THE bHLH/PAS TRANSCRIPTION FACTOR *SIM1* IS A NOVEL OBESITY GENE

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DEDICATION

I would like to thank my family for all of their support and especially my parents and brothers: Kristopher and Sean.

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by

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Obesity is epidemic in the United States and other developed countries. Obesity is a major risk factor for type II diabetes, hypertension, hyperlipidemia and osteoarthritis.

I report a unique girl with early-onset obesity (47.5 kg, +9.3 s.d. above mean at age 67 months) and a de novo balanced translocation between chromosomes 1 and 6. She has normal energy expenditure and a voracious appetite. I show that her translocation disrupts a transcription factor gene, *SIM1*, on chromosome 6q16.2. I also present data that *Sim1* haploinsufficiency causes obesity in mice. Animals heterozygous for a *Sim1* null allele fed a standard chow diet (4% fat) developed obesity around the time of sexual maturity, were 33-45% heavier than wild-type littermates by 5 months of age, and had increased adiposity by DEXA scans. In contrast, the human subject developed obesity by two years of age, well before puberty. To investigate whether differences in dietary fat consumption might explain this discrepancy in human and mouse phenotypes, I fed mutant mice and wild type littermates a “Westernized” diet (35% fat). Heterozygous *Sim1* mice fed this diet became obese prior to 6 weeks of age. The obesity was also more severe, especially in females, who by 8 weeks of

age weighed 72% more than controls compared with 13% on a low fat diet. Heterozygous *Sim1* mice maintained on a 4% fat diet ate more than controls over a 5 day period (Δkcals 12-14%), and became even more hyperphagic when acutely challenged with increased dietary fat (Δkcals 46-68% over 5 days). This altered behavior was evident within the first day of exposure to the high fat diet: during this time, heterozygous *Sim1* mice failed to significantly change the mass of food consumed, whereas wild-type littermates decreased their food consumption by >15%. These data suggest that *Sim1* is critical for the acute and chronic homeostatic response to elevated dietary fat. This data demonstrates that normal *Sim1* gene dosage is critical for proper regulation of feeding behavior and body weight regulation.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Holder JL, Habbak RA, Pearlson GD, Aylward EA, Pulsifer M and Warren AC. “Reduced survival of apolipoprotein E4 homozygotes in Down’s syndrome?” Neuroreport **7** (15-17): 2455-6.

Holder J and Warren AC. “Prevalence of Alzheimer’s disease and Apolipoprotein E allele frequencies in the Old Order Amish.” Journal of Neuropsychiatry and Clinical Neurosciences **10** (1): 100-2.

Holder JL Jr, Butte NF and Zinn AR. “Profound obesity associated with a balanced translocation that disrupts the SIM1 gene.” Human Molecular Genetics **9** (1): 101-108.

Holder JL Jr, Zhang L, Kublaoui BM, DiLeone RJ, Oz OK, Bair CH, Lee YH and Zinn AR. “Sim1 gene dosage modulates the homeostatic feeding response to increased dietary fat.” American Journal of Physiology: Endocrinology and Metabolism **287** (1): E105-13.

LIST OF FIGURES

LIST OF TABLES

Chapter 1

Introduction

The Growing Medical Problem of Obesity

The prevalence of obesity is rising in the United States and other developed countries. Recent analysis of the National Health and Nutrition Examination Survey (NHANES IV) demonstrates that obesity of adults nearly doubled from 1976 to 1999 (Fig. 1) (Kopelman 2000). Even more alarming is the recent rise in obesity and overweight in children in the United States. According to the NHANES data, the percentage of overweight children six to eleven years old jumped from four percent in 1965 to thirteen percent in 1999. This is more than a three-fold increase in less than half of a century. This phenomenon is not limited to the United States. One extreme example is in urban Samoa where it is estimated that 75% of adult women and 60% of adult men are clinically obese (Kopelman 2000). Less extreme examples include most western European countries. From the World Health Organization's Monitoring trends and determinants of cardiovascular disease (MONICA) study, over fifty percent of the European population is either overweight or obese (Kopelman 2000).

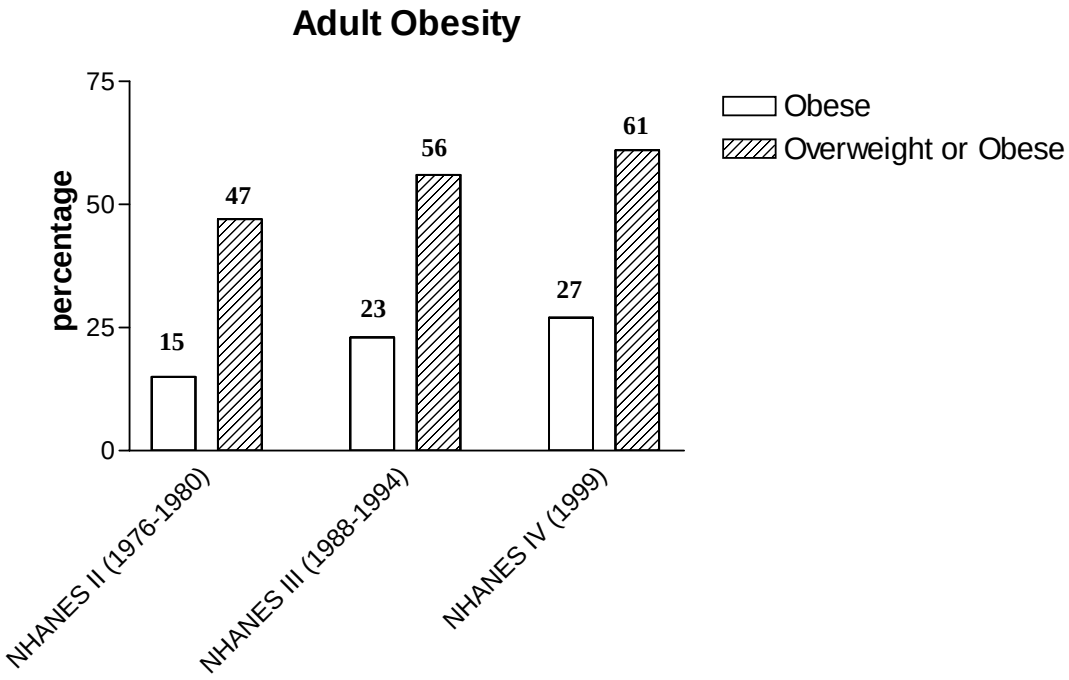


Figure 1 Increased prevalence of adult obesity in the United States

A twenty percent increase in body weight is associated with increased incidence of non-insulin dependent diabetes mellitus (NIDDM), hypertension, osteoarthritis, and hyperlipidemia. Obesity is in fact the number one predisposing factor to NIDDM. The mechanism of this predisposition is only partially understood but is thought to involve elevated serum free fatty acids (FFAs) which can prevent uptake of insulin by the liver and cause a transient increase in production of insulin by the pancreas. This rise in serum insulin results in down-regulation of insulin receptors in peripheral tissues such as muscle and insulin resistance. Either through depletion of pancreatic beta cells or a direct toxic effect of FFAs, insulin levels are eventually unable to compensate for insulin resistance in obese individuals resulting in hyperglycemia and NIDDM.

Obesity is also a major predisposing factor for cardiovascular disease (Kopelman 2000). The Framingham Heart Study reported that adults less than fifty years old there is a 2.4-fold increased risk of heart failure in women and a 2.0-fold increased risk of heart failure in men who are obese. Obesity is therefore a risk factor for two of the most common and devastating diseases, NIDDM and cardiovascular disease. With the dramatic increase in overweight and obesity of the past several decades, understanding the molecular mechanisms of weight homeostasis and using the information for rational drug design is of paramount medical importance.

Genetics of Obesity

Although the problem of obesity has been on the rise for the at least the past fifty years, some of the genetic determinants of feeding behavior and weight regulation have only recently been identified. Work determining the endocrine control of feeding behavior began with classic parabiotic experiments with mice carrying the spontaneous mutant alleles *obese* (*ob/ob*) and *diabetes* (*db/db*). Both mutant mice display a syndrome of early-onset severe obesity, hyperphagia, stunted growth, infertility and diabetes. In the first of these experiments, the circulatory systems of *ob/ob* and wild-type mice were connected (Coleman and Hummel 1969; Coleman 1973). This resulted in complete reversal of the phenotype of *ob/ob* mice. They became normophagic, normoglycemic and lost weight. This suggested that a circulating factor was absent in *ob/ob* mice and could be replaced in the parabiosis experiment. When a similar parabiosis experiment was performed with *db/db* mice, no

change in phenotype was observed in the mutant mice. In this experiment, however, the wild-type mice became anorexic. It was hypothesized that *db/db* mice were resistant to a circulating factor that controlled food intake and parabiosed wild-type mice became anorexic in response to elevated circulating levels of this factor.

In 1994, the gene mutated in the *obese* allele was determined to be leptin (Zhang, Proenca et al. 1994). Leptin is a circulating polypeptide secreted by adipocytes in proportion to their fat content. A year later, the gene mutated in the *diabetes* allele was identified as the leptin receptor (Tartaglia, Dembski et al. 1995). This confirmed the predicted function of these genes by the parabiotic experiments.

Another classic spontaneous mutant mouse model of obesity, A^y, has elucidated another molecular pathway critical for weight regulation. A^y mice are hyperphagic, obese and have a yellow coat color (Miller, Duhl et al. 1993). The mutation in these mice has been identified as a chromosomal inversion causing the promoter of a ubiquitously expressed gene to be place upstream of the *Agouti* gene which is normally expressed only in the skin and acts as an antagonist for the melanocortin 1 receptor. In the A^y mouse, Agouti is ubiquitously over-expressed and antagonizes two homologues of the melanocortin 1 receptor in the brain, the melanocortin 3 and 4 receptors (Mc3r and Mc4r). A role for both of these receptors has been confirmed in mice deficient for these receptors by homologous recombination to generate null alleles in mice (Huszar, Lynch et al. 1997; Chen, Marsh et al. 2000). *Mc4r* deficient mice largely recapitulate the A^y obesity phenotype: they are hyperphagic, obese and hypometabolic. *Mc3r* deficient mice have a more subtle phentoype: only the male deficient mice become slightly larger than wild-type mice at twenty-four weeks of age. *Mc3r* deficient

mice, however, are significantly hypometabolic and have elevated fat mass with depressed lean mass.

One last spontaneous mouse model of obesity, *tubby*, revealed a new type of obesity gene. The gene mutated in the *tubby* allele was identified in 1996 (Kleyn, Fan et al. 1996). Despite isolation and complete sequencing of the tubby gene, the molecular function of tubby was elusive as its amino acid sequence was not significantly similar to any protein of known function. A breakthrough in the understanding of the potential function of tubby came with its crystallization and elucidation of its structure (Boggon, Shan et al. 1999). Tubby and its homologues have a conserved domain at the carboxy-terminus known as the tubby-domain. The tubby-domain is able to bind double stranded DNA while the amino-terminus can activate transcription. When tubby is transiently transfected into cells, it is primarily localized to the nucleus. Combined, these data suggest that tubby is a transcription factor critical for proper feeding behavior and weight regulation, and feeding regulation in the hypothalamus requires factors beyond neuropeptides and neuropeptide receptors.

While cloning these spontaneous mouse mutations has significantly improved our understanding of the molecular mechanisms of weight regulation, scanning large numbers of obese individuals have revealed few mutations in the human orthologues of these genes. Only rare mutations in inbred kindreds have been identified in leptin (Montague, Farooqi et al. 1997) and leptin receptor (Clement, Vaisse et al. 1998). Mutations in *MC4R* appear to be more common in obese children (Farooqi, Yeo et al. 2000) than leptin or its receptor but still probably only accounts for 2-4% of pediatric obesity.

Many groups have attempted to map quantitative trait loci in human with respect to obesity related phenotype such as body mass index (BMI), serum leptin levels or waist circumference. Through these studies, only five loci with significant linkage have been identified, and of these, only one at chromosome 2p21 has been confirmed in an independent population (Rankinen, Perusse et al. 2002). Although the gene responsible for this linkage has not been identified, proopiomelanocortin (*POMC*) is located within the linked region and is considered an excellent candidate gene. *POMC* encodes a precursor protein for several neuropeptides including α-MSH which is a natural agonistic ligand for MC3R and MC4R. Indeed, there are two reports of humans with null mutations in *POMC* associated with obesity (Krude, Biebermann et al. 1998; Krude and Gruters 2000) and mice deficient for *Pomc* are obese (Yaswen, Diehl et al. 1999).

Classic approaches for identifying genes responsible for genetic susceptibility to obesity in humans as discussed above have not yielded large numbers of new candidate genes associated with obesity. Novel approaches to this problem, such as discussed in this work, could prove a fruitful approach for identifying new obesity genes.

Hypothalamic Control of Feeding Behavior

The role of the hypothalamus in feeding behavior was first established through classic lesion and stimulation experiments performed in 1940 (Elmquist, Elias et al. 1999). In the first of these experiments, a medial aspect of the hypothalamus, encompassing portions of the ventral medial hypothalamus (VMH) and arcuate nucleus of rats was lesioned. This resulted in a syndrome of obesity, hyperphagia and hypometabolism. In a separate set of experiments,

a more lateral aspect of the hypothalami of rats, encompassing the lateral hypothalamic area (LHA), was lesioned. This resulted in aphagia and weight loss often so severe that the rats had to be force fed in order to survive. It was also demonstrated that electrical stimulation of the VMH caused rats to reduce their food intake while stimulation of the LHA caused hyperphagia. This led to the "dual-center" hypothesis that an area encompassing the VMH acts as a satiety center and that the LHA acts as a hunger center.

