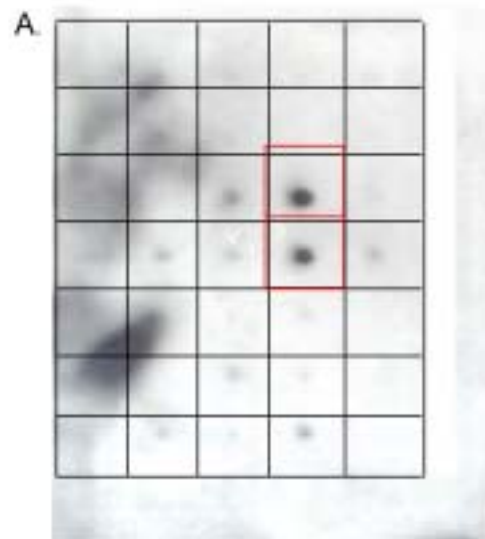
A.



B.

brain	eye	liver	lung	kidney
heart	skeletal muscle	smooth muscle		
pancreas	thyroid	thymus	submax. gland	spleen
testis	ovary	prostate	epididymis	uterus
embryo 7 days	embryo 11 days	embryo 15 days	embryo 17 days	
yeast total RNA	yeast tRNA	E. coli rRNA	E. coli DNA	
poly r(A)	Cot1 DNA	mouse DNA	mouse DNA	

Figure 21. Wild Type expression of Nipsnap4. (A.) Results from hybridization of *Nipsnap4* to an mRNA Master Blot (Clontech), 1 week exposure. Major sites of expression were the submaxillary gland and the epididymus (red boxes). (B.) Key for interpretation of expression data from the mRNA Master Blot. (C.) Highest levels of expression of *Nipsnap4* in the tissues tested was only seen in mouse testis, but weak expression was also indicated in human lymph node, fetal liver, bone marrow, and peripheral blood leukocyte (D.).



B.

brain	eye	liver	lung	kidney
heart	skeletal muscle	smooth muscle		
pancreas	thyroid	thymus	submax. gland	spleen
testis	ovary	prostate	epididymis	uterus
embryo 7 days	embryo 11 days	embryo 15 days	embryo 17 days	
yeast total RNA	yeast tRNA	<i>E. coli</i> rRNA	<i>E. coli</i> DNA	
poly r(A)	Col1 DNA	mouse DNA	mouse DNA	

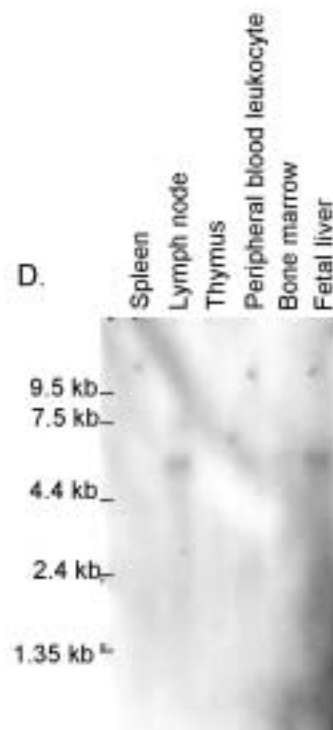
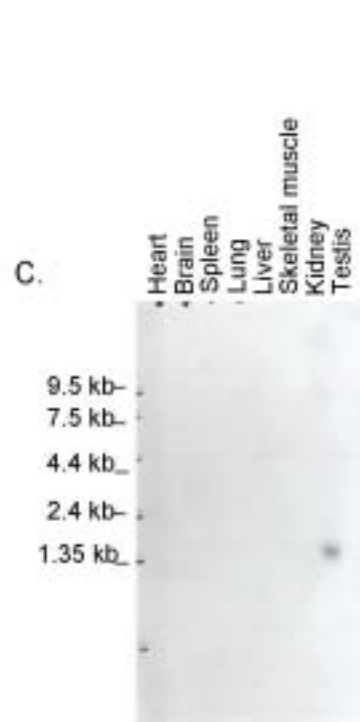


Figure 22. RT-PCR analysis of 3' Nipsnap3. Primers spanning exons 6-7 of *Nipsnap3* were used for RT-PCR with 12Gso/12Gso and C3Hf/Rl liver cDNA. Both 12Gso/12Gso and C3Hf/Rl generate an intact transcript for *Nipsnap3*.



determine the relationship between a recessive mutant locus and a previously characterized recessive mutant locus. This method can be used to determine whether the two mutations are allelic (representative of mutations within the same gene) or if they are not related (and therefore complement each other to yield animals with normal phenotypes). Complementation testing may also reveal that two genes are nonallelic but noncomplementing, indicative of genetic interaction, for example when loci on different chromosomes are members of the same genetic pathway. Phenotypic analysis of the offspring from a cross between mice carrying both mutations will determine their relationship. For example, if the two mutations are allelic (or nonallelic noncomplementing), a double mutant animal would express the aberrant phenotype. In the case of allelism, this genotype could be represented as $t1/t2$ for the particular locus affected in mice with the mutations $t1$ and $t2$. However, if the mutations are not complementary, the double heterozygote for these mutations would have a normal phenotype because of the presence of a wild type allele at both loci (Silver, 1995).

One of the mouse skeletal mutants mapping to chromosome 9, tail-kinks (tk ; Table 1), mapped within the 12Gso breakpoint interval until later FISH mapping events narrowed the region to 1 Mb. tk homozygotes have short, very kinked tails, and minor aberrations in the vertebral column. The phenotype can be observed as early as E10, when differentiation of the cervical sclerotome is absent. Penetrance in this mutant is complete, but genetic background can influence viability (Gruneberg, 1955; Lilly, 1972). Although the tk phenotype is much less severe than that of 12Gso, the two mutations do share common traits: the presence of kinky tails and aberrations in the vertebral column. For this reason, the two stocks were subjected to complementation testing. No other classical mouse mutations map near either 12Gso breakpoint region.

Phenotypic and genotypic examination of 12Gso X tk offspring indicated that although some of the pups did indeed exhibit a modest phenotypic effect related to that of tk (although not so severe as 12Gso/12Gso), not all of the 12Gso heterozygotes were phenotypically identifiable. Because of this, the allelism tests were inconclusive and more breeding was done to determine allelism because the compound heterozygote phenotype could be affected by background strain, as are both homozygote phenotypes.

12Gso heterozygote male F1 offspring of these allelism crosses were then backcrossed to their mothers, and offspring from these crosses were then examined. First generation offspring from these crosses were karyotyped by the mouse whole chromosome paint method (8 12Gso/+, only one with a slightly kinked tail; 10 +/+). For the second and third generations, genotype could partially be determined by phenotype, using coat color and tail phenotype as a guide. As the *tk* phenotype in this stock is linked to *dilute*, the *tk* mice have a dilute gray coat color as well as tails with kinks. 12Gso heterozygotes have straight tails, and either a black or brown coat color. F2 production was ceased when none of 25 F2 offspring exhibited evidence of allelism (13 12Gso/+ with brown or black coat and straight tails; 12 *tk/tk* with dilute gray coat color and kinked tails). F3 production ceased after 19 offspring, with equivalent results to that of the F2 generation (10 12Gso/+ with brown or black coat and straight tails; 9 *tk/tk* with dilute gray coats and kinked tails).

Mapping the Mmu 9 chromosomal breakpoints

FISH experiments on Mmu9 narrowed the region to approximately 4 Mb, between the markers between the markers of *Mod1* and *Rasgrf1* (Figure 23). BACs were selected using the same databases employed for Mmu4. FISH experiments narrowed the breakpoint interval to approximately 1 Mb, between the BAC clones RPCI-23-67P8 and RPCI-23-94L9, which are estimated to map 87.8 and 88.8 Mb from the centromere of Mmu9, respectively (Figure 24).

As previously mentioned, the Mmu9 region is relatively gene-poor (Figure 24). The most proximal gene, *Snap91*, is mainly expressed in brain tissue with active synaptogenesis or synapsis maturation, functioning in calcium-dependent proteolysis (Zhou *et al.*, 1992). However, there is elevated expression in two related cell lines: HL60, a lymphoblast line derived from promyelocytic leukemia, and MOLT-4, a cell line derived from T-cell acute lymphoblastic leukemia. *Tbx18* is one of a large family of T-box containing transcription factors, so named because they encode a DNA-binding domain first characterized in the brachyury (*T*) gene product. Members of this gene family are involved in developmental processes (Papaioannou and Silver, 1998; Smith,

Figure 23. Initial 12Gso chromosome 9 FISH. The 12Gso chromosomal breakpoint lies between the markers of *Mod1* and *Rasgrfl* on Mmu9. **A.** Simultaneous hybridization of RPCI-23-6D7 (*Scg3*) and RPCI-23-124J8 (*Mod1*) indicated that the clones are not separated by the chromosomal break. **B.** Cohybridization of RPCI-23-124J8 (*Mod1*) and RPCI-23-118J13 (*Rasgrfl*) indicated that the two markers have been separated by the translocation. **C.** Cohybridization of the clones RPCI-23-118J13 (*Rasgrfl*) and RPCI-23-142P8 (*Rbp1*) indicated that these markers were not separated by the translocation. **D.** Map of Mmu9, E3.1 – E3.2. Distances between markers are indicated in both the numbers of centimorgans and Mb from the centromere. The Mmu 9 region now known through FISH results to contain the chromosomal breakpoint is noted in red.