The dual-center hypothesis has been modified more recently. It was demonstrated that more precise lesions limited to the paraventricular nucleus of the hypothalamus (PVH) also leads to obesity and hyperphagia although little effect was seen with regard to metabolic rate (Weingarten, Chang et al. 1985). The PVH has outputs to the autonomic nervous system and particularly to the preganglionic neurons of the gastrointestinal tract, suggesting a possible role of the PVH in satiety through changes in gastric motility and gastric secretion.

A molecular understanding of the neurobiology of feeding behavior did not begin until the identification of leptin as the gene mutated in the *ob/ob* mouse and its receptor as the gene mutated in the *db/db* mouse. As previously discussed, leptin is secreted by the adipocytes in relation to their triglyceride content. The leptin receptor is expressed ubiquitously, and there are several isoforms due to transcriptional splice variants. One of these variants dubbed the long form or R_b form has a more tightly regulated expression pattern. Within the CNS, the long form is most highly expressed in the hypothalamus (Mercer, Hoggard et al. 1996). The long form of the leptin receptor is the only isoform with an intracellular domain that has a signaling capacity. Leptin has clear molecular effects

within the arcuate and other nuclei of the hypothalamus. ICV injection of leptin causes Fos induction within the arcuate as well as the VMH, Dorsomedial hypothalamus (DMH) and PVH (Elmquist, Ahima et al. 1997). Fos is an immediate-early gene whose presence in neurons is thought to signal elevated neuronal activity. Although the long form of the receptor has been reported in regions of the hypothalamus outside of the arcuate, it is believed that the increase in Fos immunoreactivity in the PVH, VMH and DMH is due to signaling from the arcuate.

ICV injection of leptin also causes changes in expression of neuropeptides with known functions in feeding behavior. Leptin causes decreased expression of neuropeptide Y (*Npy*) (Schwartz, Seeley et al. 1996) and agouti related peptide (*Agrp*) (Ahima and Hileman 2000). Npy is a potent orexigenic molecule as determined by ICV injection into rodents (Elmquist, Elias et al. 1999). Receptors for Npy are found throughout the CNS including in the hypothalamus where three receptors, Npy 1,2 and 5 receptors, are expressed (Kushi, Sasai et al. 1998; Sainsbury, Schwarzer et al. 2002). Agrp is coexpressed with Npy within many neurons of the arcuate nucleus and is also a potent orexigenic molecule and acts as a natural antagonist for melanocortin receptors including Mc3r and Mc4r (Elmquist, Elias et al. 1999). Leptin injection also results in elevated expression of two neuropeptides, Pomc and Cocaine and Amphetamine Regulated Transcript (Cart) expressed in the arcuate nucleus. As discussed earlier, Pomc is the prepropetide for α-MSH which is the natural agonistic ligand for both Mc3r and Mc4r. Activation of Mc4r by α-MSH is anorexigenic. Through ICV injection studies in rodents, it has also been determined that Cart is an anorexigenic molecule (Abbott, Rossi et al. 2001).

Combined, these pharmacologic and molecular studies suggest a pathway from the periphery through the hypothalamus and back to the periphery. Elevated food consumption causes elevated fat storage and thus elevated leptin secretion from adipocytes. Leptin activates neurons of the arcuate nucleus depressing expression of orexigenic peptides Npy and Agrp and elevating expression of Pomc and Cart. This has a net effect of elevated activity of Mc3r and Mc4r and less activation of Npy receptors. The result is that food intake is perturbed, possibly through action on gastric motility and gastric acid secretion via PVH control of the autonomic nervous system.

Unfortunately, the above model greatly simplifies the complexity of the hypothalamus. It largely ignores the LHA which is known to be critical through lesioning experiments discussed above. Melanin concentrating hormone (Mch) is produced by neurons of the LHA and by ICV experiments is known to be an orexigenic neuropeptide (Qu, Ludwig et al. 1996). Mice deficient for *Mch* are hypophagic and lean (Shimada, Tritos et al. 1998) while mice that transgenically overexpress *Mch* develop obesity (Ludwig, Tritos et al. 2001). These experiments suggest a critical role for *Mch* in proper weight balance. Where *Mch* fits in the above model however is not clear. Leptin deficient mice have strong induction of *Mch* (Qu, Ludwig et al. 1996) and there are leptin receptors in the LHA (Fei, Okano et al. 1997). But activation of *Mch* production could be indirect as there are Mc4rs in the LHA (Mountjoy, Mortrud et al. 1994) and the arcuate does innervate the LHA. A comprehensive list of orexigenic and anorexigenic neuropeptides and their expression patterns are listed in Table I.

Table I

Orexigenic Peptides	**Expression in mice**	**Anorexigenic Peptides**	**Expression in mice**
Orexin	LHA	Leptin	Adipocytes
Neuropeptide Y	Arcuate Nucleus	α-Melanocyte Stimulating Hormone	Arcuate Nucleus
Agouti related peptide	Arcuate Nucleus	Cholecystokinin	Small intestine
Ghrelin	Stomach	Glucagon like peptide-1	Small intestine
Melanin Concentrating Hormone	LHA	Gastrin releasing peptide	LHA
Galanin	Amygdala, supraoptic nucleus (SON), PVH and LHA	Peptide YY	Pancreas
		Corticotropin releasing hormone	PVH
		Urocortin	PVH, SON
		Cocaine and amphetamine regulated transcript	Arcuate, PVH, LHA, others
		Thyrotropin releasing hormone	PVH
		Oxytocin	PVH
		Neuromedin U	VMH, gut

SIM1

SIM1 is a mammalian homolog of the *Drosophila* transcription factor Single-minded, a prototypical member of the bHLH-PAS (basic Helix-Loop-Helix + Period, Aryl hydrocarbon receptor, Single-minded) family of proteins. Homozygous loss of function mutations in *Drosophila* Single-minded result in the failure of formation of midline central nervous system structures (Thomas, Crews et al. 1988). Two mouse genes, *Sim1* and *Sim2*, were cloned by nucleotide sequence homology (Fan, Kuwana et al. 1996). Both homologs are also present in humans, and the predicted mouse and human SIM1 proteins show 96% amino acid identity (Chrast, Scott et al. 1997).

Dimerization of bHLH-PAS proteins with other bHLH-PAS proteins such as ARNT (aryl hydrocarbon receptor nuclear translocator) or ARNT2 is necessary for their function (Probst, Fan et al. 1997). Specificity of dimerization is mediated at least in part through the PAS domains (Zelzer, Wappner et al. 1997).

By Northern blot analysis, mouse *Sim1* is expressed in adult kidney (Ema, Morita et al. 1996; Fan, Kuwana et al. 1996). Whole mount *in situ* hybridization of embryonic day 16.5 mouse embryos revealed expression restricted to the kidney tubules, spinal cord, and parts of the midbrain and forebrain. Particularly interesting is *Sim1*'s expression in anterior hypothalamic nuclei of the forebrain. Michaud and Fan determined that *Sim1* is highly expressed in the supraoptic, paraventricular and anterior periventricular (aPV) nuclei of the hypothalamus in newborn mice (Michaud, Rosenquist et al. 1998). Both the SON and PVH have neuroendocrine functions. The PVH produces corticotropin releasing factor (CRF) and thyrotropin releasing hormone (TRH) while both the PVH and SON produce oxytocin and

arginine vasopressin (AVP). The aPV produces somatostatin which also impinges on the pituitary. Further expression studies have been performed and will be presented in Chapter 5.

Targeted inactivation of *Sim1* confirmed its critical role in development of neuroendocrine lineages in the hypothalamus (Michaud, Rosenquist et al. 1998). In mice lacking Sim1, the SON and PVH are hypocellular and lack at least five types of secretory neurons (identified by the lack of expression of oxytocin, vasopressin, thyrotropin-releasing hormone, corticotropin-releasing hormone, and somatostatin) due to failure of terminal neuronal differentiation. These animals die shortly after birth, presumably due to multiple hypothalamic-pituitary axis (HPA) deficits. There is no discernable phenotype in other tissues. No growth abnormality in heterozygotes was reported, but this does not preclude a human *SIM1* heterozyogous phenotype, as there are examples of other genes that are haploinsufficient in humans but not in laboratory mice, e.g. endothelin B receptor (Hosoda, Hammer et al. 1994; Puffenberger, Hosoda et al. 1994) and Sonic hedgehog (Roessler, Belloni et al. 1996). Identification of a human with a *SIM1* mutation and her phenotype will be discussed in Chapter 3, and detailed studies of *Sim1* heterozygous mice are presented in Chapter 5.

Chapter 2

Materials and Methods

Clinical studies

Studies were approved by the Institutional Review Boards at UT Southwestern Medical School and Baylor College of Medicine, and informed consent was obtained for all studies.

Body weight was measured with a digital balance and height was measured with a stadiometer. Skinfold and circumference measurements were taken at various sites of the body. Dual-energy x-ray absorptiometry (DXA, Hologic 4500A, Madison, WI) was used to measure fat mass, fat-free mass, and total body bone mineral content.

A three-day food intake record was kept by the child's parents, after instruction by a registered dietitian. The food intake records were converted to nutrient intakes (energy, protein, fat and carbohydrate) using the Minnesota Nutrition Data System.

Energy expenditure was measured in a metabolic research unit by calorimetry or in the free-living state by the doubly labeled water method.

Calorimetry. Energy expenditure was measured for 24 hr in a room respiration calorimeter. The operation and calibration of the calorimeters have been described previously in detail (Moon, Vohra et al. 1995). Energy expenditure was computed at 1-min intervals from O_2 consumption and CO_2 production and used to calculate 24-hr total energy expenditure, basal metabolic rate, sleeping metabolic rate, and respiratory quotient.

Doubly labeled water. Total energy expenditure over a 14 day period was calculated from the fractional turnover rates of ^{2}H and ^{18}O following oral ingestion of 100 mg/kg H_2O and 125 mg/kg ^{18}O as water. Isotope dilution spaces were used to compute total body water. Baseline urine samples were collected. Subsequently, one daily urine sample was collected at home for the next 14 days. The ^{2}H and ^{18}O abundances of the urine samples were measured by gas-isotope-ratio mass spectrometry. Carbon dioxide production (VCO_2) was calculated from the dilution spaces and fractional turnover rates of ^{2}H and ^{18}O using the multipoint slope-intercept method. Fractionated insensible water losses were calculated from ventilatory volume and body surface area, both expressed as functions of CO_2 production. Total energy expenditure was calculated using the Weir equation.

Molecular studies

Epstein-Barr virus-immortalized cell lines were generated from SW116 and her parents by standard methods. The balanced translocation was verified by two-color fluorescent *in situ* hybridization (FISH) as previously described, using whole chromosome paints for chromosomes 1 and 6. A somatic cell hybrid retaining the der(1) chromosome was constructed by fusing the patient's lymphoblastoid cells to thymidine kinase-deficient Chinese hamster cells (Mohandas, Sparkes et al. 1979) using polyethylene glycol 4000 (Life Technologies, Rockville, MD). Colonies were selected in the presence of hypoxanthine-aminopterin-thymidine. After >10 serial passages, DNA was extracted from clones and tested by PCR for various chromosome 1 and 6 STS markers.

BAC clones were isolated by PCR screening pooled DNAs from Research Genetics (Huntsville, AL). YAC clone y852C9 was obtained from a local copy of the CEPH library. YAC clone yA35F11 was isolated by PCR screening pooled DNAs from the Washington University library. YACs and BACs were used for FISH as previously described (Guillen, Lowichik et al. 1997). PAC clones were isolated from an arrayed library by filter hybridization. BAC and PAC ends were sequenced using either a Thermo Sequenase cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden) or an ABI PRISM 310 automated sequencer (Perkin Elmer Applied Biosystems, Foster City, CA) according to the manufacturers' instructions.

Exon trapping experiments was performed using BAC clones b261L4 and b219G7 as described (Inoue, Hess et al. 1999). PCR and Southern blotting were performed using standard protocols. Genomic DNA from SW116 or her parents was digested with *Xba*I and hybridized with a *Kpn*I/*Xba*I fragment from chromosome 1 (GenBank AL049861, nt. 83014-85386). After exposure to a phosphorimager screen, the filter was stripped and reprobed with a *Hind*III fragment from chromosome 6 (GenBank Z86062, nt. 54268-54318). Primer pairs GACCCCTTCACTCTGCTGTAACC and TCGCCGAGCCCTGTGGAGAC or CATCTTTTGTCTCCCCTCCTGAAC and CAAAAGGGTACTCTAGCCGACTCC were used to PCR-amplify der(6) or der(1) junction sequences, respectively. Products were directly sequenced using an ABI 310 sequencer. SSCP was performed using MDE gel matrix (FMC Bioproducts, Rockland, ME) according to the manufacturer's instructions. Primers used to amplify *SIM1* exons were:

CTGGGAACACCACTCTCATTTTGA and

AGAAGAAAGGGGGAACAAGACACA (exon 1),

TCAGACCCTCAAAGCTTATGTGTT and

CAGGTCCGGGTTCAGTGG (exon 2),

GCCCCCTACCCCTGCTTCC and

TGGCTTCATCTTCGTGGTA (exon 3),

GGGGAAAAACCACAAGCGGACTGC and

CCACGGCGACGGCGACATC (exon 4),

CTTGCTTCCCGCCTCCTCTGACTC and

AGCTTCCCTTCGTTCCTCTC (exon 5),

GCCGCCCTCAGGCTAGGA and

TGTGGCTGAGTCTCCCTCCCTATC (exon 6),

CAGCGGATGCGCCAAGGTTG and

TCCTGCAGGGATTGCTCTC (exon 7),

GGGGTGGGTGAAGGGGTCTCA and

CAGGCAGGCTGGTTCACC (exon 8),

AAAAAGAAAGTTGCAAAACAG and

ATGGTGGCTGATTAAGGGCTTTGT (exon 9),

CAATGAGACCTTAAGGGTGCTTGTAG and

TGGAGTTCGGGAACCCTTTCAC (exon 10),

ACATCATGTGAGCCTGTTTCAAATA and

CATAGTAAATGCTGGTAATGGGGTAT (exon 11).

The exon 10 and 11 products were digested with *Hha*I or *Mnl*I respectively prior to SSCP.

Cell Culture, Western blotting and Reporter assays

All cell culture transfections were performed using Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to manufacturers instructions. Western blotting was performed using standard protocols. (Sambrook J, Fritsch EF and Maniatis T: 1989)

The anti-ARNT2 antibody was purchased from a commercial source (Santa Cruz Biotechnology, Santa Cruz, CA) as was the anti-HA (Babco, Richmond, CA) and anti-V5 (Invitrogen) antibodies. Goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to horse radish peroxidase (HRP) were also commercially produced (Bio-Rad, Hercules, CA). Presence of HRP was detected using the ECL Plus kit (Amersham Pharmacia, Buckinghamshire, UK).

The pGL3/6XCME plasmid was a generous gift of Stephen Crews (University of North Carolina) as was the dSIM cDNA. The Sim1HA and ARNT2 expression plasmids were gifts from Chen-Ming Fan (Carnegie Institute, Baltimore, MD). The ecdysone inducible expression plasmids (pVgRXR, pIND and pIND/LacZ) were commercially purchased (Invitrogen).

The Luciferase assay was performed according to manufacturers instructions (Promega, Madison, WI), as was the beta-galactosidase assay (Invitrogen).

Generation of knockout mice; genotyping

Mice heterozygous for a *Sim1* null allele were generated by Ying-Hue Lee (Sinica Academy, Taiwan) using standard techniques. The targeted allele contained three lox P sites, one lox P site was within the 5' UTR of exon 2 of *Sim1* and two flanked a PGK-Neo cassette within intron two. Mice heterozygous for this allele were mated to transgenic mice with the Cre recombinase under transcriptional control of the EIIa promoter (Jackson Laboratories). This resulted in mice carrying an allele with a single lox P site and the coding region of exon 2 deleted constituting a null allele.

Genotyping was routinely performed by PCR using primers
5'-TTTCTGTGCTGCTGGGGTAGGTTT-3',
5'-CATTCGTGTCTTCCCGGAGCAAACTTC-3' and
5'-CGAGGAATTCCGATCATATTCAAT-3'
under standard conditions. The null allele resulted in a product of 160 bp and the wild-type allele generated a product of 377 bp.

Growth and feeding studies

Mice were kept on a 12 hour light/dark cycle, lights on at 6:00am. Animals of each gender were group-housed for measurements of length and body weight and individually caged for at least 5 days prior to measurements of food intake. Nose to anus length was determined at necropsy.

Mice were either fed a low fat chow diet (LF) (Teklad diets, Madison, WI) that is 2.94 kcal/g with 46.8% available carbohydrate, 4.0% available fat and 24.0% available protein or a

high fat diet (HF) (Research Diets, New Brunswick, NJ) that is 5.24 kcal/g with 26.3% available carbohydrate, 34.9% available fat and 26.2% available protein. Feeding efficiency was calculated by dividing the change in mass (mg) by the food intake (kcal) for a seven day period on both the HF and LF chow.

Wheel Running Assay

Mice were housed individual for at least seven days prior to data collection. Wheel turns were measured every five minutes and collected for five days on both the LF and HF chow. Each revolution was counted by magnetic switch closures with magnets placed on the revolving wheel. The data acquisition system was by Data Systems International.

Dual energy X-Ray absorptiometry (DEXA)

Animals of six months of age were anesthetized with a mixture of ketamine, xylazine, and acepromazine. Body fat content and bone mineral density were measured using a Lunar PIXImus densitometer calibrated with an aluminum/lucite phantom as described by the manufacturer (GE Medical Systems, Madison, WI).

Serum insulin and leptin assays

Animals were sacrificed by CO_2 asphixiation at either six months of age for mice fed the LF diet or at four months of age for mice fed the HF chow. Blood was obtained by exanguination and serum collected. All mice were sacrificed between 1:00 pm and 2:30 pm. Serum glucose was determined using the One Touch Basic glucometer (Johnson and

Johnson, Milpitas, CA). Insulin levels were determined using Linco Laboratories (St. Charles, MO) Rat Insulin RIA kit. Leptin levels were determined using R&D Systems (Minneapolis, MN) mouse Leptin EIA Kit.

Chapter 3

Profound obesity associated with a balanced translocation that disrupts the *SIM1* gene

SW116: A Case of Early-Onset Obesity

Numerous family, twin, and adoption studies over the past sixty years indicate that the heritability of obesity is on the order of 0.4 to 0.8 (Stunkard, Sorensen et al. 1986; Stunkard 1991); in most cases the pattern of inheritance is polygenic. Genetic studies in humans and laboratory animals have mapped quantitative trait loci that influence body weight and diabetes susceptibility (Rankinen, Perusse et al. 2002), but the molecular nature of these genes is as yet unknown.

By contrast, studies of monogenic obesity in mice have greatly advanced our knowledge of the endocrine and metabolic pathways regulating body weight. Five mouse spontaneous obesity mutations have been cloned: *diabetes*, *fat*, *obese*, *tubby*, and *yellow*. The most intensively studied of these are *obese*, which encodes leptin (Zhang, Proenca et al. 1994), and *diabetes*, which encodes the leptin receptor (Tartaglia, Dembski et al. 1995). *Fat* encodes carboxypeptidase E, an enzyme necessary for normal processing of neuropeptides such as Pomc that is involved in central control of feeding behavior as well as processing of hormones such as proinsulin that regulate peripheral energy metabolism (Naggert, Fricker et al. 1995). *Yellow* is a mutation causing ectopic expression of the *Agouti* gene, which encodes a competitive antagonist of α-MSH signaling (Miller, Duhl et al. 1993).

Predictably, screening of morbidly obese humans has identified rare mutations in some of these same genes, including leptin (Montague, Farooqi et al. 1997) and the leptin receptor (Clement, Vaisse et al. 1998). Human mutations associated with obesity have also been discovered in genes encoding other elements of these pathways, such as proopiomelanocortin (Krude, Biebermann et al. 1998), prohormone convertase I (Jackson, Creemers et al. 1997), which cooperates with carboxypeptidase E in prohormone processing, and the melanocortin 4 receptor (MC4R) (Farooqi, Yeo et al. 2000), a key hypothalamic target of α-MSH. Clinical and metabolic studies of these rare human patients have complemented studies of laboratory rodents in defining the roles of these molecules in energy balance and revealed some interesting species differences, e.g. the association of leptin deficiency with hypercortisolemia in mice but not humans.

In this chapter, I report clinical and molecular studies of a unique patient with obesity and a balanced 1p;6q chromosome translocation. The results suggest an unsuspected role for a transcription factor in the regulation of food intake. The transcription factor, SIM1, is critical for formation of the supraoptic and paraventricular hypothalamic nuclei in mice. The latter nucleus is well known to be involved in energy homeostasis. Our data suggest that SIM1 plays a role in this function.

RESULTS

Clinical studies

The proband (SW116) was referred to a pediatric geneticist at age 18 months because of excessive growth. A prenatal karyotype performed for advanced maternal age revealed a *de novo* balanced translocation between the short arm of chromosome 1 and the long arm of chromosome 6, karyotype 46,XX,t(1;6)(p22.1;q16.2). She was delivered at term by Cesarean section after an uncomplicated pregnancy. At birth she weighed 3.7 kg (NCHS Z-score +1.5 SD) and was 53 cm long (+1.3 SD). Accelerating growth was noted at age 3 months, and by 25 months she weighed 19.8 kg (+5.2 SD) and was 96 cm tall (+3.1 SD) (wt. for ht. +3.5 SD). She was not dysmorphic, and apart from her obesity, there were no features suggestive of Prader-Willi, Bardet-Biedl, or other well-defined syndromes. An umbilical hernia present during infancy resolved without treatment.

The proband's height curve has remained stable at +3 SD (Fig. 3), while her weight and weight-for-height curves continue to deviate upward (Fig. 2 and 4). Her rate of weight gain is comparable to that of girls with mutations in leptin, leptin receptor or MC4R (Fig. 5).

Figure 2

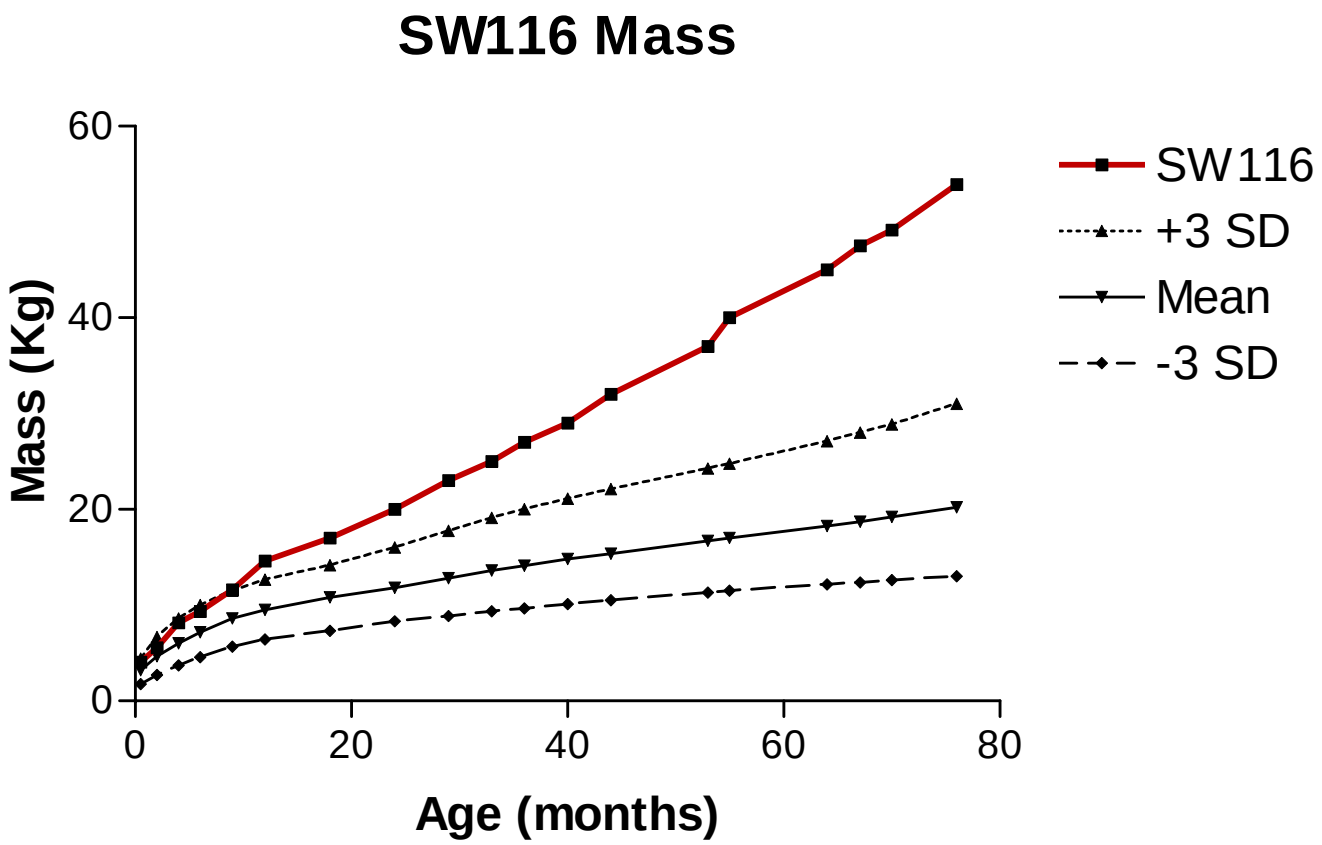

SW116 Mass
Mass (Kg)
60
40
20
0
0
20
40
60
80
Age (months)
SW116
+3 SD
Mean
-3 SD

Figure 2 SW116 has early-onset obesity.

SW116's mass was determined at the indicated ages and compared with standard values from NCHS. Her mass has been greater than three standard deviations above the mean since eighteen months of age.