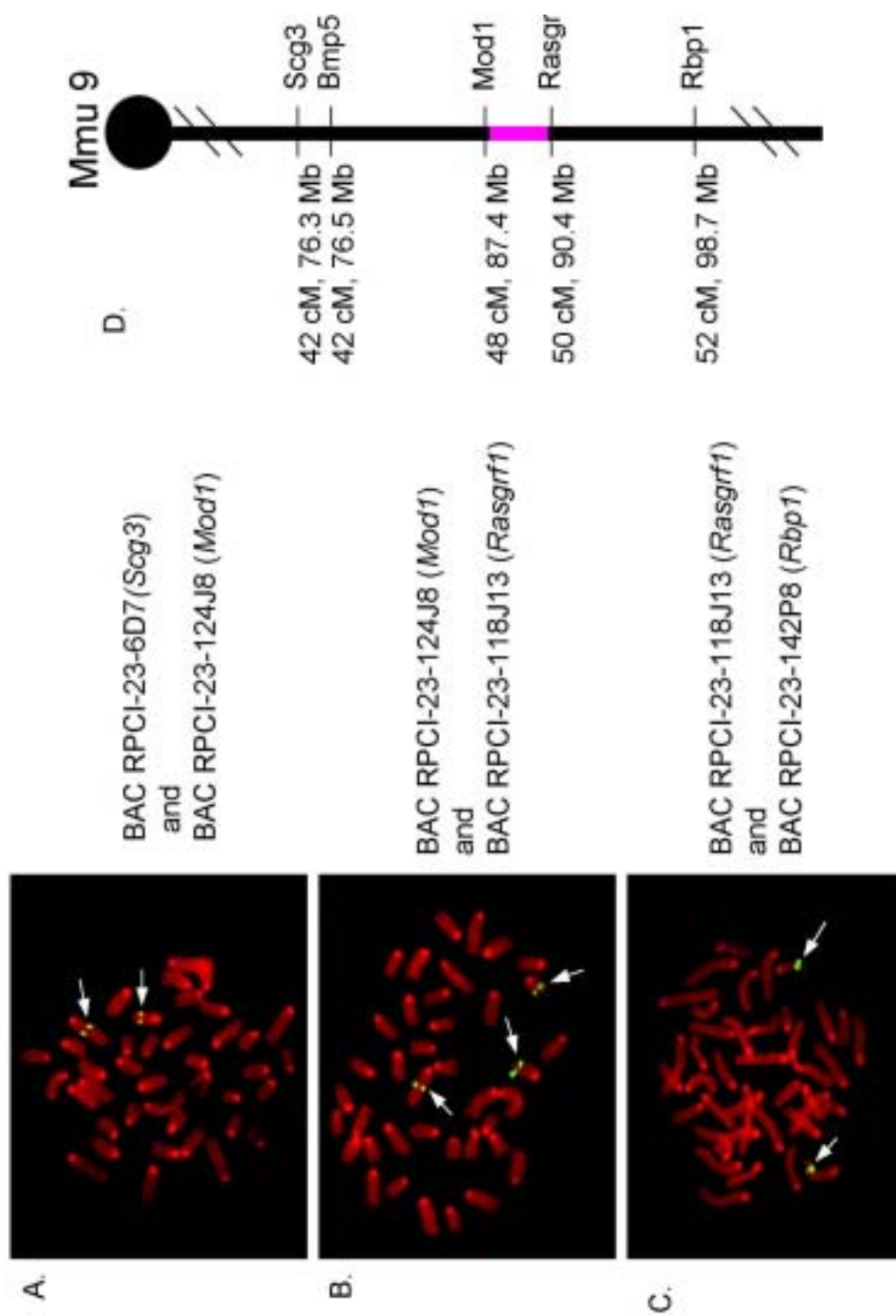
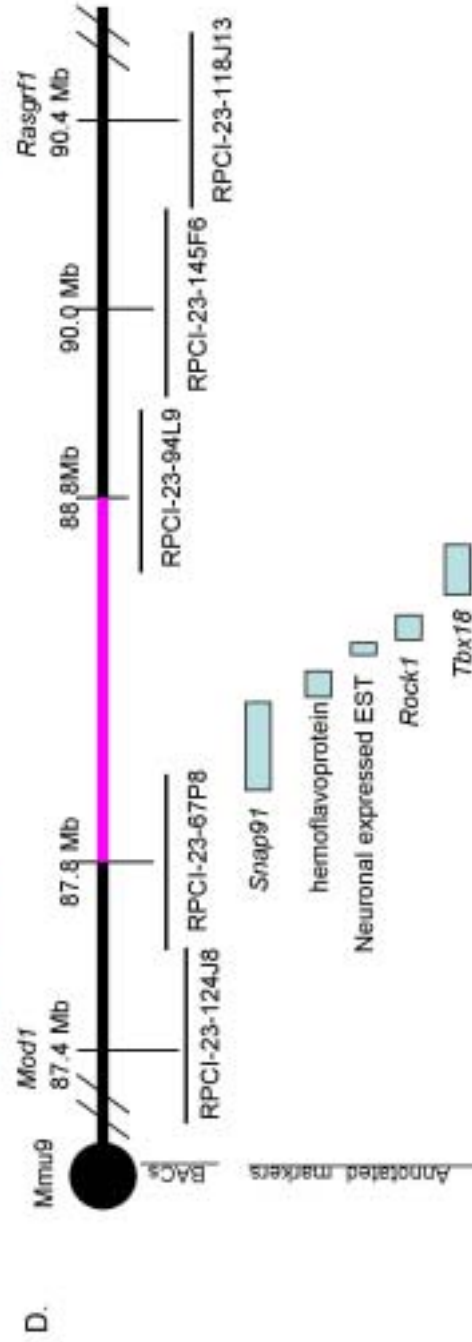
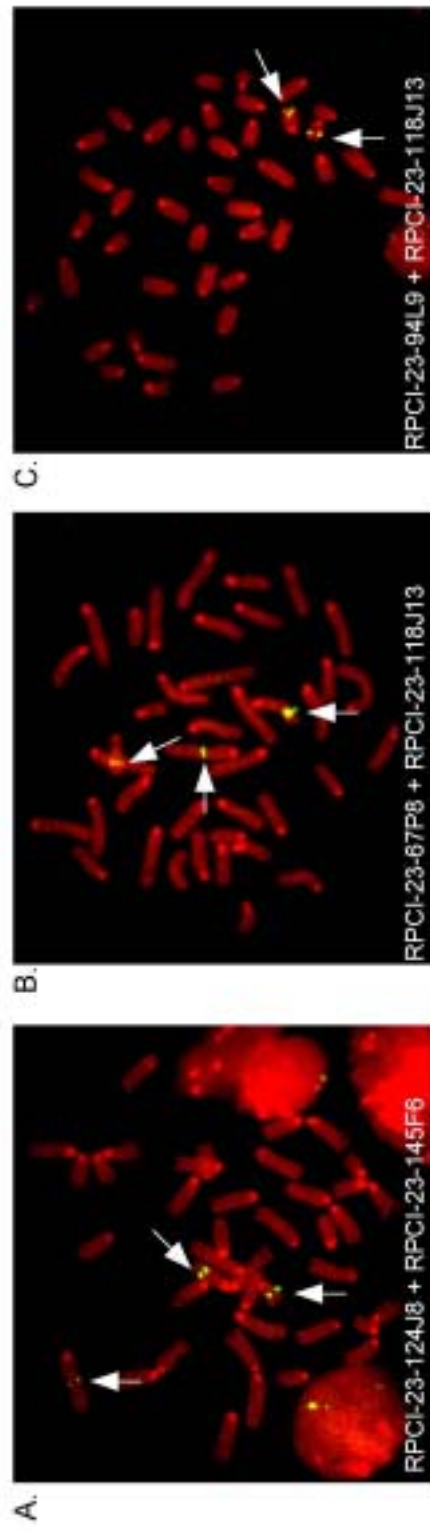


Figure 24. Narrowing the 12Gso chromosome 9 breakpoint. The Mmu 9 breakpoint of 12Gso is contained within a 1 Mb region, flanked by BACs RPCI-23-67P8 and RPCI-23-94L9. **A.** Hybridization of the proximal BAC RPCI-23-124J8 (*Mod1*) and BAC RPCI-23-145F6 produced three signals, thus indicating the region spanned the chromosomal breakpoint. **B.** The overlapping region, flanked by the BAC markers RPCI-23-67P8 and RPCI-23-118J13 (*Rasgrf1*), also indicates the presence of the breakpoint between these two markers. Interpretation of the data from **A.** and **B.** would presume the breakpoint maps between RPCI-23-67P8 and RPCI-23-145F6. **C.** FISH with BAC RPCI-23-94L9 and RPCI-23-118J13 (*Rasgrf1*) allows for the further reduction of the estimated breakpoint region to that flanked by BAC RPCI-23-67P8 and RPCI-23-94L9, a distance of 1 Mb. **D.** Map of the Mmu 9 critical region, including the map positions of BAC markers used in FISH analysis and nearby gene markers. The 1 Mb region to which the 12Gso breakpoint maps is indicated in red. This 1 Mb region is relatively gene-poor; only 5 ESTs are evident in the region: *Snap91*, a hemoflavoprotein-related EST, two uncharacterized ESTs, and *Tbx18*.



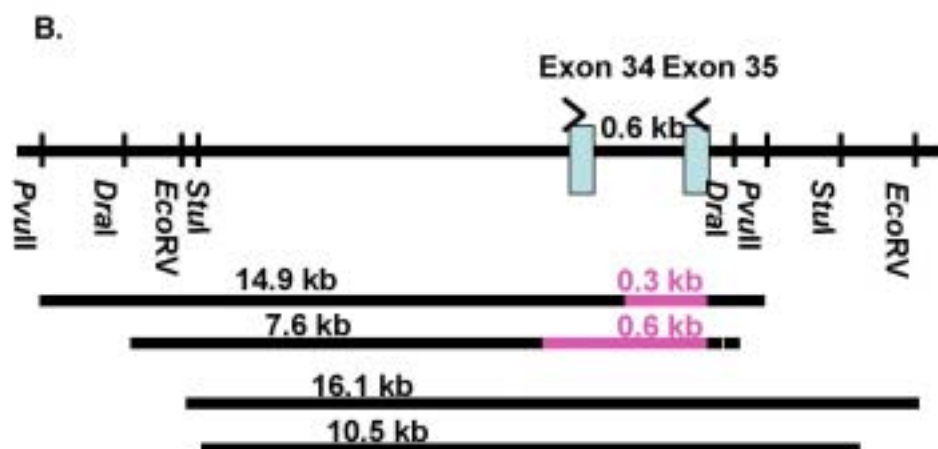
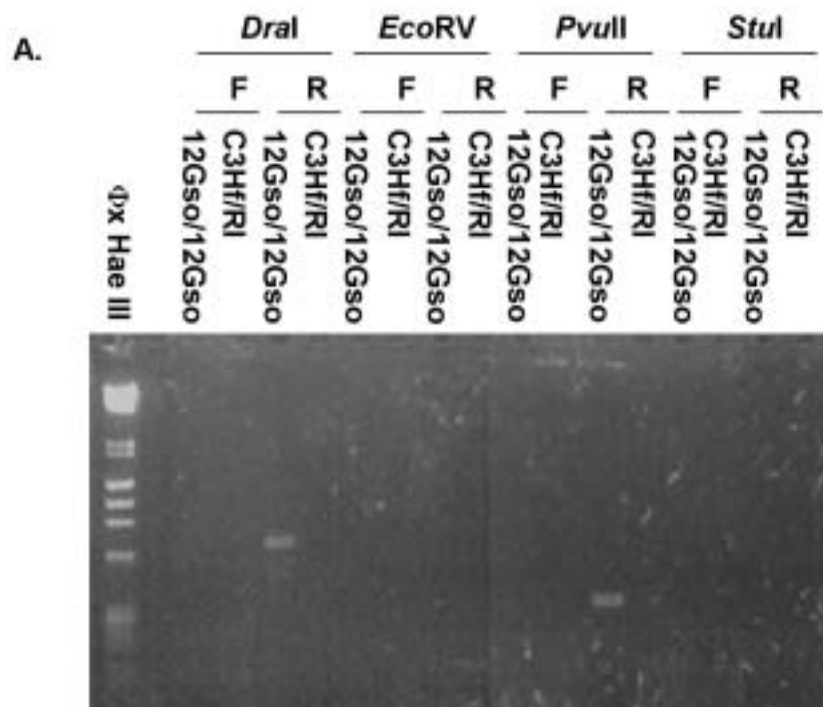
1999). In the mouse, the *Tbx18* gene is expressed from E7.75 to E13.5 in the anterior portions of the somitomers in the presomitic mesoderm, and in the sclerotome of the epithelialized somites. Transcripts were also detected in the developing heart and limb, the genital ridge, and the vibrissae (Kraus *et al.*, 2001).

One of the uncharacterized ESTs from chromosome 9, *Rock1*, is thought to be involved in transcription and encodes regions of M-repeats, protein motifs common in many myosin heavy chains, and a rho domain, found in some members of the *ras* family of oncogenes. Libraries from which this EST was isolated include osteoclast, bone, cartilage, neonatal kidney, and 11-day embryo. Another of the ESTs from chromosome 9 encodes a transmembrane domain, and library tissues are mostly brain-related. The final EST of the chromosome 9 region is related to flavohemoproteins and encodes an oxidoreductase FAD-binding domain and cytochrome b5 domains. This EST has been isolated from numerous libraries, some of which are bone, B-cell and tumor.

Analysis of a fragment containing the t(9;4) chromosomal breakpoint

To clone the Mmu9 breakpoint, primers derived from the Mmu4 junction sequences were used to carry out a "Genome Walk" across the breakpoint in 12Gso homozygote DNA. This method permits PCR generation of sequences extending from a gene-specific primer to a linker sequence ligated to the end of a restriction enzyme site located within 6 kb from the primer sequence. A primer designed from *Abca1* exon 35 was used to walk "upstream" relatively to the gene into adjoining Mmu 9 sequences. The results from the Genome Walker experiment provided two PCR products representing restriction fragments in 12Gso/12/Gso DNA that were not present in C3Hf/RI DNA (Figure 25). The wild type fragments in this region, as predicted by published DNA sequences, were large (~10 kb) and expected to exceed to limits posed by the kit (Figure 25). No Genome-walking fragments from the use of *Abca1*-exon 35 primers were expected in wild-type DNA. The two resulting fragments in 12Gso/12Gso DNA, therefore, could represent breakpoint-spanning fragments, and were thus isolated,

Figure 25. Results from the Genome Walker experiment. Abbreviations: F, forward primer from exon 34; R, primer set D reverse primer. **A.** Use of the Genome Walker kit (Clontech) resulted in the production of two different fragments from libraries of digested 12Gso/12Gso DNA (*Dra*I and *Pvu*II libraries; arrows). Band intensities of the 300 and 600 bp fragments were amplified for greater visibility. **B.** Schematic of the expected and resulting products from the Genome Walker experiment. Arrows designate primers used for the genome walk. Black bars represent the various fragments expected from +/+ DNA, and the resulting fragment generated in 12Gso is labeled in red within the black bars.



subcloned, and sequenced. BLAST analysis of the sequence from the smaller fragment indicated that this fragment did contain sequence both from *Abca1* and Mmu9. The *Abca1* sequence ended 46 base pairs upstream of *Abca1* exon 35, and the Mmu9 sequence matched public mouse DNA sequence roughly 85 kb from *Tbx18* and roughly 300 kb from the *Rock1* EST. There was a 2 base pair ambiguity in the sequence, which could have originated from either chromosome, but no complex insertions or deletions are apparent at the translocation breakpoint (Figure 26). As the breakpoint junction maps to the location predicted by FISH analysis, these large rearrangements were not expected. Complete analysis of the breakpoint sequence for the possible presence of small rearrangements awaits the cloning of the reciprocal breakpoint junction.

Figure 26. Double-pass sequence of the t(9;4) PvuII fragment generated in the Genome Walker experiment. Alignment of the t(9;4) breakpoint clone sequence (top line) with homologous sequence from a region of Mmu 9 mapping approximately 88.5 Mb from the centromere (middle line, green). and *Abca1* (bottom line, blue). The t(9;4) sequence is color-coded to match alignments. There are two ambiguous base pairs at the junction (red); these base pairs could have derived from either Mmu4 or Mmu9.