Figure 3

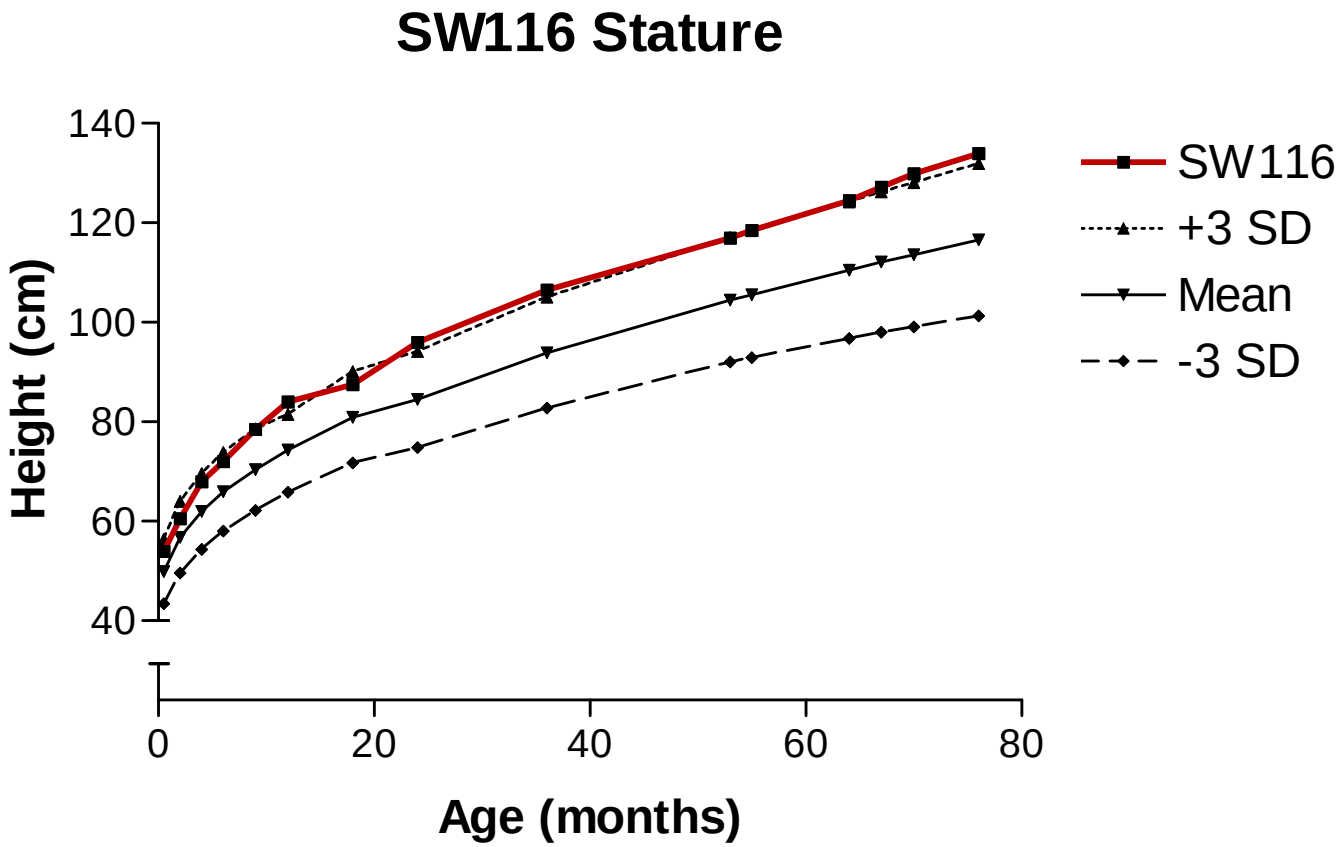

SW116 Stature
Height (cm)
140
120
100
80
60
40
0
20
40
60
80
Age (months)
SW116
+3 SD
Mean
-3 SD

Figure 3 SW116 has elevated linear growth**.**

SW116's length was determined at the indicated ages and compared with standard values from NCHS. Her length has been at or greater than three standard deviations above the mean since eighteen months of age.

Figure 4

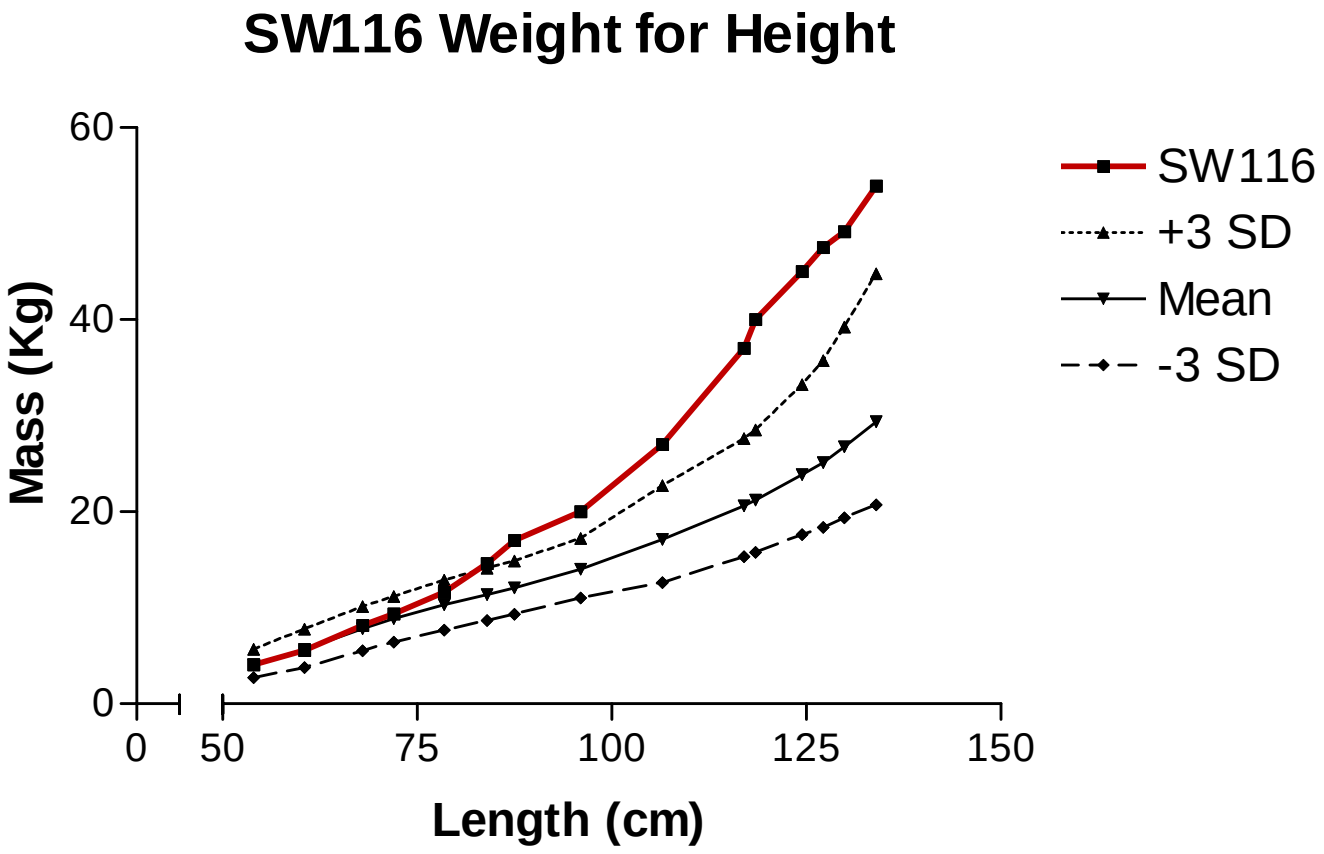

SW116 Weight for Height
Mass (Kg)
60
40
20
0
0
50
75
100
125
150
Length (cm)
SW116
+3 SD
Mean
-3 SD

Figure 4 SW116 has elevated mass when corrected for height.

SW116's weight for height was determined at the indicated ages and compared with standard values from NCHS. Her weight for height has been greater than three standard deviations above the mean since 85 cm of height.

Figure 5

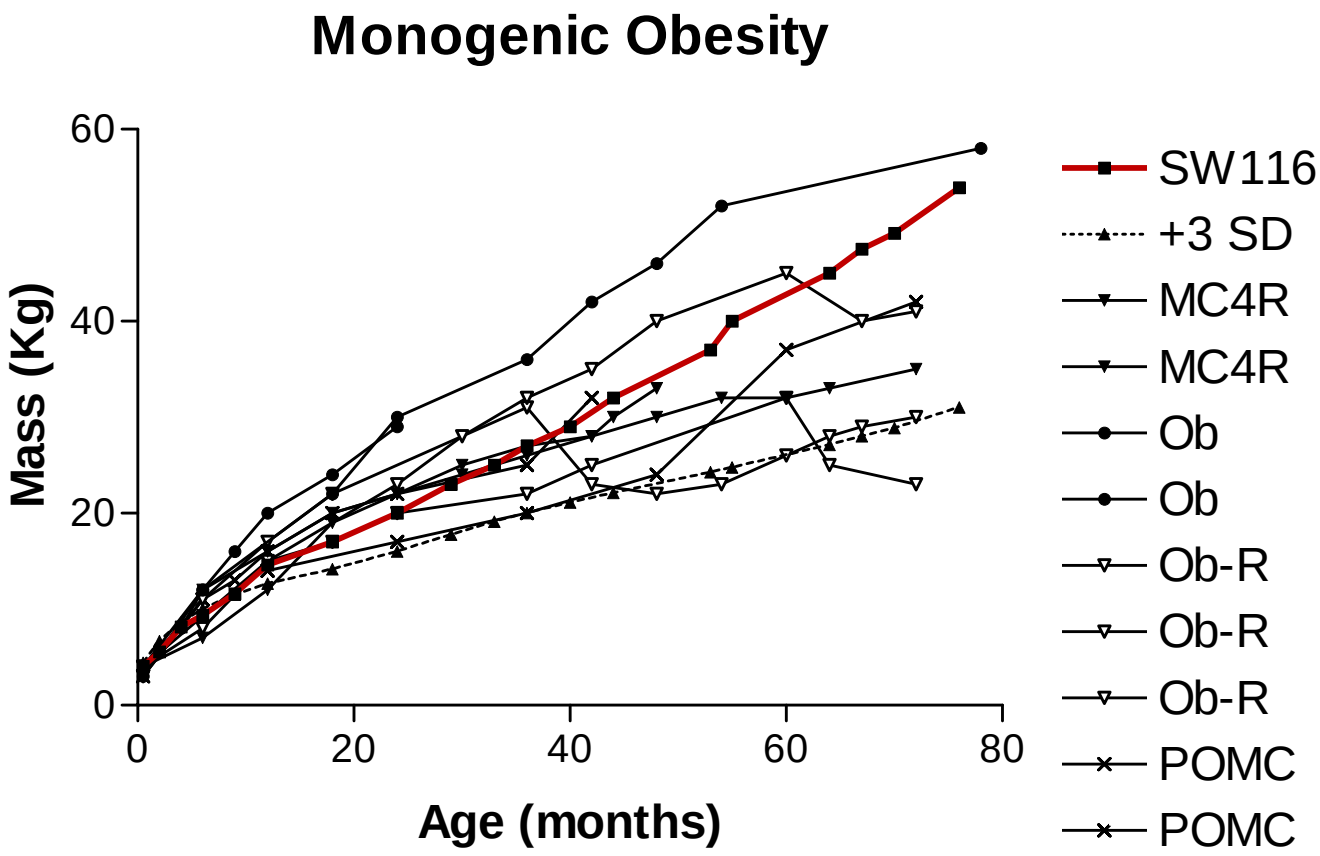

Monogenic Obesity
Mass (Kg)
0
20
40
60
0
20
40
60
80
Age (months)
SW116
+3 SD
MC4R
MC4R
Ob
Ob
Ob-R
Ob-R
Ob-R
POMC
POMC

Figure 5 SW116's mass is similar to other monogenic obesities.

SW116's mass is plotted versus other children with early-onset severe obesity due to mutation(s) in the indicated genes. Leptin, Leptin receptor and POMC mutations are recessive. MC4R mutations are dominant.

The father of SW116 was 188 cm tall and weighed 104 kg (BMI=29.4), the mother was 160 cm tall and weighed 54 kg (BMI=21.1), and a male sib at age 51 months was 111 cm tall (+1.5 SD) and weighed 22.4 kg (+2.5 SD) (wt. for ht. +1.9 SD). Abdominal CT and pituitary MRI of the proband were normal. Lab tests revealed slightly elevated serum insulin (16 µU/ml, normal 2-13); normal thyroid stimulating hormone, glucose, growth hormone, somatomedin-C, calcium, and phosphate; and slightly depressed cortisol (5.9 µg/dl, normal 8.7-22.4) concentrations. Repeat tests at age 53 months were normal, except for a radiographic bone age measurement of 82 months. Serum leptin concentrations were 16 and 24 ng/ml at 38 and 53 months of age, respectively, commensurate with her obesity (Garcia-Mayor, Andrade et al. 1997; Verrotti, Basciani et al. 1998).

An oral glucose tolerance test was administered to SW116 based on her elevated serum insulin level. SW116 was fasted overnight and provided an oral glucose load the following morning. Whole blood was serially collected over the next several hours. Three hours after administration of the oral glucose solution, SW116's blood glucose level peaked at 183 mg/dL (normal < 140 mg/dL). Individuals with impaired glucose tolerance peak with a blood glucose levels of between 140 and 200 mg/dL, and individuals with diabetes peak with a blood glucose level above 200 mg/dL. Our subject's serum insulin peaked two hours after administration of the oral glucose load at 194 μU/mL (normal < 80 μU/mL). Together, these data suggest our subject is insulin resistant but is not diabetic. As obesity is the major predisposing factor for insulin resistance and non-insulin dependent diabetes mellitus, SW116's obesity has likely caused her insulin resistance.

The mother noted mild hyperphagia beginning around age 4 years. There has been no evidence of developmental delay, preschool difficulties, or precocious puberty. Recently, detailed clinical analyses of several individuals with mutations in the melanocortin 4 receptor have been reported. Much of the phenotype unique to those who are obese due to MC4R mutations is shared by SW116. In addition to obesity and increased velocity of linear growth, both have significantly increased bone age. For SW116, bone age at chronological age of 5 years and 10 months was 9.5 years (>3 years advanced). For four out of five children reported by Stephen O'Rahilly with mutations in the MC4R, the range of advancement in bone age was 1.0-4.9 years. Advanced bone age has been reported to be associated with pediatric obesity, but in O'Rahilly's group of 243 obese children, the average advancement

of bone age was 1.7 years. Both SW116 and most children with mutations in the MC4R have substantially greater bone age advancement.

Both SW116 and children with MC4R mutations also have increased bone mineral density (BMD). The average Z-score for obese MC4R mutant children was +2.32, while it was +0.72 for normal family members. SW116's BMD Z-score was +2.3 at 67.1 months.

SW116's body composition, food intake, and energy expenditure were studied at age 67 months. She weighed 47.5 kg (+9.3 SD) and was 127.2 cm tall (+3.2 SD) (wt. for ht. +6.3 SD). Fat mass was 52% as determined by dual-energy X-ray absorptiometry, with generalized distribution. This value lies outside the values, 9-43%, of the 318 healthy girls, ages four to sixteen, measured previously.

A three-day food intake record by the mother indicated energy intake of 5017 kJ/d. Her basal and sleeping metabolic rates, measured by 24-hr calorimetry, were slightly greater than predicted by equations derived from calorimetry of 318 girls (Fig. 6). 24-hr respiratory quotient was normal at 0.86. Total energy expenditure in the free living state measured by the doubly-labeled water method was 8314 kJ/d, significantly greater than her reported energy intake. The ratio of total energy expenditure to basal metabolic rate, an index of physical activity level, was 1.45, which is within the range of measurements (1.3 to 2.5) observed for a series of 101 normal 8-year old girls. Observation of her *ad libitum* consumption revealed an aggressive, voracious appetite.

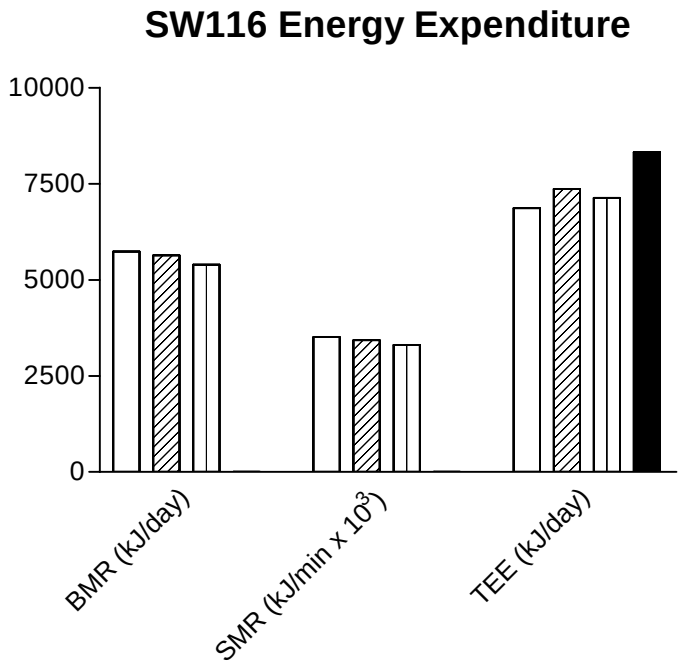


Figure 6 SW116 has normal energy expenditure

Basal metabolic rate (BMR), sleeping metabolic rate (SMR) and total energy expenditure (TEE) were measured by 24 hour calorimetry for SW116 (open bars) and compared with values predicted from her gender, age, race and body weight (hatched) or from her fat-free mass and fat mass (vertical stripe). Black bar, SW116's TEE as measured by the doubly-labeled water method.

Molecular studies

Review of the cytogenetic literature revealed three patients (Turleau, Demay et al. 1988; Villa, Urioste et al. 1995; Gilhuis, van Ravenswaaij et al. 2000) with complex phenotypes that included early onset obesity and small interstitial deletions of 6q potentially overlapping SW116's breakpoint (Fig. 7).

I hypothesized that SW116's unusual growth was due to haploinsufficiency of a gene on 6q. A number of CEPH megaYACs from the Whitehead Institute contig were tested by FISH against the patient's cell line and identified one clone, y852C9, that gave signals on both the der(1) and der(6) chromosomes as well as from chromosome 6. Sequence-tagged sites (STS's) mapping to this YAC clone were used to isolate a smaller Washington University YAC, yA35F11, that also crossed the breakpoint by FISH, and allowed the breakpoint to be mapped between D6S475 and WI6516. BAC clones were then isolated using markers WI6516, AFM176xg9, and D6S475, and the ends of these clones were then sequenced to generate additional STS markers. A BAC and PAC contig (Fig. 8) was constructed by iterative library screening, end sequencing, and STS content mapping. BAC b325C19 crossed the breakpoint by FISH (Fig. 9) and the del(6)(q16.2q21) subject reported by Villa et al. (Villa, Urioste et al. 1995) was deleted for this probe.

Figure 7

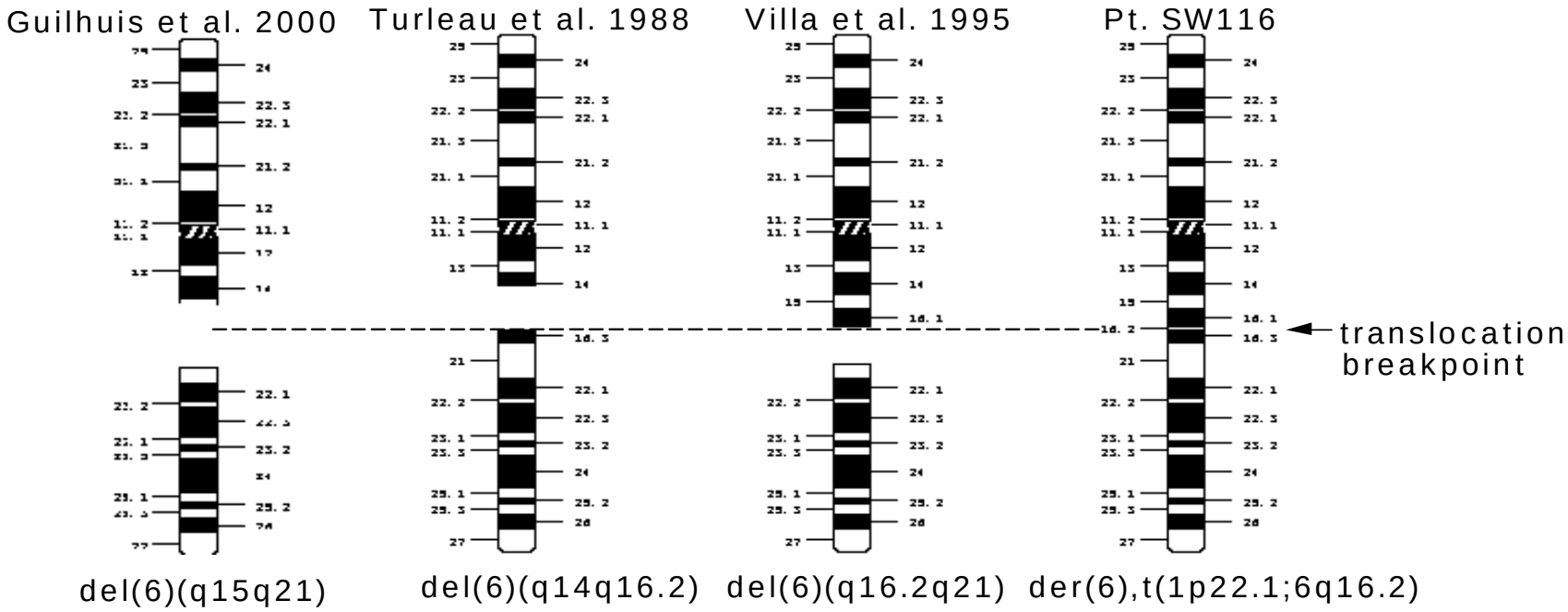

Guilhuis et al. 2000
Turleau et al. 1988
Villa et al. 1995
Pt. SW116
translocation breakpoint
del(6)(q15q21)
del(6)(q14q16.2)
del(6)(q16.2q21)
der(6),t(1p22.1;6q16.2)

Figure 7 Cytogenetic abnormalities encompassing 6q16 associated with obesity.

Ideograms of chromosome 6 depicting SW116 translocation breakpoint (arrow) and three interstitial deletions associated with obesity.

Figure 8

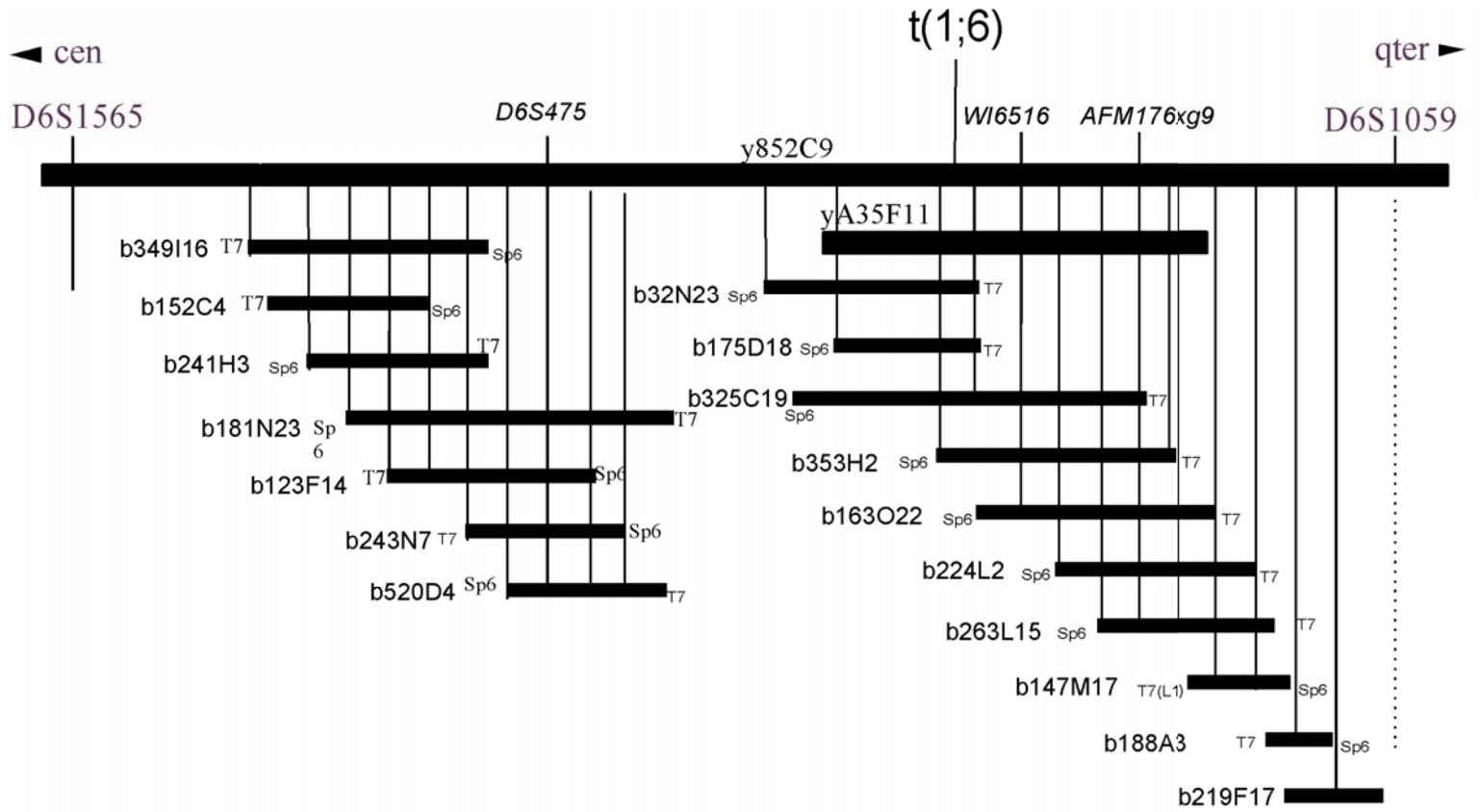

t(1;6)
cen
qter
D6S1565
D6S475
WI6516
AFM176xg9
D6S1059
y852C9
yA35F11
b349I16 T7 Sp6
b152C4 T7 Sp6
b241H3 Sp6 T7
b181N23 Sp6 T7
b123F14 T7 Sp6
b243N7 T7 Sp6
b520D4 Sp6 T7
b32N23 Sp6 T7
b175D18 Sp6 T7
b325C19 Sp6 T7
b353H2 Sp6 T7
b163O22 Sp6 T7
b224L2 Sp6 T7
b263L15 Sp6 T7
b147M17 T7(L1) Sp6
b188A3 T7 Sp6
b219F17

Figure 8 Chromosome 6 physical map of BACs near the translocation breakpoint.

t(1;6) indicates the location of the breakpoint. PCR and Fluorescence In Situ Hybridization (FISH) were used to map the order of BACs with respect to the breakpoint and to one another. Two Yeast Artifical Chromosome (YAC) clones crossing the breakpoint are also shown (thick lines).