CTGGAAGGAATAGGTTNTTGTATTATCTTTAATTGAAAGCAATGCTCC	t(9
CTGGAAGGAATAGGTTNTTGTATTATCTTTAATTGAAAGCAATGCTCC	Mm
-----	Abc
ACCACTGCACCTACAGCAGGTTTTGTGCACTCTCCCCCTGGTGAATGG	t(9
ACCACTGCACCTACAGCAGGTTTTGTGCACTCTCCCCCTGGTGAATGG	Mm
-- -----	Abc
GGCAGGAGATAAGAA TA AGTTTCTGAAGTTTCCATGCACGTTTGTG	t(9
GGCAGGAGATAAGAA TA -----	Mm
----- TA AGTTTCTGAAGTTTCCATGCACGTTTGTG	Abc
CATATACGCTTACTTCTTGAAG	t(9
-----	Mm
CATATACGCTTACTTCTTGAAG	Abc

CHAPTER 4: DISCUSSION

The goal of the 12Gso project

While the original purpose of the 12Gso project has not altered since its inception, the focus of this project has expanded as new knowledge about the mutation has come to light. When work on 12Gso began, only basic information was available: the mouse carried a reciprocal translocation and exhibited profound skeletal deformities. A few small but obscure clues to the reduced fitness were also available, such as the high dead implant rate, decreased homozygous mutant survival, failure of liveborn mutants to thrive, and the scruffy coat that accompanies many mouse immune disorders. As this research progressed, however, this mutant undertook new roles as a model to support research in hematopoietic development and lipid metabolism.

This thesis work has been devoted to the characterization and development of 12Gso, a unique mouse model exhibiting skeletal malformations, aberrant hematopoiesis, and disruption in lipid transport. The focus of this work included phenotypic and genetic studies, as well as physical mapping and molecular analysis of the translocation breakpoint sites. The long-term goals of this work are to determine and characterize the gene(s) disrupted by chromosomal breakage in 12Gso mice, and to utilize those gene(s) as a basis for uncovering the mechanisms that underlie inherited forms of lymphoproliferation, skeletal defects and development, and lipid transport in mammalian systems.

Analysis of the skeletal phenotype of 12Gso homozygotes

These studies have linked outward morphology of homozygous 12Gso mice to specific skeletal anomalies including rib and vertebral fusion and improper vertebral formation and spacing. By contrast, the animals have a relatively normal appendicular skeleton. These skeletal deformities are strikingly similar to those presented in members of the *Notch-Delta* segmentation pathway, hinting that the likeness between these mutants and 12Gso may not be superficial, but could reflect nearly identical types of disruptions in basic skeletal development. These data strengthen the likelihood that the

12Gso mutation disrupts a gene that represents another link within that developmental cascade. Even if 12Gso does not prove to be a member of this developmental pathway, the skeletal phenotype associated with this mutation is strongly indicative of segmentation defects. More specifically, the two characterized mutations that most resemble 12Gso are *Dll3^{pu}* and *Lfng^{-/-}*, both of which exhibit a lack of segment borders and random rostrocaudal polarity in the null state. This is in contrast to several other members of the *Notch-Delta* pathway, which either present segment borders or completely nonpolarized or polarized somites (Table 5). From this we can hypothesize that the gene(s) affected in 12Gso may be closely related to either the *Delta* or *fringe* genes or interacting partners of these loci.

The aberrant skeletogenesis presented in 12Gso homozygotes is phenotypically similar to the NBCC and spondylocostal dysostosis disease phenotypes outlined previously. However, as the genes responsible for these phenotypes are known (*Ptch* and *Dll3*, respectively), and neither of these are candidates for 12Gso, the gene responsible for the skeletal phenotype in 12Gso is likely to represent a novel disease mechanism. The 12Gso homozygous phenotype may still represent the manifestation of a gene within a pathway that is also disturbed by some of these known human and mouse mutations, either as an upstream or downstream effector. Database searches currently indicate no abnormal skeletal phenotype linked to 6q14, the homologous region to which the 12Gso breakpoint has been narrowed on Mmu9. However, Shwachman-Diamond syndrome, although mapped to HSA7, was documented in a single patient with a t(6;12)(q16.2; 21.2) translocation (Masuno *et al.*, 1995). This syndrome carries not only the skeletal phenotype of chondroplasia, but also bone marrow aberrations that frequently become leukemic (Pringle *et al.*, 1968; Smith *et al.*, 1996). It is possible that while this patient may not have actually presented Shwachman-Diamond syndrome, the phenotype could be related to those documented for 12Gso homozygotes.

Table 5. Targeted alleles of Notch-Delta pathway genes involved in mammalian skeletogenesis.

<i>Notch-Delta</i> pathway gene	Protein function	Knockout phenotype
<i>Notch1</i>	Receptor	Embryonic lethal; irregular somite border
<i>Dll3</i>	Ligand	Profound vertebral aberrations; lack of somitic segment borders; random polarity
<i>Dll1</i>	Ligand	Profound vertebral aberrations; lack of somitic segment borders; lack of polarity
<i>Lfng</i>	Glucosyl transferase	Reduced viability before birth and until weaning; profound vertebral aberrations; lack of somitic segment borders; random polarity
<i>Mesp2</i>	Transcription factor	Early lethality; skeletal anomalies; lack of somitic segment border; caudalized polarity

The abnormal bone marrow phenotype in 12Gso/12Gso is leukemic

Most mouse models of B-cell hyperproliferation derive from genes specifically targeted by transgenics or knockouts, so finding novel genes associated with this family of diseases is rare, and is mostly limited to human case studies. Because 12Gso does not develop the hepatosplenomegaly normally associated with human leukemias, the leukemia may not have been identified had the mice not been sectioned to examine bone structure. The marrow of a 12Gso homozygote was first examined using the acidic/basic combination of an H & E stain to study the composition of the bone marrow and liver. Upon examination, it was noted that a marked cellularity was present, and the cells bear morphological similarity to B-cells. Further examination of this mouse and several other 12Gso homozygotes at various ages confirmed this, and the darkly staining nuclei and diffuse congregations of these cells are associated with the histology of chronic lymphocytic leukemia.

As the signals from hematopoietic antigens tested on the 12Gso/12Gso and +/- samples were relatively equivalent, more sensitive methods would be needed to ascertain any quantitative differences in expression. And although hepatosplenomegaly is distinctly lacking in 12Gso homozygotes, the grossly normal organ pathology does not rule out the possibility of 12Gso being a model for CLL, as 12Gso homozygotes do present a clonal expansion of mature B-cells such as defined by the epidemiology of CLL. If 12Gso truly represents a model of CLL, it would be one of the few mouse models that exist for this common leukemia, and one with a previously uncharacterized disease mechanism, as the 12Gso chromosomal breakpoints do not overlap with known genes associated with classical pathways contributing to the manifestation of this disease.

Numerous deletions mapping to the region between HSA 6q13-q21, which shares homology with the 12Gso Mmu9 breakpoint region, have been documented with relation to many different forms of leukemia, including ALL, AML, and CLL, and are often associated with a poor prognosis. This information would lead to the assumption that

the *Mmu9* gene affected in 12Gso/12Gso mice is likely to be associated with the bone marrow hypercellularity. However, a known gene that would indicate involvement in a leukemic phenotype has not been specifically mapped to this region, so elucidation of the gene responsible for this phenotype in 12Gso homozygotes would provide a valuable resource for dissecting the mechanisms underlying these common human diseases.

Penetrance of the 12Gso homozygous phenotype

Preliminary data showed that the reduction in number of viable mutants in early breeding studies at LLNL was related to background genetic effects, as mutant production again increased after backcrossing the 12Gso translocation onto an inbred C57BL/10J background for four generations. These data indicate that the penetrance of the phenotype is variable, as different extremes are seen on different backgrounds. The translocation homozygotes do not survive well on a B6C3 background, and though generally less viable, survive past birth on BLH and C57BL/10J backgrounds. Studies done on many other mouse mutations have also indicated the importance of background when examining phenotype (e.g., Gruneberg, 1954; Green, 1955; Letts *et al.*, 1995) and these studies indicate that genetic modifiers of the 12Gso/12Gso lethality and skeletal defects also differ between mouse strains.

It was for this reason that several generations of offspring were generated during complementation testing with *tk*, even when it seemed apparent that the mutation was not allelic by the first generation. However, after three generations, no real indication of allelism was present, indicating the two mutations are complementary. Although allelism with *tk* would have more readily given a greater amount of information regarding the 12Gso phenotype, as well as to more quickly pinpoint the *Mmu9* translocation breakpoint, it appears that these two mutations, though similar, are not allelic. No other known skeletal mutations map near the 12Gso breakpoint; the closest mutations other than *tk* are *kyphoscoliosis* and *Bmp5^{se}* both of which map more than 6 cM outside the breakpoint interval, and neither of which are closely related to the 12Gso phenotype. If *Tbx18*, or another undiscovered gene is the candidate for the 12Gso skeletal phenotype, allelism testing for this gene would need to be done in an alternate

manner. A mouse knockout of *Tbx18* (or the gene suspected) could be generated, or the presence of this affected gene in mutagen-treated (such as ENU or EMS treatment) collections of either mice or ES cell lines (Chen *et al.*, 2000; Munroe *et al.*, 2000) would be a useful resource for testing candidacy, thus providing possible evidence for the association of this gene with the 12Gso phenotype.

The relationship between translocation breakpoints and gene(s) associated with the mutant phenotypes

One of the questions frequently asked when examining a phenotype derived from mutagenesis schemes is with regard to the possibility of a second, linked mutation that is responsible, either in part or in whole, for the mutant phenotype. While this scenario cannot be entirely ruled out at this point in the 12Gso project, the methodology used to screen offspring has greatly reduced the probability of this occurrence. First, animals were selected for further breeding experiments based on karyotype, rather than phenotype. Because the karyotypic presence of the translocation is required for establishment as a carrier, one-sixth of the progeny from carrier matings (reduced from one-fourth because of unbalanced complement in a portion of the embryos) should receive a copy of the translocation from each parent. If manifestation of the phenotype is consistent with the inheritance of two copies of the translocation, these translocation homozygotes should be phenotypic mutants. In keeping with this, all mice born with the skeletal phenotype were genotyped as homozygous for the translocation, and prenatal death was also clearly associated with inheritance of two copies of the translocation. Conversely, none of the non-affected mice genotyped carried two copies of the translocation. These results support the concordance of the phenotype with the genotype, even if specific and solitary involvement of the translocation is not proven.

To deduce the actual possibility of a second, linked mutation being associated with the phenotype, analysis of the genomic region surrounding the breakpoint was done to determine the quantity of remaining founder DNA surrounding the breakpoint. However, our data indicate that the initial mutagenic lesion occurred on the C3Hf/Rl chromosome of the mutagenized founder male and not on the 101/Rl chromosome. No

101/R1-specific alleles were detected in the 20 cM region surrounding the breakpoint. Because C3Hf/R1 and later, B6C3, were used to maintain the 12Gso stocks its is impossible to determine where founder DNA ends and maintenance stock DNA begins, at least on chromosome 4. Because direct measurement of the amount of founder DNA is impossible to determine, we must use the rough estimate provided by Silver's equation. These calculations predict that only 10 cM of DNA from the originally mutagenized chromosome remain in present stocks. If 1 cM is equivalent to approximately 2 Mb (Silver, 1987), this distance can be estimated as an averaged 20 Mb.

Chi-square analysis predicts that the maximum possible distance between the translocation and the mutation can be estimated, with 95% certainty, to be less than 11 cM. While this distance may be large, the size of the sample to be analyzed certainly has a large influence on these numbers. As numbers of liveborn mutants increase, and with the assumption that the 12Gso/12Gso karyotype will accompany the phenotype, the estimated interval should decrease. However, it must be noted that these data are assuming no there is no recombination suppression deriving from the translocation breakpoint.

At this time, the issue of recombination suppression must be addressed, especially since the projected estimates of founder DNA are based on theoretical and not observed data. Yeast is frequently looked to for models of translocations because of the frequency of occurrence in the species, and recombination suppression almost invariably accompanies translocations in yeast (Herman, 1978; Rosenbluth and Baille, 1981; McKim, 1990). This reduction of crossover events is also seen in *Drosophila* translocation heterozygotes, albeit not to the same extent (Roberts, 1970; Roberts, 1976; Hawley, 1980). However, this generalization may not be made for mice. Studies with regard to synaptonemal complexes and crossover events have indicated the arm ratios of the synaptonemal complexes in Giemsa (G)-dark bands is much shorter than that of G-light bands (Solari *et al.*, 1980), possibly due to differential packaging as evidenced by synaptic participation differences between heterochromatin and euchromatin at pachynema (Stack, *et al.*, 1984). Indeed, differences in condensation, replication, and GC content are known to exist within the G-dark and G-light bands (Bickmore and

Sumner, 1989), so differential packaging of the different bands is not an unreasonable assumption. The degree of synapsis is inversely related to the amount of crossing over: crossover could preferentially occur in the G-light bands, where synapsis is limited to homology (Ashley, 1988). These findings are especially relevant with regard to chromosomal translocations, and represent the foundations of a theory put forth by Ashley (1988). This theory states that when both chromosomal breakage events in a translocation occur in G-light bands, there is no recombination suppression, but if one or both of the chromosomal breakage events occur in a G-dark band, evidence of recombination suppression does not exist. This theory was further tested by Ashley and Cachiero (1990), and the definition was further refined to include the bands designated as “hatched” (Nesbitt and Francke, 1973) to behave as G-light bands and those designated as “stippled” to behave as G-dark bands when synaptic behavior is analyzed. With all of this in mind, it must be noted that the translocation breakpoints of 12Gso, 4B3 and 9E3.2, both occur in G-light bands, and in accordance with theory, should not be subject to significant levels of recombination suppression. This said, it must be noted that recombination suppression cannot be completely ruled out, as certain mouse regions, including that of the *t* region (Justice and Bode, 1988), are subject to repression.

Our data predict that if a second mutation is responsible for the 12Gso phenotype, it must be closely linked (within 20 Mb) to the translocation breakpoint sites. Given the low frequency of mutational events expected from the mutagenic regimen in which the 12Gso mutation arose, the existence of a second mutation is highly unlikely, although the possibility must be taken into account in further studies with this mutant strain. At least, these studies provide support for the notion that mapping the translocation breakpoints will bring us near the site of the gene(s) that are disrupted by mutation in this mouse strain. While disruption of the gene is supportive evidence with respect to candidacy, the possibility of another affected gene cannot be ruled out, even if this second locus is not disrupted directly by the translocation or a second mutation. Instances of position effects have been reported in species ranging from *Drosophila* to human, but the distance of the affected loci from the translocation (inversion, deletion, etc.) is usually less than 300 kb (Bedell *et al.*, 1996). While the exact mechanism by

which position effects occur is elusive, current theories propose that the effects are usually caused by either position effect variegation, in which aberrant repression of a translocated gene is caused by condensation of the proximal heterochromatin (Eissenberg, 1989; Henikoff, 1990), by more subtle, long-range chromatin effects (Townes and Behringer, 1990; Romao, 1991), methylation (Bartholomew and Ihle, 1991), or by long-range transcriptional elements (Feingold and Forget, 1989; Elder, 1990). However, this is unlikely (discussed below).

Localization of the 12Gso chromosomal breakpoints

The molecular analysis of the 12Gso translocation was initiated with the placement of markers on Mmu 4. At that time, the maps and probes for Mmu 4 were more complete and accessible than those available for Mmu 9. Many of the probes required for this project were not at that time mapped in mouse; these probes were obtained by the hybridization of clones from the homologous human regions to the mouse IB panels. The mapped gene marker probes were then used to isolate BAC clones for FISH mapping. Several probes corresponding to gene markers were initially used in FISH mapping of Mmu4, scattered at variable distances along the length of the chromosome. These FISH experiments gradually narrowed the breakpoint region to one in which we had no probes available at the time, between flanking markers *Baat* and *Lv.*, to BACs selected from the public databases, and finally, a single BAC clone that spanned the breakpoint (RPCI-23-353G1). The sequence of this BAC was used to generate primers for more accurate localization of the chromosomal breakpoint on Mmu4. These primer sets eventually exhausted the region of the BAC sequence outside of the *Abca1*, so RT-PCR was used to traverse the *Abca1* gene. The breakpoint was indeed found in the *Abca1* gene, between exons 34 and 35.

The possibility of position effects and the altered expression of genes neighboring the breakpoint were discussed previously (see above). However, as the chromosomal break occurred within *Abca1*, expression levels and patterns should be indicative of the candidacy of this gene, and expression analysis of neighboring genes would also determine if multiple genes are affected by the Mmu 4 chromosomal

breakage events. If immediately neighboring genes are not affected, other genes further upstream or downstream of *Abca1* are unlikely to be affected without the existence of a second mutation. What must also be considered is that the phenotypic effects seen in 12Gso may not be due to the disruption of only one gene. Because there are two chromosomes broken, there still exists the possibility of two genes being affected by this translocation. For this reason, FISH analysis on both sides of the chromosomal break was necessary. The 1 Mb size of the Mmu9 region, established by FISH analysis, represented a manageable distance for analysis of additional candidate genes.

Molecular analysis of breakpoint region

Because the breakpoint has been specifically localized, cloned, and its sequence is in the process of being analyzed, access to the answers to several questions is now possible. The first of these questions is asked with regard to why the translocation occurred at that precise location, and what genomic features preclude breakage and nonhomologous recombination between the two chromosomes.

The mechanisms that drive the formation of translocations remain elusive, and vary slightly depending on whether the derived translocation is somatic or germline. The possibilities presented in somatic cells are much less complicated than those in germline cells, and include homologous recombination, such as an exchange between repetitive elements, or nonhomologous recombination, which could either be a random event (e.g., double-stranded breakage events from radiation damage that are consequently repaired) or nonrandom (e.g., enzyme cleavage). Regarding germline-derived translocations, which can be compared to those produced in the mouse heritable translocation test, the translocation could occur in either sex, in pre-meiotic, meiotic, or post-meiotic stages, and could then either be homologous, random nonhomologous, or nonrandom nonhomologous. It has been generalized that most mammalian translocations are a result of nonhomologous recombination. Studies have shown that, at least in mouse and human, the distribution of chromosomal translocation breakpoints has been relatively random, albeit slightly biased towards occurrence in G-light bands (Warburton, 1991; Stubbs *et al.*, 1997).

However, analyses of many different translocation breakpoint regions have identified very short spans of identical base pairs (often in the form of repeats) which lie within the critical breakpoint regions on both chromosomes involved in the translocation, and may actually serve to stabilize the translocation. Repetitive regions in particular have been strongly implicated as they are genomically unstable and susceptible to the double-stranded breakage associated with translocations, as well as leaving chromosomal “ends” which can more easily undergo interchromosomal recombination than unique sequence (Bodrug *et al.*, 1991; Giacalone and Francke, 1992; van Bakel *et al.*, 1995; Kehrer-Sawatzki *et al.*, 1997; Ishikawa-Brush *et al.*, 1997; Edelmann *et al.*, 1999; Masuno *et al.*, 1995, Budarf *et al.*, 1995; Chernova *et al.*, 2001). Other observed “markers” for translocations which hint at recombination mechanisms include the C-like octamer (Krowczynska *et al.*, 1990; Sowerby *et al.*, 1993), Z-DNA derived from alternating purine/pyrimidine residues (Boehm *et al.*, 1989; Love *et al.*, 1989), topoisomerase I and II consensus recognition sites (Bae *et al.*, 1988; Laemmli *et al.*, 1987; Spitzner and Muller, 1988; Sander and Hsieh, 1989), immunoglobulin heptamers (Chen *et al.*, 1989) or a-protein recognition sites (Sowerby *et al.*, 1993). Another interesting point to note is related to the sequence at the actual breakpoint junction. In the vast majority of breakpoints in the human constitutional translocations that have undergone analysis, there is a small loss of DNA and the common accompaniment of a small addition of DNA from a source other than the breakpoint region. Presumably, this occurs because of an initial degradation of the free ends generated upon double-stranded breakage of the chromosomes, followed by a repair mechanism which would have used the additional nucleotides to “fill in” the gap during repair of the break (Richardson and Jasin, 2000; Bodrug *et al.*, 1991). The additions are rarely over 100 bp, in good agreement with the repair theory. These deletions, while typically less than 100 bp, do not always follow this model: at least two recorded cases have reported larger deletions of 518 bp (Krebs *et al.*, 1997) and 5000 bp (Giacalone and Francke, 1992).

The sequence of the t(9;4) breakpoint region is now known, and as of current analysis, does not appear to contain any of the aforementioned elements to indicate

possible mechanisms for translocation, nor does it indicate deletions or rearrangements. However, because the cloned fragment is very small, further verification by PCR will be needed to assess the integrity of the chromosome 9 DNA. The sequence of this fragment did verify the occurrence of the chromosomal break in *Abca1*, in the intron between exons 34 and 35. The sequence on the Mmu9 side of the breakpoint will need much more scrutiny in order to determine the causative factors for the skeletal phenotype. The sequence is not interrupted in the middle of a mapped gene, but this does not preclude the separation of regulatory elements for either *Tbx18* or *Rock1* from the respective gene by the chromosomal rearrangement, or that either gene does not extend further into this poorly mapped region. There are several methods available to test these theories. First and foremost, embryonic expression may now be tested because sequence is available at the breakpoint junction. Now that the means are available to screen embryos from heterozygote crosses, experiments such as whole mount and/or section *in situ* hybridization, Northern analysis, RT-PCR, and microarray analysis can be performed. Second, if sequence analysis indicates the presence of a regulatory element, luciferase assays may be used to test the expression driven by the regulatory element. The indication that the break does not occur in an Mmu9 gene also assumes that current maps are correct, and a gene does not map to this region. However, available mouse sequence data is far from complete at this time, and the possibility of another gene mapping to this region still exists. Generation and analysis of sequence data from this region should provide more clues to support or refute the presence of a disrupted gene, which would be subjected to similar expression analysis as mentioned for *Tbx18* and *Rock1*.

Preliminary evidence for Abca1 as the candidate gene associated with the reduced fitness of 12Gso/12Gso mice

In *Abca1* knockout mice, homozygous mutant offspring of heterozygote crosses are generated at a rate of 8.3%, much decreased from the expected Mendelian ratio (McNeish *et al.*, 2000; Christiansen-Weber *et al.*, 2000). The same is true for 12Gso: initial karyotyping results indicated that the homozygotes were either dying prenatally, or dying just after birth so as not to be counted in initial litter tabulation. Further

analysis of earlier, prenatal stages indicated that, at least on a B6C3 background, mutants were being generated at expected rates through at least the last day of gestation. This observation would indicate that mutants were indeed dying by some means shortly after birth.

As PCR and cloning results indicated that the chromosomal break occurred within the *Abca1* transcription unit, and RT-PCR experiments indicated that a truncated transcript is produced from this gene, closer scrutiny of *Abca1* as a candidate for some aspects of the 12Gso phenotype was therefore imperative. In studies of targeted mouse *Abca1* null mutations (Christiansen-Weber *et al.*, 2000; Orso *et al.*, 2000) the percentage of homozygote mutants born live was significantly lower than expected, and postnatal viability was also substantially decreased. In fact, survival rates for *Abca1*^{-/-} offspring correlate well with decreased 12Gso homozygote survival rates on a B6C3 background (12Gso, B6C3 background: 7.4%; *Abca1*^{-/-}, C57BL/6J background: 8.3%). The hypothesis presented as an explanation for the reduced viability was that altered steroidogenesis caused severe placental malformation leading to embryonic runting and fetal and neonatal loss. The aberrant steroidogenesis in *Abca1*^{-/-} mice manifested as levels of progesterone and estrogen levels that were each decreased more than 50% in homozygous females. This information would implicate *Abca1* as being associated with the early death of 12Gso homozygous mice and could possibly correlate with the infertility we have noted for all mutant mice of either sex.

Abca1 encodes a large transmembrane protein involved in cholesterol homeostasis. Hydropathy plots of the protein indicate that ABCA1 and other members of the ABC-A superfamily share common unique features, including the presence of two ATP binding cassettes, a regulatory domain positioned between these two cassettes flanked by two hydrophobic regions, and a highly conserved N-terminal hydrophobic region (Schmitz and Langmann, 2001). The role of ABCA1 is thought to be involved in the ATP-dependent transport of lipids to lipid-free apolipoprotein A-I (Fielding and Fielding, 2001; Schmitz and Langmann, 2001) during a process called reverse cholesterol transport. Reverse cholesterol transport begins with cholesterol efflux, one

mechanism of which is ATP-dependent and facilitated, such as the microsomalization pathway of efflux mediated by ABCA1 and ApoA-I (Fielding and Fielding).

Apolipoprotein A-I (ApoA-I) is the major component of high-density lipoprotein (HDL), the function of which is the removal of excess tissue cholesterol (Chiesa *et al.*, 1998; Oram, 2002). Evidence for a relationship between ABCA1 and ApoA-I is confirmed in Tangier disease, which is linked to a mutation in the *Abca1* gene. ApoA-I is produced at consistent levels in Tangier disease patients, but is degraded much more rapidly. This ability of ApoA-I to accept phospholipids is thus reduced, leading to the serum HDL deficiency (Oram, 2002). Though originally the Tangier disease findings indicated a direct role for *Abca1* in phospholipid and free cholesterol transport out of the cell, current data has alluded to an indirect transferal of phospholipids to ApoA-I, without the necessity of direct binding of this protein to ABCA1 (Fielding and Fielding, 2001). However, the exact mechanism for ABCA1-dependent reverse cholesterol transport has not been clearly elucidated.