Figure 9

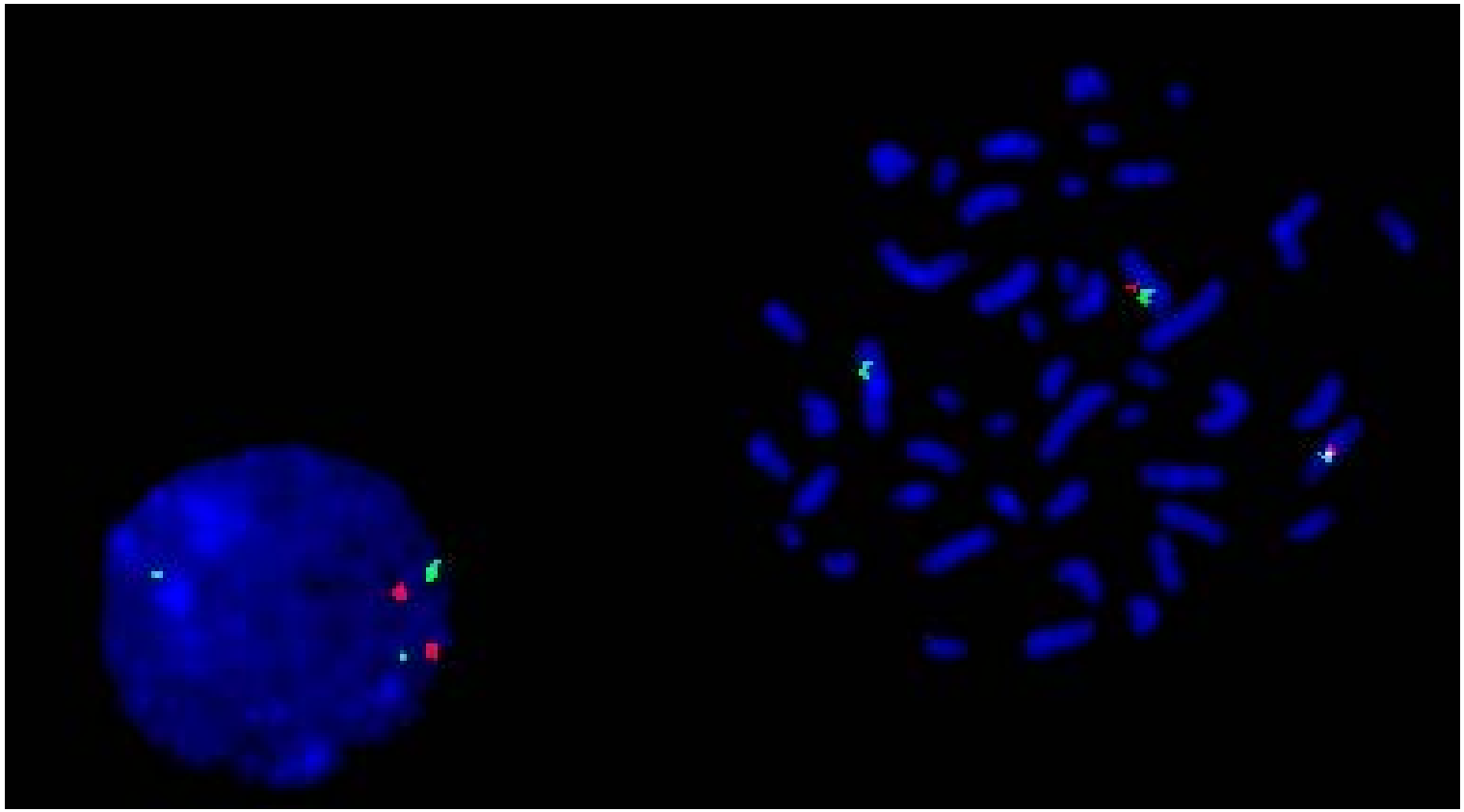


Figure 9 A chromosome 6 BAC crosses SW116's translocation breakpoint

Fluorescent in situ hybridization of b325c19 (green) and a chromosome 6 centromere probe (red). Note three green signals: two are associated with the chromosome 6 centromere representing the unaffected chromosome 6 and the derivative(6), one is not associated with the chromosome 6 centromere representing derivative(1).

To facilitate further breakpoint mapping, a somatic cell hybrid was made selectively retaining the der(1) chromosome. STS content mapping using this hybrid allowed me to position the chromosome 6 breakpoint on our contig and to map the chromosome 1 breakpoint between markers WI7492 and D1S1673. Three BAC clones containing D1S1673 were isolated and STS's generated from end sequences (Fig. 10). All three clones crossed the breakpoint by STS content mapping and by FISH.

Database searches revealed a match between one end of BAC clone b353H2 which crosses the 6q breakpoint and a 177 kb contiguous genomic sequence from 6q16 deposited in GenBank (Accession Z86062). Additional genomic sequence from BAC clone b21C21 crossing the 1p breakpoint was generated by The Sanger Center (Accession AL049861). I used these genomic sequences to design additional primers used to fine-map the breakpoints by PCR. Restriction fragments from either side of the translocation detected a novel der(6) junction fragment (Fig. 11*a*).

Figure 10

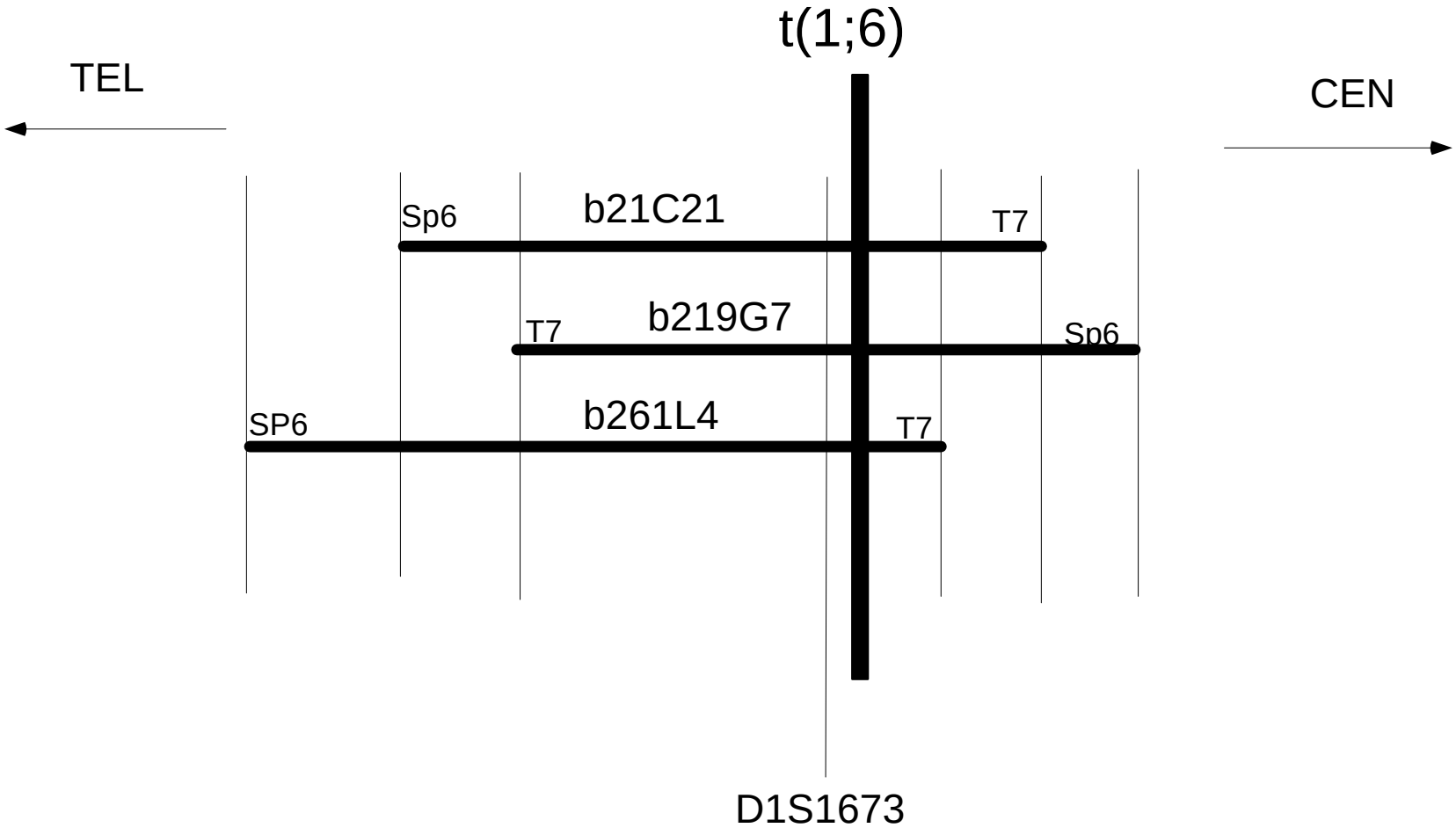
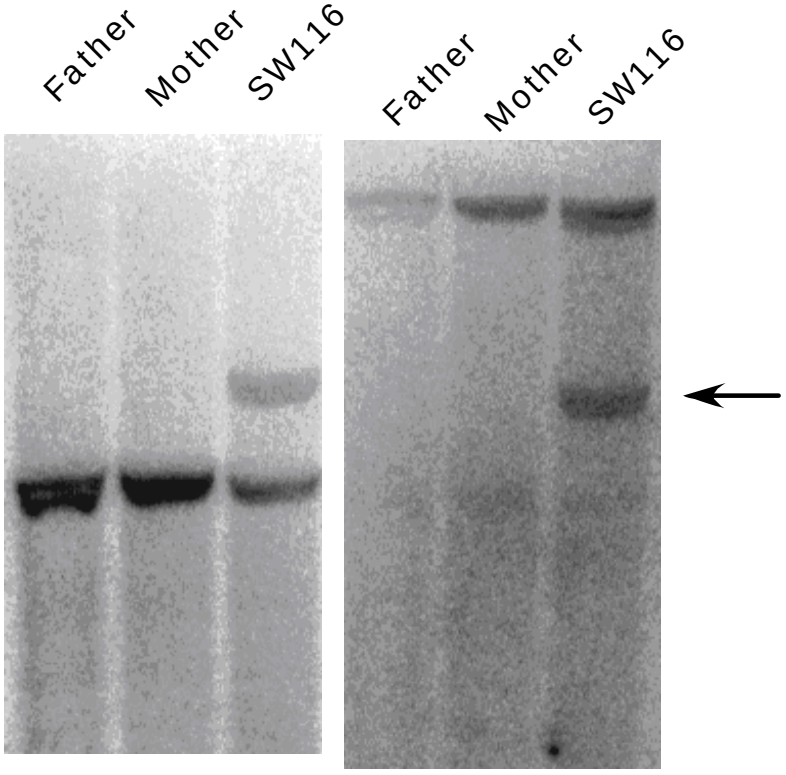


Figure 10 Chromosome 1 physical map of BACs crossing SW116 translocation breakpoint.

BACs b21C21, b219G7 and b261L4 were identified by PCR with STS D1S1673. BAC orientation was determined by PCR with primers developed from BAC end sequencing. b21C21 was determined to cross the translocation breakpoint by FISH and Southern blotting. t(1;6) indicates location of the translocation breakpoint.

Figure 11

(a)


Father
Mother
SW116
Father
Mother
SW116

(b)

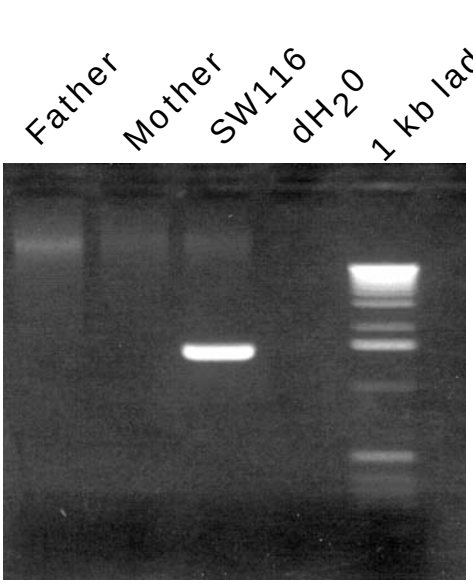

Father
Mother
SW116
dH2O
1 kb ladder

Figure 11 Cloning SW116's translocation breakpoint

(a) Southern blotting with genomic DNA digested with XbaI from SW116 and her parents. The first panel was probed with a DNA fragment from the first intron of *SIM1*. An abnormally migrating band was present in the proband, but not present in the father or mother (arrow). (b) Using a primer from chromosome 1 and a primer from chromosome 6, the der(6) translocation breakpoint was PCR amplified. The product of this reaction was present only in the proband and not in the father or mother.

Similar results were obtained for the der(1) chromosome, indicating that the translocation was not accompanied by a large concomitant deletion. I PCR-amplified and sequenced both junctions (Fig. 11*b*) and found that the translocation deleted only a single base pair of chromosome 6 and none of chromosome 1(Fig. 12).

```
chr 6    TCCTTGGTGTTTCCCCTTAAGATCTTTGGAGGCTTTGCCGCAAACTTTCTC
der (6)  TCCTTGGTGTTTCCCCTTAAGATCT  gctcactgcagaattctctaacatt
der (1)  gtttatcctgttggattacattcat  TGGAGGCTTTGCCGCAAACTTTCTC
chr 1    gtttatcctgttggattacattcat gctcactgcagaattctctaacatt
```

Figure 12 Alignment of SW116's translocation breakpoint junction fragments with consensus human chromosome 1 and 6 sequence.

The translocation disrupts *SIM1*

BLAST analysis of > 84 kb of contiguous genomic sequence from BAC clone b21C21, as well as exon trapping experiments using clone b219G7 failed to identify any 1p22 transcription units disrupted by the translocation. By contrast, sequence and mapping data indicated that the 6q breakpoint lies within a known gene, *SIM1*. Inspection of the intron/exon structure of *SIM1* indicated that the translocation breakpoint falls within the first intron and separates the 5' flanking sequence and the first exon from downstream exons (Fig. 13).

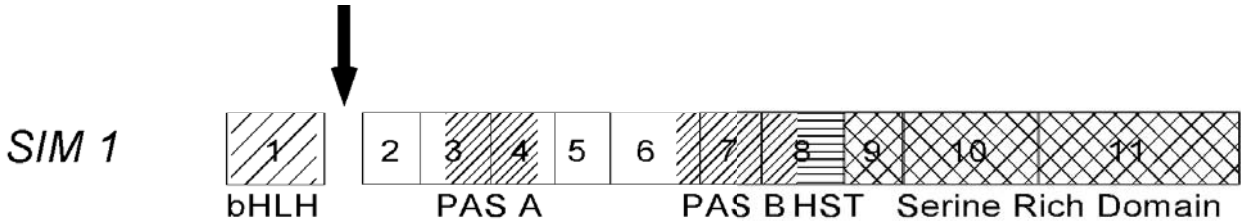


Figure 13 SW116's chromosome 6 translocation breakpoint lies within intron 1 of human *SIM1*.