Mutations in this gene that result in the encoding of a nonfunctional or truncated protein result in defective lipid transport, which has pleiotropic effects on the rest of the anatomy. As a result of aberrant lipid transport, *Abca1* targeted null mutant mice exhibit reduced platelet count, hemorrhagic diathesis, fat-soluble vitamin deficiency, impaired leukocyte-platelet aggregation, increased production of macrophages, megakaryocytes, and erythroid precursors, increased immune complexes, low serum cholesterol, lipid deposition in specific tissues such as liver, bronchopulmonary dysplasia resulting in respiratory distress, placental malformation, runting and fetal distress, aberrant female steroidogenesis, cardiomegaly with hypertrophied ventricles, congested liver, spleen, and thymus, and glomerulonephritis (McNeish *et al.*, 2000; Orso *et al.*, 2000; Christensen-Weber *et al.*, 2000). When examining the array of genes for which expression is significantly down-regulated or absent in the 12Gso homozygote bone marrow, we can see that 12Gso presents the genetic hallmarks of thrombosis and hemorrhaging, chronic inflammation, macrophage proliferation, and serum HDL deficiency, a definite parallel to observations of *Abca1*^{-/-} mice. Whether or not this gene is related to the bone marrow hypercellularity remains to be seen. While *Abca1* can

cause an increase in the number of erythroid precursors, megakaryocytes, and macrophages, excessive presence of these cell types have not been examined in the bone marrow.

The microarray analysis on 12Gso/12Gso tissue provides preliminary evidence for the notion that the absence of functional *Abca1* plays a significant role in the lack of fitness of 12Gso homozygous mice (Table 2). As has been noted in microarray studies of *Abca1*-deficient mice (McNeish *et al.*, 2000), several key genes involved in cholesterol and lipid metabolism are significantly down-regulated in 12Gso mutant tissues. Included in this group are several apolipoprotein genes (Table 4): *Apoa1* and *Apoa2*, the lack of which drastically decreases the serum levels of high density lipoprotein (HDL; Franceschini *et al.*, 1988; Yui *et al.*, 1988; Warden *et al.*, 1993), and *Apoa5*, the lack of which is involved in increased very low density lipoprotein (VLDL) and increased triglyceride production (Pennacchio *et al.*, 2001). In patients of Tangier disease, the human form of *ABCA1* mutations, and the mouse targeted *Abca1* mutations, blood serum presents an almost complete lack of HDL components and increased triglyceride production (Pietrini *et al.*, 1985; Orso *et al.*, 2000; McNeish *et al.*, 2000). The decreased expression of these genes in the 12Gso/12Gso tissue encourages the hypothesis that *Abca1* disruption is likely to be a cause in the lack of fitness exhibited by 12Gso homozygotes. As indicated in several cases of Tangier disease, the absence of full-length transcripts for this gene are sufficient to eradicate the normal function of the gene. This lack of function in a truncated product would explain the low survival rates of 12Gso homozygous offspring (Bodzioch *et al.*, 1999; Rust *et al.*, 1999; Brooks-Wilson *et al.*, 1999).

The initial microarray studies gave evidence of other genes with protein products belonging to other functional categories that may also be significantly affected in 12Gso/12Gso tissue samples. A disturbance in inflammation response, as also seen in examples of *Abca1* deficiency, is evident by the severe decrease in *alpha-1-microglobulin/bikunin precursor (Ambp)* expression. Lack of normal *Ambp* function allows the chemotaxis of neutrophils to endotoxin-activated blood sera, which eventually spirals into a full-blown inflammation response. Another gene in this category, *urate*

oxidase (Urox), is severely downregulated in 12Gso. The gene is by itself nonfunctional in humans, but mice with this deficiency die before 4 weeks of age (Wu *et al.*, 1994). Although it is not known at the time whether this gene specifically relates to *Abca1*, neither *Urox* gene expression or product levels were tested in mice deficient for *Abca1*, though the similarity in offspring viability in the two mutations is striking. The decreased expression levels of genes in the 12Gso/12Gso involved in thrombosis and hemorrhage are in accordance with the data presented for *Abca1* null mutations. These *Abca1*-deficient mutations manifest bleeding disorders and myocardial infarction, both of which could be induced by the same anti-thrombosis genes showing decreased expression in 12Gso/12Gso bone samples: *antithrombin III (At3)*, *a-2 microglobulin*, *thrombin*, *plasminogen*, and *vitronectin*. The *Cyp3* family of genes, also appearing downregulated in 12Gso/12Gso bone, is involved in the catalysis of hydroxylation of various steroids, including testosterone, progesterone, and cortisol (Wrighton and Stevens, 1992). Again, the appearance of the dysregulation of these genes in 12Gso/12Gso tissue provides yet another parallel between affected genes in 12Gso and the protein expression pattern in *Abca1*-deficient mice. The presence of the neurological genes in the 12Gso/12Gso microarray data is also interesting. While careful analysis of these genes must be performed because of the innervation of the tissue used in the microarray, these genes could be indicative of the peripheral neuropathy seen in patients with Tangier disease. The downregulation of numerous genes encoding liver enzymes is also intriguing, as many of these enzymes can contribute to liver disease, as in the case of *glycine-N-methyltransferase (Gnmt)*, or to aberrant transport from the liver to peripheral tissues (*retinol binding protein 1, Rbp1*). Although gross pathology of the brain and liver has already been examined and appears normal in 12Gso homozygous mice, these microarray data suggest that subtle abnormalities in the lungs, liver, thymus, intestine, kidneys, adrenal glands, and reproductive tissues of the mutants may be noted on more detailed examination. Such detailed analyses are presently under way.

While neither *Abca1*, nor the lack thereof, has been documented in the bone marrow lymphocytes of patients or animals with *Abca1* mutations, current knowledge does not indicate a role for this gene in B-cell lymphoproliferative disorders. However,

Abca1 deficiency has been associated with the recruitment and proliferation of macrophages and the increase in cellularity of megakaryocytes and erythroid precursors (Orso *et al.*, 2000). Other studies have indicated not only an increase in these leukocytes, but also an expansion of T-helper cells (van Eck *et al.*, 2002). However, all of these factors could be associated with inflammation, and not necessarily with lymphoproliferative disease.

If *Abca1* is responsible for the reduced fitness observed in 12Gso/12Gso mice, the most obvious human diseases for which 12Gso can serve as a model are Tangier disease and the allelic high-density lipoprotein deficiency, both of which derive from mutations in *Abca1*. Just as in the families with these diseases, which exhibit a truncated or null *Abca1* protein due to point mutations, deletions, or frameshifts, the 12Gso/12Gso mouse also presents a truncated transcript of the gene, one which separates the both the two ATP-binding sites and the two transmembrane domains, and crude analyses estimate the break is likely to map within or near the conserved regulatory domain. This disruption is further down stream in the coding sequence than the three knock out mutations described; one of these mutations targeted the first ATP-binding cassette (Orso *et al.*, 2000), one disrupted exons 18 – 22 (Christiansen-Weber *et al.*, 2000), and the third deleted 5.7 kb of the 7.4 kb transcript produced by *Abca1*.

Just as speculations can be made in the comparison of 12Gso homozygotes with targeted *Abca1* mouse mutations, the same logic exists for comparison to Tangier disease. Patients with Tangier disease report deposition of lipid in thymus and intestine, enlarged liver, spleen, and lymph nodes, peripheral neuropathy, one documented case of visual impairment, accumulation of cholesterol esters in the macrophages, and a case of myocardial infarction (Engel *et al.*, 1967; Pietrini *et al.*, 1985; Pressly *et al.*, 1987; Chueng *et al.*, 1993; Brooks-Wilson *et al.*, 1999; Schmitz *et al.*, 1999). The defects in lymphatic tissues offer a possible link between Tangier disease and therefore, *Abca1* deficiency, with the lymphoproliferative disorder we have observed in 12Gso homozygous mice. While these defects may be due to an increase in T-helper cells in these tissues (van Eck *et al.*, 2002), there still exists the possibility that there is bone marrow involvement as well. On a molecular level, patients with Tangier disease have

low levels of plasma HDL and its components apolipoproteins A-I and A-II (Cheung *et al.*, 1993). When comparing these diagnostics to the preliminary expression data presented in the microarray experiment, we see that downregulated 12Gso/12Gso gene expression could indicate a correlation with genetic components of this disease.

Identification of a possible candidate gene for the 12Gso skeletal phenotype on Mmu9

As an association of *Abca1* with bone marrow hypercellularity has not been reported, and knockout-mutant mice or human mutations suggest no involvement of this gene in somite determination or segmentation, it seems likely that the candidate for these phenotypes should lie on Mmu9. Mapping of the Mmu9 breakpoint also identified an excellent candidate for the skeletal phenotype. This gene (*Tbx18*) is but one member of a closely related family of transcriptional regulators containing T-box elements. Transcriptional regulators in general play a prominent role in development, and this family is no exception. Mutations in members of this family include the brachyury phenotype (*T*), Holt-Oram disease (*TBX5*; Basson *et al.*, 1997), and ulnar-mammary syndrome (*TBX3*; Li *et al.*, 1997; Bamshad *et al.*, 1997). While mutations in this gene have not been documented, expression patterns are consistent with the 12Gso phenotype.

The gene for *Tbx18* was considered a particularly strong candidate, as it is expressed most highly in the developing somitic tissue. Expression of *Tbx18* is also notably high in the developing heart and limb bud, and these structures do not provide evidence of anomaly in 12Gso. However, there is a distinct possibility of functional overlap between genes in the T-box family of transcription factors and it is possible that some expression sites of the gene will not be reflected in mutant phenotypes associated with the *Tbx18* gene. *Tbx18* and its paralogue, *Tbx15*, overlap in expression patterns with respect to the appendicular skeleton; both genes are expressed at high levels in the developing limb bud, with no apparent involvement of the apical ectodermal ridge (Kraus *et al.*, 2001; Agulnik *et al.*, 1998). However, a distinct difference in expression pattern between these genes is seen when examining the axial skeleton. *Tbx18* exhibits a distinct metamereric expression pattern confined to the anterior compartment of the developing somites, in contrast to a lack of expression of *Tbx15* in any but the most

anterior somites, which develop into head structures. To corroborate this data, expression patterns of *Tbx15* and *Tbx18* in zebrafish were examined, and it was found that *Tbx15* was absent in tail somites at any developmental stage, but both were expressed in the developing fin (Bengemann *et al.*, 2002). Association of *Tbx18* with a developmental pathway has yet to be assigned, and cannot be determined by the examination of family members. *Tbx18* is closest in homology to family members *Tbx8*, *Tbx15*, *Tbx14*, and *Tbx22*, and within the same phylogenetic branch as *Tbx1* and *Tbx13*. Little is known about the upstream and downstream genes for most of these, but *Tbx1*, a DiGeorge syndrome candidate gene, is regulated by *Shh* in mouse and chick pharyngeal arch development (Garg *et al.*, 2001). Expression of the less similar *brachyury* (*T*) and *Tbx6* genes are each induced by *Bmp4* (Finley *et al.*, 1999) and by *Wnt3a* (Yamaguchi *et al.*, 1999) in paraxial mesoderm specification. *T* expression in the notochord is activated by *Suppressor of Hairless* [*Su(H)*; Corbo *et al.*, 1997; Corbo *et al.*, 1998], an intermediary gene between *Notch* and the *Enhancer of Split* complex in *Notch-Delta* signaling (Bailey and Posakony, 1995). While it cannot be assumed that *Tbx18* may be a member of one of these established pathways, the connection between these pathways and other *Tbx* genes, especially those involved in somitogenesis, encourages the hypothesis that a mutation in *Tbx18* could certainly cause the skeletal phenotype presented in 12Gso homozygotes.

Future experiments proposed for studying the 12Gso mouse

Phenotypic and molecular analysis of the *Abca1* disruption

Our preliminary results have indicated that disruption of *Abca1* is likely to play a role in the 12Gso homozygous reduced fitness phenotype, suggesting many future experiments for analysis of this mutation in impending studies. Initial experiments would include analysis similar to those used for the current gene knockout models that exist for this locus. Because of the lack of grossly abnormal tissues, sites which would be most affected by the *Abca1* mutation (e.g. thymus, kidney, and liver) will be closely scrutinized for the types of subtle defects detected in *Abca1* null mutant mice and in human Tangier disease patients. Because of the indications presented in these data,

sectioned tissues of both 12Gso/12Gso and 12Gso/+ mice will be analyzed for cellular hyper- or hypoproliferation, abnormal cell differentiation, and changes in lipid content. The presence of neurological-associated genes in the microarray data could correlate to subtle changes in brain signaling, similar to the peripheral neuropathy observed in Tangier disease (Engel *et al.*, 1967; Pietrini *et al.*, 1985). While general 12Gso/12Gso behavior is counter indicative to brain involvement, the lack of available homozygotes, abnormal skeletal morphology, and general failure to thrive makes comprehensive behavioral tests difficult with 12Gso homozygous mice. Consequently, brain chemistry may indeed be slightly altered; brain sections hybridized with various probes for neurotransmitters and receptors such as those indicated in the microarray may be useful in determining the extent of brain involvement. Analysis of blood plasma will be a crucial aspect of the complete analysis of this mutation. As many of the components affected by this mutation are expressed in the plasma (e.g. *ApoA1*), blood analysis would indicate further possible protein interactions, as well as affected loci whose expression is not located in the bone or bone marrow. Included in this blood analysis would be a quantitation of HDL and triglyceride levels, as this is strong proof of *Abca1* deficiency. All these experiments should also include samples from heterozygote mice; serum data reported for targeted mouse *Abca1* heterozygotes and Tangier disease relatives make it probable that expression levels could be altered in 12Gso heterozygotes as well.

A major area of future study, however, will be to determine whether or not *Abca1* has a role in the hypercellularity of the bone marrow. Though documented cases of marrow involvement are scarce, the lack of information may only be due to lack of observation. One way in which the role of *Abca1* may be tested for contribution to lymphoproliferation would be complementation testing, involving crosses of 12Gso carriers to animals carrying *Abca1* targeted null mutations. These animals are available from Jackson Laboratories and complementation studies will soon be in progress. Complete analysis of the marrow from animals that are homozygous *Abca1* null, and from offspring of a 12Gso x *Abca1*^{+/-} that have been determined to be doubly heterozygous for the two mutations should help determine the contribution of *Abca1* gene truncation to this aspect of the 12Gso phenotype (see below). As a corollary to

this, transgenic mice overexpressing the *Abca1* gene are also available, and breeding experiments with these mice should complement the genetic deficiencies indicated in 12Gso/12Gso lipid efflux. The bone marrow of the offspring from the crosses with the transgenic strain can be examined for hyperplasia. The combination of these two breeding experiments should confirm or refute the contribution of *Abca1* to the 12Gso/12Gso bone marrow phenotype.

Determination of the root causes for the lack of viable male 12Gso mutants

Of the 14 12Gso homozygotes generated, only 3 of the offspring were male, and both of these were sickly and died within a few weeks after birth (data not shown). Though the sample size is too small to state conclusively that a gender bias does exist, the current numbers could represent a general trend in which the reduced fitness is further exacerbated in males. One clue to this puzzle may be given by the downregulation of many genes encoding products involved in steroidogenesis downstream of *Abca1*, including *hydroxysteroid 17- β dehydrogenase 1* (less than 10-fold decrease; data not shown), a gene associated with the processing of estrone into estradiol (Tremblay *et al.*, 1989), but is closely related to *hydroxysteroid 17- β dehydrogenase 3*, the gene implicated in human pseudohermaphroditism (Geissler *et al.*, 1994). This aspect of the 12Gso phenotype has not been explored, and would certainly deserve some future focus. While *Abca1* could be involved in this process, it is also possible that a second gene residing on Mmu9 may play a role, or it may derive from an artifact due to breeding schemes, specifically the fact that maintenance of the stock always derives from the male, which may be transmitting other loci from the Y chromosome. A full accounting of any possible gender bias in live-born mutants must therefore await both a more complete molecular and genetic description of the 12Gso mutation and an increase in numbers of 12Gso/12Gso produced.

Verification and specification of leukemic phenotype

We cannot yet point conclusively to *Abca1* as the culprit for bone marrow hypercellularity, and another disrupted gene may indeed play a role in this aspect of the

12Gso phenotype. To clarify the role of *Abca1* and other potentially contributing genes, a thorough immunophenotyping of the 12Gso/12Gso bone marrow, thymus, fetal liver, and blood will need to be carried out. Part of the reason that the current analysis is incomplete is that only a small number of anti-mouse antibodies for the diagnostic set of relevant CD antigens are available for use on paraffin sections. In addition, there is a much greater danger of RNA degradation in bone samples than in softer tissue, and difficulties may be encountered in RNA-based studies of the relevant marker genes. The most accurate way to immunophenotype the bone marrow is by FACS analysis, for which numerous anti-mouse antibodies are available. This analysis should be able to determine B- and T- cell ratios, the presence or absence of hematopoietic cells, and the developmental stage of the major lymphocyte population present in the tissue. Once these diagnostics are analyzed and a determination is made as to the form of hypercellularity, additional experiments can proceed. For example, determination of which genes are up or down regulated can be tested using documented human cases of leukemia as a guide.

A combination of antigenic markers is already available to examine the bone marrow and liver for B and T cell lineages and stages. These markers include CD117, CD18, CD5, CD23, CDw75, and CD45RO. CD117 is a marker for hematopoietic stem cells and very early lymphocytic development, we can assume that the nucleated cells do not derive from the undifferentiated cell populations. The second marker (CD18) is expressed on all leukocytes, and has a role in cellular adhesion. The third marker (CD5) is mostly expressed on T cells, but also on a small subpopulation of B-cells (termed B1a cells) and in chronic lymphocytic leukemia. The CD23 antigen is expressed on mature activated B-cells, the cellular stage whose population expands in B-CLL. CDw75 is also expressed on mature B-cells and is involved in intercellular adhesion. The final antigen (CD45RO) is expressed on most T-cell populations and thymocytes. Once alternative forms of analysis are available, a more accurate description of the 12Gso bone marrow phenotype can be made.

Expression analysis of Mmu9 candidate genes in 12Gso homozygotes and determination of developmental pathway partners

Future studies will also focus on the candidacy of *Tbx18* with regard to the skeletal, and possibly, the hematopoietic phenotypes. Because this gene has a limited window of expression, like many other genes expressed in the early embryonic stages, determination of aberrant expression has not been possible before breakpoint cloning was completed. As the embryos produced in 12Gso carrier timed matings have mostly appeared normal (LRC, unpublished), visual identification of mutants to use as templates for expression studies has been impossible. Although some embryos appear runted and developmentally delayed, and these are likely to be mutants (especially considering the *Abca1* embryonic phenotype), we cannot be certain that the delayed development and growth is due to the mutation of *Abca1*. Now that breakpoint spanning sequences are available, genotypic analysis of the embryos may be done using PCR analysis on yolk sac DNA associated with each of the isolated embryos. A more reliable genotyping scheme will involve detection of mutant Mmu4-Mmu9 junction fragments with PCR, and this should be possible within the next several months now that Mmu4 breakpoint sequences are cloned. By using two different sets of primers, one that crosses the breakpoint and one that spans the region on either normal chromosome 4 or 9, genotype of these embryos would be accessible. Wild type embryos should show a product only from that primer set which spans the normal region, and not the breakpoint. Heterozygotes should produce a product with both primer sets, and homozygotes should produce a product only with the primer set that spans the breakpoint.

Once embryos have been genotyped, gene expression analysis can proceed either in the form of whole mount or section ISH. While whole mount *in situ* hybridization can provide a more global picture of gene expression and can be more visually striking to the untrained eye, section *in situ* has many other advantages. The use of sections allows the application of multiple probes to a single embryo, as well as the ability to zoom in the exact cellular type that expresses the gene of interest. The difficult task in section *in situ* hybridization, with regard to somitic development, is encompassing all of the different structures involved in somitogenesis. Sagittal sections of an embryo that slice directly

through the midline, and thus encompassing structures like the notochord, may be difficult to come by. This can be at least partially addressed by transverse sections through the midline of the body, thus including the neural tube and notochord. Each form of *in situ* has pros and cons, and a mix of the two procedures would be most informative.

Even if *Tbx18* does not turn out to be the gene whose disruption is associated with 12Gso skeletal phenotype, this same procedure should be followed with other genes in the breakpoint region. The 12Gso skeletal phenotype, and all of the accumulated data from mutants expressing similar phenotypes, predicts that the gene will be expressed primarily, if not exclusively, during embryonic stages. To study the mechanism of action of the candidate gene, it will be particularly valuable to study expression of various somitogenesis markers. Members of the *Notch-Delta* pathway are excellent candidates for analysis of segmentation and somitogenesis, as these genes are expressed in a temporal spatial pattern that clearly defines anterior and posterior compartments of the somites (e.g. Conlon *et al.*, 1995; Dunwoodie *et al.*, 1997; Hrabe de Angelis *et al.*, 1997; Kusimi *et al.*, 1998; Zhang and Gridley, 1998; Evrard *et al.*, 1998). In a similar vein, markers in the *Hh* pathway would be useful in determining perturbation of dorsoventral polarity (Marti *et al.*, 1995). Other markers, such as *Pax1*, should be used to determine whether or not the phenotype presents notochordal involvement (Timmons *et al.*, 1994; Wallin *et al.*, 1994), and other markers, such as either *MyoD* (Hannon *et al.*, 1992) or *Mesp* (Sawada *et al.*, 2000) should be used to analyze possible involvement of the myotome. By using a series of these markers, a clear picture should evolve as to the exact function and pathway of the 12Gso candidate gene.

The downstream genes regulated by this gene can also be determined in ways very similar to those currently in use to elucidate the effects of *Abca1* in 12Gso, such as the use of microarrays and complementation testing.

Generation and testing of alternate alleles to verify the phenotype as related to the candidate genes

In order to provide conclusive evidence that a particular gene is the relevant one with regard to the phenotype, other alleles of the gene such as those generated by transgenesis, gene knockout, or targeted mutagenesis will need to be analyzed. While gene knockout or transgenesis may be necessary for testing the skeletal candidate gene for 12Gso, another option is available for providing solid evidence of the phenotypic contribution of the *Abca1* gene in 12Gso: complementation testing. Mice that carry a null mutation in *Abca1* are available from Jackson Laboratory, and by crossing these mice with 12Gso, confirmation of the contribution provided by this gene to the 12Gso phenotype (including the possible role of *Abca1* in the bone marrow phenotype) can be investigated. Compound heterozygotes would be tested as in the experiments listed for further phenotypic characterization (p. 138, above). Crosses with transgenic overexpressers of *Abca1* (Cavalieri *et al.*, 2001) can also be performed; the offspring from these crosses should be complemented at the *Abca1* locus. If all hypotheses put forward for *Abca1* are correct, that is, *Abca1* is responsible for early lethality and biased sex ratio, offspring homozygous for the translocation should exhibit the skeletal phenotype in equivalent gender ratios, and the mutants should have increased longevity. Because of complementation of the early phenotype, these mutants should also be generated at the expected ratio of 1 in 6.

Alternate alleles of *Tbx18*, if more evidence points to the involvement of this gene in the 12Gso homozygous phenotype, would need to be found or generated as well to test and verify the candidacy of the putative role for this gene in the 12Gso/12Gso skeletal phenotype. While knockouts or transgenics of this gene are not currently available, experiments could be conducted to generate a knockout for complementation crosses with 12Gso. By the same token, collections of mutagenized ES cells are available for screening (Chen *et al.*, 2000; Munroe *et al.*, 2000), and it is possible to take advantage of this resource to derive an allelic series of mutations by which the candidacy of *Tbx18* may be tested.

Final considerations

Because 12Gso is the first translocation mutant in the LLNL collection to be characterized so completely, it can serve as a prototype for the analysis of the other uncharacterized mutants within the colony. More complete protocols have now been established for the mouse translocation projects where a candidate gene is not readily obvious or where tissues expressing candidate genes are difficult to access for expression studies, as was the case with 12Gso. These features made 12Gso one of the more challenging of the translocation mutants that we might have attempted to tackle. The importance and excitement of finding novel genes associated with early lethality, leukemia, and skeletal defects with likely roots in defective early somitogenesis made the challenge worthwhile. With the influx of mouse sequence data into the public databases, finding genes associated with other mouse mutants, including those in our translocation colony, is rapidly being transformed. The pace of gene discovery has changed remarkably over the course of this endeavor, even over the course of the past year. When the 12Gso project began, many of the gene markers used in mapping were yet to be mapped to mouse chromosomes, and were necessarily subjected to IB mapping before ever being used to isolate BAC clones for FISH analysis. The rapidly changing databases now have mapped contigs of BAC clones, which essentially eliminate the need for BAC library screening. Much has changed in this aspect of translocation projects, but the steps and precautions outlined by the 12Gso project will aid in specific recovery of these breakpoints from mutant DNA and the more rapid recovery of affected genes.