To determine whether our proband's other allele of *SIM1* was normal, I PCR-amplified and sequenced all *SIM1* exons and splice sites. She is heterozygous for a silent C to T substitution at nt. 1328 in exon 9 of the coding sequence. I found no other mutations. Thus SW116 is presumably heterozygous for a loss-of-function *SIM1* mutation. Attempts to detect *SIM1* transcripts by RT-PCR of RNA from control and patient lymphoblastoid cells were unsuccessful.

SIM1 mutations are probably not a common cause of early-onset obesity

I performed mutation detection using single stranded conformational polymorphism analysis and limited DNA sequencing on samples from 45 markedly obese children referred to a pediatric endocrinologist. I found no other coding or splice site mutations. The C to T silent substitution in SW116 appears to be a common polymorphism. Eight of the 45 obese children were heterozygous for the T allele, and the remaining subjects were homozygous for the C allele. By comparison, 9 of 33 controls were heterozygous for the T allele, and the

remainder were homozygous for the C allele. There was no significant association of obesity with this polymorphism (χ^2=0.53, 0.25<P<0.5).

DISCUSSION

I identified a mutation in the *SIM1* gene in a girl with profound obesity and increased linear growth. Her weight gain must involve occult food consumption, since measured energy expenditure was normal and was significantly greater than her reported caloric intake. I hypothesize that *SIM1* haploinsufficiency causes her phenotype. If this hypothesis is correct, obesity due to *SIM1* mutations would be expected to show autosomal dominant inheritance. This prediction cannot be tested in our kindred, since the translocation occurred *de novo*. No additional *SIM1* mutations were identified by SSCP in 45 other children with marked obesity, but such mutations may be identified by screening larger numbers of subjects with more sensitive techniques. Linkage of obesity to 6q16.2 has not been reported in large genetic studies, but these studies have not specifically examined subjects with severe, early onset obesity. The physical map of 6q16.2 and the availability of *SIM1* genomic sequences should facilitate testing for associations between obesity and the *SIM1* gene.

The possibility that one or both of the *SIM1* gene fragments are expressed as part of a fusion gene cannot be excluded, although no other transcription units are in close proximity to the breakpoints. It is also possible that a truncated *SIM1* transcript produces a dominant negative protein. The reported interstitial 6q deletions associated with early-onset obesity are consistent with our haploinsufficiency model. Screening additional patients with early onset obesity might reveal additional microscopic or submicroscopic deletions of *SIM1*.

Position effects on expression of nearby genes could also account for the phenotype of the proband. Inspection of the human transcript map revealed no other obvious candidate genes near either translocation breakpoint. The leptin receptor gene (*LEPR*) maps to 1p31,

over 20 Mb from the 1p breakpoint, and this locus appeared to be intact by FISH. The only human obesity-related trait that has been genetically mapped near either breakpoint is a quantitative trait locus for 24-hr respiratory quotient linked to *D1S550* on 1p31-p21 in a study of Pima Indian (Norman, Tataranni et al. 1998). However, this locus is unlikely to be relevant to our subject's phenotype, since her 24-hr respiratory quotient was normal.

It is also possible that the phenotype of the proband and her translocation are coincidental. Indeed, her father and brother, who do not carry the translocation, are large. However, unlike the proband, the father's obesity developed during adulthood, and the brother's growth was not sufficiently abnormal to bring him to medical attention. Nevertheless, as with any complex trait, genetic background and environmental factors undoubtedly contribute to the severity of the phenotype.

Sim1 continues to be expressed in the PVH in adult mice, suggesting that *Sim1* participates in post-developmental HPA function(s). These functions are presently obscure, since SIM1's transcriptional targets are not known. A system for potentially identifying transcriptional targets of SIM1 will be presented in Chapter 4. Expression of *SIM1* in the PVH is intriguing with regard to obesity, since this nucleus is critical for the integration of signals governing appetite and energy expenditure (Elmquist, Elias et al. 1999). Lesions in the rat PVH result in obesity, and microinjection into the PVH of virtually all known orexigenic neurotransmitters or neuropeptides stimulates feeding. Conversely, microinjection of anorexic peptides such as CRF or leptin attenuates post-fasting food intake.

PVH neurons also express the *Mc4r* (Mountjoy, Mortrud et al. 1994), neuropeptide Y receptors Y1 (Jacques, Tong et al. 1996) and Y5 (Gerald, Walker et al. 1996), galanin and

galanin receptors 1 and 2 (Gundlach and Burazin 1998), CRF and CRF receptor (Richard, Rivest et al. 1996), and the orexin 2 (hypocretin) receptor (Trivedi, Yu et al. 1998). This nucleus appears to be an important mediator of these anorexigenic and orexigenic signals. Mutations in the *MC4R* gene have been shown to cause obesity and increased linear growth in both mice and humans (Huszar, Lynch et al. 1997; Farooqi, Yeo et al. 2000). The increased linear growth phenotype is not a general feature of monogenic obesity, since leptin deficiency does not increase linear growth in humans (Montague, Farooqi et al. 1997) and in fact impairs linear growth in mice. In this regard, our proband's tall stature and the coincident expression of *SIM1* and *MC4R* in the PVH suggests that both genes may belong to the same physiologic or even molecular pathway regulating growth and energy balance (Table II). Alternatively, our proband's stature may reflect increased growth velocity without change in final height, as has been noted in other obese children and ascribed to increased insulin levels (De Simone, Farello et al. 1995).

Table II. Comparison of SW116 with individuals with mutations in leptin or *MC4R*

	SW116	leptin	*MC4R*
Age of onset	early-onset	early-onset	early-onset
Linear growth	accelerated	normal	accelerated
Food seeking	yes	yes	yes
BMR	normal	normal	normal
Fasting insulin	elevated	elevated	elevated
Fasting glucose	normal	normal	normal
Bone age	+3.7 years	+3.5 years	+1.0-4.9 yers
Bone Mineral Content	+2.3 SD	??????	+2.3 SD

Previous genetic and pharmacologic studies have identified a number of signaling molecules important for hypothalamic regulation of energy balance (Robinson, Dinulescu et al. 2000), but few studies have examined nuclear transcription. The transcription factor Fos is induced rapidly in response to cytokine-related signals, and this induction has been used to map leptin-responsive neurons in the hypothalamus (Elmquist, Ahima et al. 1997). Mice lacking another transcription factor expressed in the hypothalamus, nHLH2, show adult-onset obesity, possibly through a quantitative defect in Pomc levels in the arcuate nucleus (Good, Porter et al. 1997). It seems likely that changes in hypothalamic gene expression mediated by transcription factors such as nHLH2 or SIM1 play a role in the long term regulation of food intake and energy expenditure.

Chapter 4

Creation of a cell culture system for identifying SIM1 transcriptional targets

Introduction

From the previous work with patient SW116, *SIM1* is a candidate novel human obesity gene. The mechanism by which haploinsufficiency of SIM1 results in obesity is unclear. As SIM1 is a transcription factor expressed in the hypothalamus, *SIM1* regulated genes might be critical for proper feeding behavior and body weight regulation. Because of SIM1's expression in the hypothalamus, it is impossible to determine what genes might have altered regulation directly in SW116. To overcome this limitation, I have created a cell culture system for identifying genes whose expression will be altered when SIM1 is conditionally overexpressed. Similar approaches have been taken for other transcription factors expressed in the CNS such as NPAS2 (Reick, Garcia et al. 2001) and Six5 (Sato, Nakamura et al. 2002). Identifying differentially expressed genes in the brain is particularly problematic because of the limited expression patterns of many genes of the central nervous system, including that of SIM1. Use of cells in culture expressing the gene of interest can potentially overcome this problem because of uniform expression. Using conditional expression systems, it is also possible to produce dramatic changes in expression of the gene of interest. In order to take advantage of these benefits, I have created a cell culture system that inducibly expresses SIM and used this system to attempt identification of SIM1 transcriptional targets.

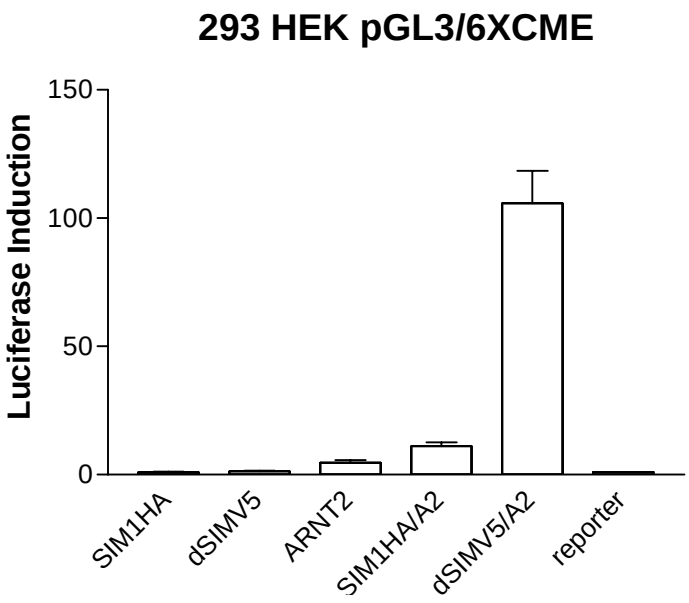
Results

SIM and ARNT2 cooperate to induce expression from a 6X CME reporter

It was previously reported that cotransfection of SIM1 and ARNT2 with a reporter construct containing 6 copies of the *Drosophila toll* 4 central midline enhancer (CME) site causes induction of the reporter gene (Moffett and Pelletier 2000). The CME was first described by Stephen Crews as the binding site for dSIM and its dimerization partner in *Drosophila*, Tango (Wharton, Franks et al. 1994). Since both dSIM and Tango have the same amino acid sequence in their basic, DNA binding, domains as their mammalian homologues, SIM1 and ARNT2 are able to bind to the *Drosophila* CME. I replicated this experiment and extended it to demonstrate that SIM1 and dSIM can dimerize with ARNT2 to induce reporter production from a construct with six copies of the CME binding site upstream of a minimal promoter and luciferase gene in both 293 human embryonic kidney cells (HEK) and a neuroblastoma cell line SK-N-BE(2)-M17 (Fig. 14). As reported by Moffett and Pelletier (Moffett and Pelletier 2000), SIM1 is a weaker transcriptional activator than dSIM in both the 293 and BE(2)-M17 cell lines.

Figure 14

(a)


293 HEK pGL3/6XCME
Luciferase Induction
150
100
50
0
SIM1HA
dSIMV5
ARNT2
SIM1HA/A2
dSIMV5/A2
reporter

(b)

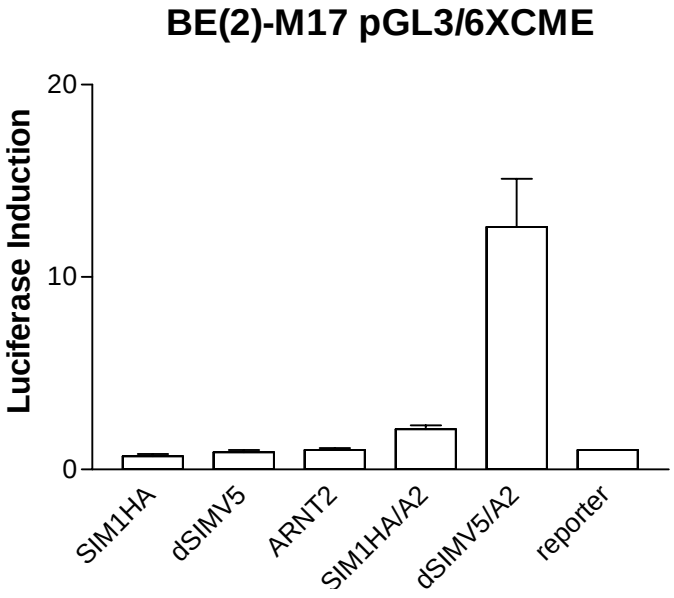

BE(2)-M17 pGL3/6XCME
Luciferase Induction
20
10
0
SIM1HA
dSIMV5
ARNT2
SIM1HA/A2
dSIMV5/A2
reporter

Figure 14 SIM1 acts as a transcriptional activator.

(a) 293 HEK cells or (b) SK-N-BE(2)-M17 human neuroblastoma cells were transiently transfected with the pGL3/6XCME reporter either alone or with expression vector(s) containing a cDNA of the gene noted. Each bar represents the mean +/- SEM.

Establishing cell lines stably expressing SIM and ARNT2

Despite the high level of induction of the luciferase promoter from pGL3/6XCME with cotransfection of either SIM1HA or dSIMV5, in transient transfections there can be substantial variability with cell to cell expression of transfected genes. To eliminate this possibility, 293 cells were engineered that inducibly express either SIM1 or dSIM. Figure 15 represents the strategy used to create cell lines expressing SIM1HA or dSIMV5 and ARNT2.