The 12Gso mutation serves not only as a primary example for similar research projects to follow, but also lays the foundation for the futures of these projects as well. Work on this mouse mutation can now progress through the many channels outlined above. These channels include analysis of the genomic regions surrounding the breakpoint, complete characterization of the genes involved in the phenotype, examination of the possible involvement of other organs, determination of interacting pathway genes and modifier loci, and last but not least, the generation and/or testing of an alternate allelic mutation, such as those in the form of a transgenic or knockout, to verify or refute the direct association of the translocation with the phenotype.

Because the 12Gso mutation is a translocation, we were consistently aware of the fact that the mutation might disrupt the activities of genes in two different chromosomal sites. The pleiotropic nature of the phenotype presented by 12Gso homozygotes made the possibility of at least two affected genes - one on Mmu4 and one on Mmu9 - even more likely. Our molecular analyses provide strong indication that this prediction is true for 12Gso, as the known disorders associated with *Abca1* mutations, including early lethality and low live birth rates, steroid imbalance, and lymphatic defects, do not include defects in development of the axial skeleton. The fact that genes in two locations contribute to the 12Gso phenotype adds a complication to the study of this mutant, but also has added some interest to the analysis of the animals and potentially, to its utility as a model. In characterizing this mutation before the molecular basis of lethality was known, we have uncovered some phenotypic manifestations of *Abca1* deficiency that may have relevance to at least some Tangier disease patients. Preliminary data collected on the 12Gso mouse indicate that additional aspects of the phenotype associated with *Abca1* mutations should be scrutinized and tested. The lymphoproliferative phenotype detected in 12Gso homozygotes may also be a useful finding with respect to understanding the causes of the human form of the disease. Whether it is eventually traced to defects in *Abca1* and lipid transport or to defects in a second, unidentified gene, the 12Gso lymphatic defect is a novel finding with high potential relevance to the study of leukemia in humans. The data suggest that the 12Gso mouse represents a model for leukemia susceptibility, with striking similarities to the phenotype associated with CLL. Our examination of the Mmu4 and Mmu9 breakpoint regions have not identified any candidate genes that are known to be associated with leukemia or that are closely related in sequence to CLL-associated genes. The *Nipsnap* genes in the Mmu4 breakpoint region were originally suspected as candidates for this aspect of the mutant phenotype because of their sequence similarity to HSPC299, which is expressed on CD34+ cells in humans (Genbank, NCBI). However, though a potential role cannot be excluded unequivocally at this time, preliminary data indicate that these genes are normal in structure and expression in 12Gso/12Gso mice. Establishing a link between any of the genes in this region and leukemia susceptibility will be a novel

discovery and will highlight a new marker involved in the disease. Publicly documented cases of the classic mechanisms for CLL have not reported linkage to human regions homologous to the breakpoint sites of 12Gso, although deletions in the homologous region have been found in numerous cases of many forms leukemia.

However, it is in the study of skeletal defects that 12Gso should become particularly useful. Although *Tbx18* is a known gene with appropriate patterns of expression and represents an excellent candidate for further study, no gene located in the Mmu4 or Mmu9 breakpoint regions has been associated with inherited skeletal abnormalities. The link between this mutation and skeletal defects will therefore lead to discovery of a new factor, and perhaps a new molecular pathway, associated with inherited scoliosis and the more serious manifestations of abnormal development of the axial skeleton in humans and mice. The mouse is a particularly useful model for studying the events of skeletogenesis because of the relative accessibility of the relevant early developmental stages in mice compared to developing human offspring. Mouse mutations that are able to elucidate the pathways and mechanisms responsible for development should provide insight into human prenatal development, and possibly aid in an effort to prevent fetal abnormalities.

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APPENDIX

FORWARD	REVERSE	SEQUENCE-F	SEQUENCE-R
ABCA1209	ABCA1211	TTGCCAAGGACCAAAGTGA TGATGA	AGGGCTGTACATGGT GATAATGGACTG
ABCA1210	ABCA1213	ACCAGTGCTCAATGTCATCC GTCTTC	GATTTCATTGATATA ACATCCCCTGAATCA
ABCA1214	ABCA1214	CTTCGAGAACACACCTTAA ACATGGAGTA	CCTGTCATACACGCCA GAGTCCTAACAT
ABCA1215	ABCA1215	GAAAGAGGCACAAACGTGTC AAACACTAG	CTAGCACCATGATGCC ATCTTCCATGAAC
ABCA1216	ABCA1216	GGTCTCCACCTCTAAATATC ATTACATTCTG	TACCAGCTTTTCTTCT TTGACTTATTGTCT
ABCA1219	ABCA1219	TGCAAACGATCTATGCTACT ATGTGAACCT	TGACGTGTTCTTTGTC TGTGATCTCATGAC
ABCA1219	ABCA1220	TGCAAACGATCTATGCTACT ATGTGAACCT	AGCGCAGACCATGAA GGCCTTTCAAAGA
ABCA1220	ABCA1220	CACTTCGGTGATTAAATGTG CCACGGAG	AGCGCAGACCATGAA GGCCTTTCAAAGA
ABCA1220	ABCA12232	CACTTCGGTGATTAAATGTG CCACGGAG	ACAGCTAAGTATGAT CTACTTCTGCAG
ABCA1236	N2363	CCAATATGGCTGGGAGTGC TACAGTGCA	CTGGTCCCATTAGATA TGGCTGTGCCAC
ABCA1236	NIP2RELUTR28	CCAATATGGCTGGGAGTGC TACAGTGCA	CTTTCTACAGGGAATA CTCTCTGTAGACTC
ABCA1A-F	ABCA1A-R	TCAACAACCTTTCAAGATGCT GACTGGAGACA	CACTGCCAAGGCACC TGAACCTTCCATTGA
ABCA1B-F	ABCA1B-R	CAGTGTGAACCTCTTCATCG GCATCAATGG	TGTCTCCAGTCAGCAT CTTGAAAGTTGTTGA
ABCA1C-F	ABCA1C-R	CTGGCATGCTATCAGTTCGT TCTTGAATGT	CACTGCCATTGATGCC GATGAAGAGGTTCA
ABCA1D-F	ABCA1D-R	ATCGTGGACCTCTTCAGAA TGGAACCTGG	GACATTCAAGAACGA ACTGATAGCATGCCA
ABCA1E-F	ABCA1E-R	TTGTGGAACCTCTTCATGAG ATTGATGACC	CCAGTTTCCATTCTGG AAGAGGTCCACGAT
ABCA1EXON 31-F	ABCA1EXON31-R	ACCGCAGACATCCTTCAGA ATCTGACAGGA	TCTTCCTGTCAGATT TGAAGGATGTCTGC
ABCA1EXON2	ABCA1EXON2	AGTCACAGCTCTGTGCTCTG G	GTTTGTCTCCTTCGAA ATGTCA
ABCA1EXON32-F	ABCA1EXON33-R	AAAGAATAAGATCTGGGTG AACGAGTTTCG	CTCGGAGGAAGTGCT TGAGAATTACTGACA
ABCA1EXON33-F	ABCA1EXON33-R	CCTGGGTGTCAGTAATTCTC AAGCACTTCCT	CTCGGAGGAAGTGCT TGAGAATTACTGACA
ABCA1EXON34-F	ABCA1EXON34-R	GAAGGTTTCATGGCAGGGCT GGATACGAA	TTCGTATCCAGCCCTG CCATGAACCTTC
ABCA1EXON50	ABCA1EXON50	GTGAACCTTTGCCAAGGACC A	AGGCTACAAAGGCAC TGCC
ABCA1F-F	ABCA1F-R	AAACCAGTTGGGAACGGGT TACTATCTGAC	GGTCATCAATCTCATG GAAGAGTTCACAA
ABCA1G-F	ABCA1G-R	GCTCAACTTTTACGAAGGCC AGATTACCTC	GGTCAGATAGTAACC CGTTCCTCAACTGGTT
ABCA1H-F	ABCA1H-R	GTTTGCCATGGTGACCATCC TACAGTGCTT	GAGGTAATCTGGCCTT CGTAAAAGTTGAGC
ABCA1I-F	ABCA1I-R	AAGAAAACGGGTGTCTACG TGCAACAGATG	GGAAGCACTGTAGGA TGGTCACCATGGCAA
ABCA1J-F	ABCA1J-R	AATGGCTCTGTGTATACCTG GAGAGAAGCT	GCACGTAGACACCCG TTTTCTTCTCAGATC
ABCA1K-F	ABCA1K-R	GTACGAGGATAACAACCTAC AAAGCCCTCTT	AGCTTCTCTCCAGGTA TACACAGAGCCATT
ABCA1L-F	ABCA1L-R	CTGCAGCACAATTTGTCCCT TCCAAGATCT	CTCCAAAGAGGGCTTT GTAGTTGTTATCCT
ABCA1M-F	ABCA1M-R	AAGTCCATCGTGTCTCGCCT GTTCTCAGAC	AGTAGATCTTGAAG GGACAAATTGTGCTG
CONTIG1.1	CONTIG1.1	CCATGTAGATCCTAAACCTG AACGATG	CTCCTTCTTTAGGCT TCAAAACATCG

FORWARD	REVERSE	SEQUENCE-F	SEQUENCE-R
CONTIG1.1	CONTIG1.3	CCATGTAGATCCTAAACCTG AACGATG	GCTTTGACCTTTCCTT TACCTTGTAGC
CONTIG1.2	CONTIG1.2	CTGCCACAGATACTCTGGA AAATCAGC	GGTGACTGACGACGT GGAAAACTTAAG
CONTIG1.3	CONTIG1.3	AAGAGCATCCACCTTTCTGG ACCTACT	GCTTTGACCTTTCCTT TACCTTGTAGC
CONTIG1.3	CONTIG1.5	AAGAGCATCCACCTTTCTGG ACCTACT	CTGTTTCCTTAGCTTG GCTGTAGTGAC
CONTIG1.4	CONTIG1.4	TTCTAGGTAAGACAAAGAA CGCTTCGTG	AGATAATAAAAACCA GGTCCCCGTCAA
CONTIG1.5	CONTIG1.5	CACTCACTGAAGACCCAGA GCTGGTTA	CTGTTTCCTTAGCTTG GCTGTAGTGAC
CONTIG1.5	CONTIG1.6	CACTCACTGAAGACCCAGA GCTGGTTA	ACCTTAAAGTCCTGGG GATAAGAGACG
CONTIG1.5	CONTIG1.7	CACTCACTGAAGACCCAGA GCTGGTTA	TCAAAAAGAGAGCTCT GTGAACCTTGATAG
CONTIG1.6	CONTIG1.6	AGCCATTGTGGTCTGTCTTC ATATCAC	ACCTTAAAGTCCTGGG GATAAGAGACG
CONTIG1.6	CONTIG1.7	AGCCATTGTGGTCTGTCTTC ATATCAC	TCAAAAAGAGAGCTCT GTGAACCTTGATAG
CONTIG1.7	CONTIG1.7	AGATAATGTGTGCATATCCA CGTGACC	TCAAAAAGAGAGCTCT GTGAACCTTGATAG
CONTIG1.7	CONTIG1.8	AGATAATGTGTGCATATCCA CGTGACC	TTTACAACTCAAATC AATTACCCGAAGA
CONTIG1.8	CONTIG1.8	GGTTCTTATTTCCTCTGGG CTATAGC	TTTACAACTCAAATC AATTACCCGAAGA
CONTIG6.1	CONTIG6.1	GAAGCTTGGCTGTCTTACCT TATGGAC	TGAGGCAGATTAGAG CATAAACATAACG
CONTIG6.1	CONTIG6.2	GAAGCTTGGCTGTCTTACCT TATGGAC	CCTGATACATCTTCCC TCTTTTAAATCG
CONTIG6.2	CONTIG6.2	ATATGTCAACTAGCCACCTC CCTACTCTT	CCTGATACATCTTCCC TCTTTTAAATCG
CONTIG6.2	CONTIG6.3	ATATGTCAACTAGCCACCTC CCTACTCTT	GCTGATCATCAATAGC CAATTGTTTAATC
CONTIG6.3	CONTIG6.3	CTCAGACTTTGTGTGGATGC TATGGTC	GCTGATCATCAATAGC CAATTGTTTAATC
CONTIG6.3	CONTIG6.4	CTCAGACTTTGTGTGGATGC TATGGTC	GAAACTGGTGTCTTG TCCATTTTCGAT
CONTIG6.4	CONTIG6.4	GTGGGAGATAAGATGACAG AATGGACG	GAAACTGGTGTCTTG TCCATTTTCGAT
CONTIG6.4	CONTIG6.5	GTGGGAGATAAGATGACAG AATGGACG	CCAGCTTGGAGACAA GAACATCAGTAG
CONTIG6.5	CONTIG6.5	GTTCTCACATGAAGTCAACA GTTGAGC	CCAGCTTGGAGACAA GAACATCAGTAG
CONTIG6.6	CONTIG6.6	GTGAAGCTGGGTGAGTATA ACGGTCAA	TGAACATCCATGAG AAGACAGGATAAGTC
CONTIG6.6END	CONTIG6.7BEGIN	GTGACAACCACTGAAGAAT TCGTGCTA	TTCTAAAAGAAAAAG ATGCCCTCAAG
CONTIG6.7	CONTIG6.7	GAGGGGCATCTTTTCTTTT AGAATATCA	TTACAAACACCAGCT AAGACCACATGT
CONTIG6.7	CONTIG6.8	GAGGGGCATCTTTTCTTTT AGAATATCA	GGCTGTAAAGTTGAA TAAGCAATAGGTGAC
CONTIG6.8	CONTIG6.8	ACCCAGCTGAGGAGAAATT GGATCTAA	GGCTGTAAAGTTGAA TAAGCAATAGGTGAC
KY	KY	ATTCCAAGGTGTCAGAAAC GGAATC	GGTGGATTTCACAGT CAGCTGAGTA
KY	KY	CTATCATAGCCATTTTCCCT CCTGTCTT	GGATGCTACTGCAGG AACCTCTTGTC
N2217	N2232	TTCACACAAGCACACATTGT ACAGTGAGTT	ACAGCTAACTTATGAT CTACTTCTGCAG
N2217	N2240	TTCACACAAGCACACATTGT ACAGTGAGTT	CAGCTCTGTGGGGTA ATGCATTTAAAAG
N2237	N2240	CTCTTCCAGTTTCTAAACAA CTGTTATCC	CAGCTCTGTGGGGTA ATGCATTTAAAAG

FORWARD	REVERSE	SEQUENCE-F	SEQUENCE-R
N2237	N2263	CTCTTCCAGTTTCTAAACAA CTGTTATCC	CTGGCATTGTCAAGGT ACACTGCAGTTA
N2248	N2253	GGGTCATCTGCAGATACAG TTTTATGCA	CTATCAGATCTACTTA TCCTCCAGTTTC
N2256	N2263	CCTAGATTTTGACACTGCAAG AGATGGCA	CTGGCATTGTCAAGGT ACACTGCAGTTA
N2256	N2274	CCTAGATTTTGACACTGCAAG AGATGGCA	CCCTGAAGACTGTGAT TTGATGCAGATA
N2270	N2274	CTCACTTAACAGCTGAACAT TTTCAGTC	CCCTGAAGACTGTGAT TTGATGCAGATA
N2270	N2294	CTCACTTAACAGCTGAACAT TTTCAGTC	CTTCTTACCACACTTG CAAGCAGTTACC
N2270	N2307	CTCACTTAACAGCTGAACAT TTTCAGTC	CACCTGCAGTGTGTTT TATAGGAAGCTT
N2280	N2294	GCACTGTGAACTGAATATAT GTGCAATC	CTTCTTACCACACTTG CAAGCAGTTACC
N2280	N2307	GCACTGTGAACTGAATATAT GTGCAATC	CACCTGCAGTGTGTTT TATAGGAAGCTT
N2294	N2307	GGTAACTGCTTGCAAGTGTG GTAAGAAG	CACCTGCAGTGTGTTT TATAGGAAGCTT
N2294	N2331	GGTAACTGCTTGCAAGTGTG GTAAGAAG	CGTTAGGAGTTTAAA GGCTAACAGGATT
N2294	N2363	GGTAACTGCTTGCAAGTGTG GTAAGAAG	CTGGTCCCATTAGATA TGGCTGTGCCAC
N2310	N2331	CCAGATCAAAAAGGAAACA TGGCAGACT	CGTTAGGAGTTTAAA GGCTAACAGGATT
N2310	N2348	CCAGATCAAAAAGGAAACA TGGCAGACT	CTTCTCCAAAGAGTAT GAATCCAGAAGG
N2339	N2348	GCTGACATTATGTTATGAGC ACATAATG	CTTCTCCAAAGAGTAT GAATCCAGAAGG
N2339	N2363	GCTGACATTATGTTATGAGC ACATAATG	CTGGTCCCATTAGATA TGGCTGTGCCAC
N2352	N2363	GGAGCTTCTGATAGAGGTA AACTTGATT	CTGGTCCCATTAGATA TGGCTGTGCCAC
N2352	NIP2RELUTR28	GGAGCTTCTGATAGAGGTA AACTTGATT	CTTTCTACAGGGAATA CTCTCTGTAGACTC
N2576	N2580	TGCAACACAGGGTGCCATT AAGAGCTGAT	TAGAAATGCCTTAGA GAAAGGCCCTGGAGG
N2576	N2589	TGCAACACACAGGGTGCCATT AAGAGCTGAT	GGCTACAAGAAATAG CACACTGACACACAA
N2576	N2612	TGCAACACAGGGTGCCATT AAGAGCTGAT	GACTGGGAGAACGAG ATGAATGGGGAAA
N2591	N2612	GCTTTCAGGGTACTTTTGTC TTGGATTT	GACTGGGAGAACGAG ATGAATGGGGAAA
N2591	N2623	GCTTTCAGGGTACTTTTGTC TTGGATTT	CTGAGAATATGCTAA GGGGATCCACAGGAT
N2614	N2618	ACTAGTAAGCCCCACACTTG GGAGACTGA	CATAATGAAGGTTTCC ATGAGAAAGTCC
N2614	N2639	ACTAGTAAGCCCCACACTTG GGAGACTGA	ACCTACTTCTCTCCAG ACCTACTTCCTC
N2621	N2623	TCCCACCTTGCTTCCTTGAG GCAGCACTTT	CTGAGAATATGCTAA GGGGATCCACAGGAT
N2621	N2651	TCCCACCTTGCTTCCTTGAG GCAGCACTTT	GCCATGCCTAATAAAA GTATGTTGTGACT
N2626	N2639	GCTCTCAATACCTAGGAAC ACATTTTAC	ACCTACTTCTCTCCAG ACCTACTTCCTC
N2626	N2651	GCTCTCAATACCTAGGAAC ACATTTTAC	GCCATGCCTAATAAAA GTATGTTGTGACT
N2641	N2651	CCTGATTTCCCTAACTTTGA GGACAAAT	GCCATGCCTAATAAAA GTATGTTGTGACT
N2657F	N2670R	TTAGGTCACTTTCTAGAGTC GTAATGCTAC	CTTAATAGGTACTTAC AACTCTTGCAAGTG
N2681F	N2683R	GCTGAATAGCAGGAATATG GTGTTCAAGCAT	AAGACAGCTATAAAT GAATACTAATGAACC

FORWARD	REVERSE	SEQUENCE-F	SEQUENCE-R
N2692F	N2695R	AAGCCTCAGGGTTGGTGGT ACACACAAAAG	CAGTGTCACTTAATGG ATGCAACTACTGCA
N2697F	N2700R	TGCACACATATAGCAAACA AGTACATGCAA	TCTCCTCTGACCTCAA GTGCTGTTACCACT
N2709F	N2715R	GTGCTGAACCTTGAGAGTTTT AACAGCTCTT	GCTGAGGGGTATTTCAT ATATTCTCGAATC
N2725F	N2725R	CTTGATGAGCATCCTCAAGG GGCTGATTCTG	CGAATCAGCCCCTTGA GGATGCTCATCAAG
N2739F	N2739R	TACTAGGCTAGTGTCAAGTA GCAAAGTGAG	CCTCACTTTGTCAACT GACACTAGCCTAGT
N2750F	N2750R	CTTGTGCTATGAAGCTTTTG TATGTGATGAA	TTCATCACATACAAAA GCTTCATAGCACAAAG C
N2761F	N2765R	TGTGCGAACAGAGTCTGTGT AGAATGGCAG	CATAAGATAGCATAT GCATTGTCTTTCCTTA C
N2770F	N2770R	GTCTTAATGTAAACTTGTGG TAATTTAGACCTA	TAGGTCTAAATTACCA CAAGTTTACATTAAGA C
N2778F	N2782R	AGCAGACAGATCTATACAG CTTCAATTACG	ACCTTCCAGGATTAAT GAGTGCCAAACTGC
NIP2RELUTR28	NIP2RELUTR28	CCAGATGTACTTGACACAG GAACTTATTG	CTTTCTACAGGGAATA CTCTCTGTAGACTC
NIP4P1	NIP4P1	CTAAGTCTTCGTGGCTTTGA CCTTTCTC	TACCTAGTCCATTGTG GCACCACAGTGG
NIP4P10	N2589	CCCAGGAGTGGAGTTGTTG GCTTGTAAGTT	GGCTACAAGAAATAG CACACTGACACACAA
NIP4P10	NIP4P10	CCCAGGAGTGGAGTTGTTG GCTTGTAAGTT	CTCTGTCAATTCTACT GGATATCCAGCCAT
NIP4P10	N2618	CCCAGGAGTGGAGTTGTTG GCTTGTAAGTT	CATAATGAAGGTTTCC ATGAGAAAGTCC
NIP4P1RVS	NIP4P2	CCACTGTGGTGCCACAATG GACTAGGTA	GGAGCTGATGTGACA TGTGT
NIP4P2	NIP4P2	GCTGAGGTACAGACAATGG A	GGAGCTGATGTGACA TGTGT
NIP4P3	NIP4P3RNEW	GGGTGTCTTACAGAAGAG G	CATAGTAGGAAGTCA TACAGTTTGGGTCTTG
NIP4P3	NIP4P5RNEW	GGGTGTCTTACAGAAGAG G	AAGTGTTAAAAGCTG CAAACCTGGAAGCTG
NIP4P4FNEW	NIP4P4RNEW	CAAGACCCAACTGTATGA CTTCTACTATG	AACTGGCTACTTTTCT GATGAAACCTGG
NIP4P5FNEW	NIP4P5RNEW	AGAAGATGAATAAGTCACC AACATACACGGA	AAGTGTTAAAAGCTG CAAACCTGGAAGCTG
NIP4P6FNEW	N2505	AACCTGGAGCATGCAAGTC CACCATGTTGT	CCTAATTGATTCTCTC GCACAAGAGACT
NIP4P6FNEW	NIP4P6	AACCTGGAGCATGCAAGTC CACCATGTTGT	GTGCATATGTACCGAC ACATACTCAAGG
NIP4P7	N2518	CCTTGAGTATGTGTCGGTAC ATATGCAC	GGAATGCACTGAAGG GAGAAAAATTGTAT
NIP4P7	NIP4P7	CCTTGAGTATGTGTCGGTAC ATATGCAC	TAGTGCATTTTCCAAA TACACACTCACTC
NIP4P7	N2573	CCTTGAGTATGTGTCGGTAC ATATGCAC	TACCAACCACTACACT ACGAGTGTAGCA
NIP4P8	N2559	GAGTGAGTGTGTATTTGGA AAATGCACTA	CTCAAGGCTGAAAAA GCATTCTAAGTGA
NIP4P8	NIP4P8	GAGTGAGTGTGTATTTGGA AAATGCACTA	ACATTTCCAGGGGTG AAGACCATCCAGGA
NIP4P9	N2573	TCCTGGATGGTCTTCACCCC TGGGAAATGT	TACCAACCACTACACT ACGAGTGTAGCA
NIP4P9	N2580	TCCTGGATGGTCTTCACCCC TGGGAAATGT	TAGAAATGCCTTAGA GAAAGGCCCTGGAGG
NIP4P9	NIP4P9	TCCTGGATGGTCTTCACCCC TGGGAAATGT	AACTTACAAGCCAAC AACTCCACTCCTGGG

FORWARD	REVERSE	SEQUENCE-F	SEQUENCE-R
NIPSNAP33'	NIPSNAP33'	CAGAATACG TTCCTGATTCC AACATCA	AATGAAGTACCAGGG CACGGAGTA
NIPSNAP3CDNA-F	NIPSNAP3CDNA-R	TATGGAGCCCTCAACAGAG TTCATGTTCTG	AGCTCAGCCCGTCCTT CAGACAATGAACTG
NIPSNAP43'	NIPSNAP43'	TGGTCACAAACTTGGAAC ACGAT	CCACTGAAATAGTTTC CTAACGACTGT
TBX18	TBX18	CTCCAGGACCCTAGGACGTT TAAC	GCATCCTTACCAACTT TCAAGTGTACC

VITA

Laura Ray Chittenden was born at Fort Riley, Kansas on August 1, 1972. She moved to Claxton, Tennessee in December of 1974, and went to grade school at St. Mary's School in Oak Ridge, Tennessee. She was the 1990 class valedictorian at Knoxville Catholic High School in Knoxville, Tennessee. From there she attended Old Dominion University in Norfolk, Virginia on partial academic and athletic scholarships, and graduated in May 1994 with a Bachelors of Science in biochemistry and a minor in biology. Her graduate studies were begun in June 1994 at the University of Tennessee Oak Ridge Graduate School for Biomedical Sciences.