Figure 15

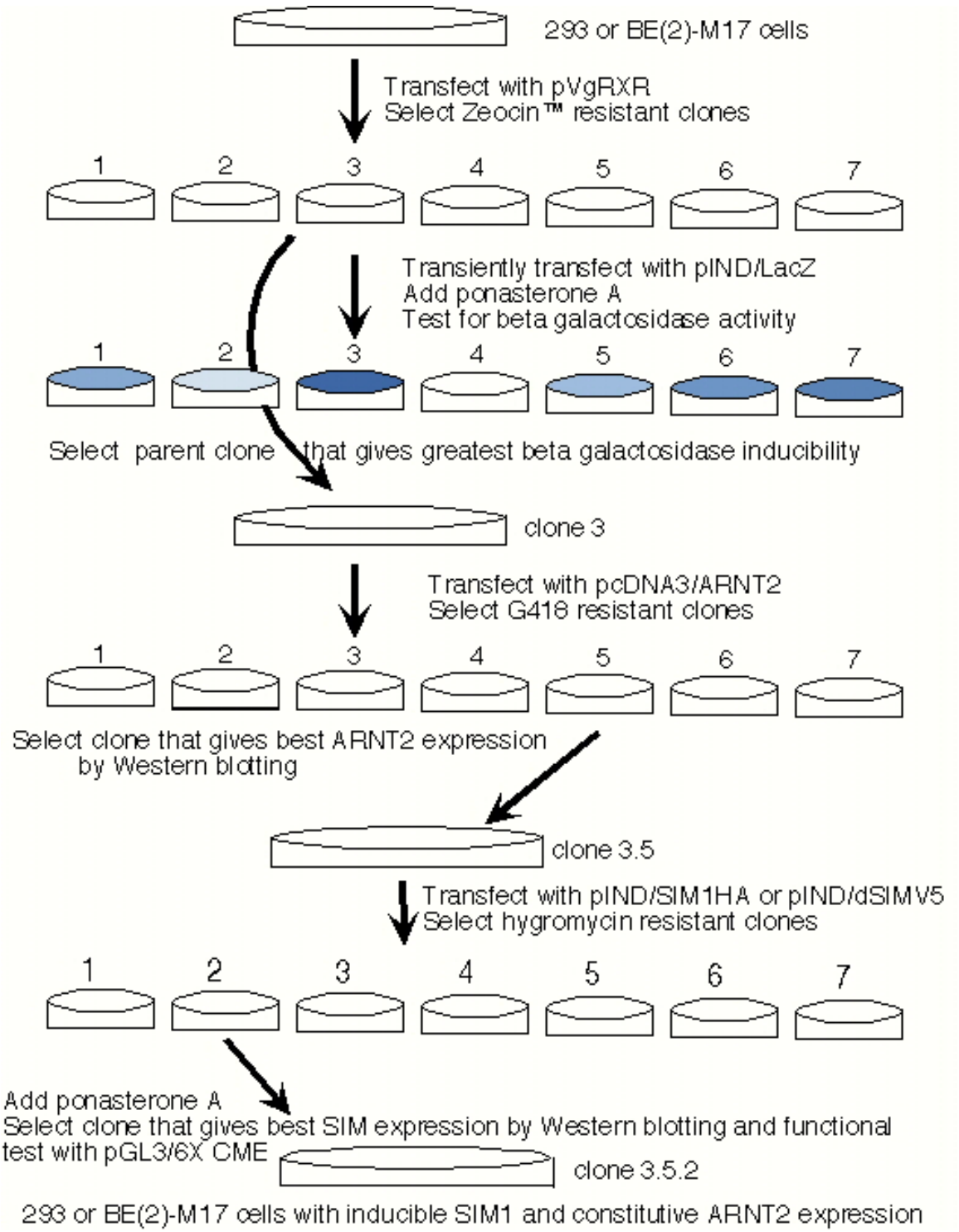

293 or BE(2)-M17 cells
Transfect with pVgRXR
Select Zeocin™ resistant clones
1
2
3
4
5
6
7
Transiently transfect with pIND/LacZ
Add ponasterone A
Test for beta galactosidase activity
Select parent clone that gives greatest beta galactosidase inducibility
clone 3
Transfect with pcDNA3/ARNT2
Select G418 resistant clones
Select clone that gives best ARNT2 expression by Western blotting
clone 3.5
Transfect with pIND/SIM1HA or pIND/dSIMV5
Select hygromycin resistant clones
Add ponasterone A
Select clone that gives best SIM expression by Western blotting and functional test with pGL3/6X CME
clone 3.5.2
293 or BE(2)-M17 cells with inducible SIM1 and constitutive ARNT2 expression

Figure 15 Experimental scheme for creation of a cell culture system with inducible expression of either SIM1 or dSIM for identifying transcriptional targets of SIM1. Iterative transfections and selection of drug resistant clones will yield cells which constitutively express ARNT2 and inducibly express dSIM or SIM1.

The ecdysone inducible expression system from Invitrogen was used to create cells with inducible expression. First 293 cells were stably transfected with a mammalian expression plasmid, pVgRXR, that constitutively expresses the ecdysone receptor, RXR and a Zeocin resistance gene. Four clones were then selected that are resistant to Zeocin. The clone (pVgRXR-4) with best conditional expression, as determined by transient transfection with a reporter plasmid, pIND/LacZ, containing beta-galactosidase under an inducible promoter with five ecdysone receptor/RXR binding sites, was used for subsequent transfections (Fig. 16).

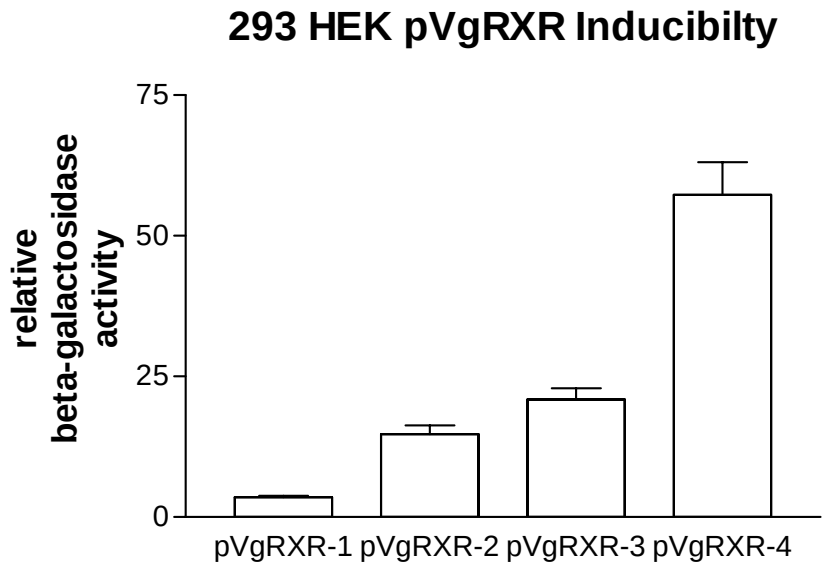


Figure 16 293 HEK cells express beta-galactosidase under control of a promoter which contains five binding sites for heterodimers of the Ecdysone receptor and RXR. Cells were incubated with either standard media or media supplemented with 10 μM ponasterone A for 48 hours. Bars are mean +/- SEM.

As shown previously, cotransfection of SIM and the 6X CME reporter alone is ineffective in activating transcription of the luciferase reporter. This is due to the requirement

of a dimerization partner such as ARNT or ARNT2. Although 293 cells express ARNT and ARNT2 by rt-PCR, neither can be detected by Western blotting for endogenous protein. Together, these data indicate that neither ARNT nor ARNT2 are endogenously expressed at adequate concentrations to dimerize with SIM when overexpressed by transfection. Therefore, it is necessary to also overexpress ARNT2 to allow adequate formation of SIM/ARNT2 dimers to activate transcription of either endogenous genes or luciferase from a reporter construct.

The pVgRXR-4 clone was therefore further transfected with pcDNA3.1/ARNT2, an expression vector that constitutively expresses mouse ARNT2 with the Cytomegalovirus (CMV) promoter. Six independent clones were selected for resistance to G418 due to expression of a nemomycin resistance gene present in the pcDNA3.1 expression vector. Expression of ARNT2 was determined by Western blotting using a commercial antibody specific to ARNT2 (Fig. 17). The same protein filter was also hybridized with an antibody specific for RPS4X, a ribosomal protein, to normalize for protein loading. Clone number two (293 4.2) had the highest expression of ARNT2 as determined by the ARNT2 to RPS4X ratio, and was used for subsequent transfections.

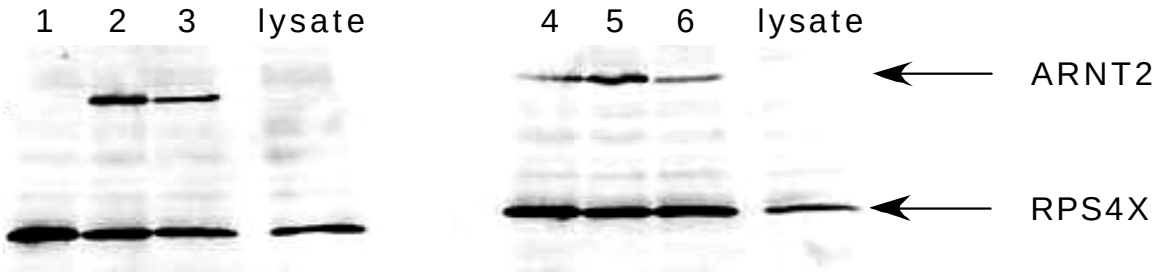
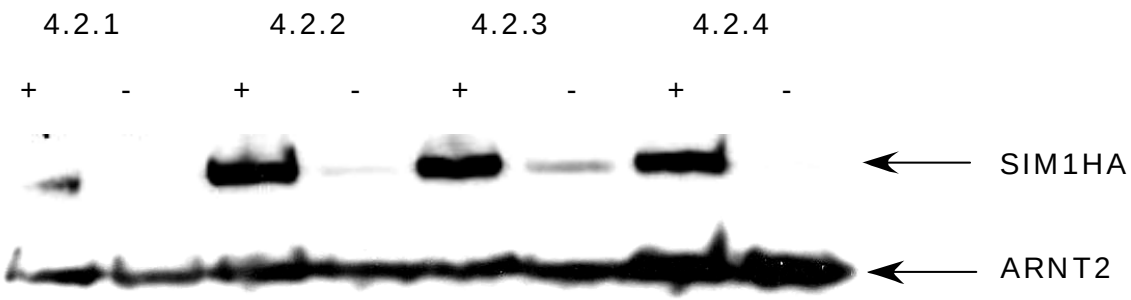


Figure 17. Constitutive expression of ARNT2 in 293 pVgRXR-4 cells stably transfected with pcDNA3.1/ARNT2. Western blotting was performed with an antibody specific for ARNT2 or for RPS4X. Note the ARNT2/RPS4X ratio. 293 4.2 has the greatest expression of ARNT2.

The 293 4.2 cell line was next transfected with an expression vector that contains five Ecdysone receptor/RXR heterodimer binding sites (pIND) with either mouse SIM1 tagged with an epitope from the influenza hemagglutin (pIND/mSIM1HA) or drosophila Singleminded tagged with V5 (pIND/dSIMV5) cloned downstream of the inducible promoter. The pIND vector contains a hygromycin resistance gene and clones resistant to hygromycin were selected. Both Western blotting with an antibody specific to HA for mSIM1HA or an antibody specific to V5 for dSIMV5 and transient transfections with the pGL3/6XCME reporter plasmid plus or minus induction by ponasterone A were performed for determining which clone had the best induction of transcriptional activity. By Western blotting, clone 293 4.2.2 and 4.2.4 had high level of induction of SIM1HA by ponasterone A. However, using the functional assay with the 6X CME/Luciferase reporter, none of the clones isolated appear to be able to strongly induce transcription from the reporter (Fig. 18).

Figure 18

(a)


4.2.1
4.2.2
4.2.3
4.2.4
+ - + - + - + -
SIM1HA
ARNT2

(b)

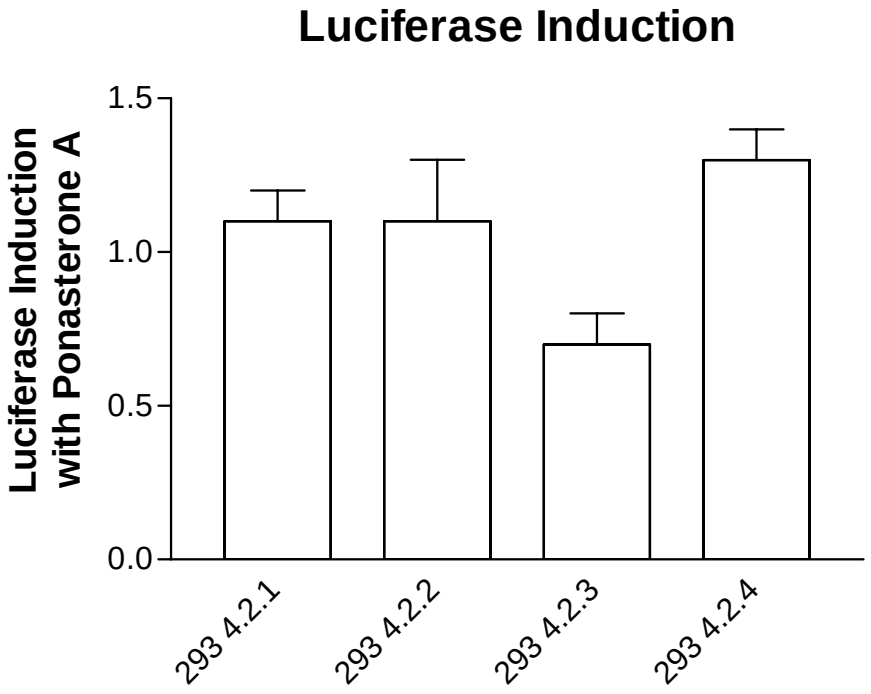

Luciferase Induction
Luciferase Induction with Ponasterone A
1.5
1.0
0.5
0.0
293 4.2.1
293 4.2.2
293 4.2.3
293 4.2.4

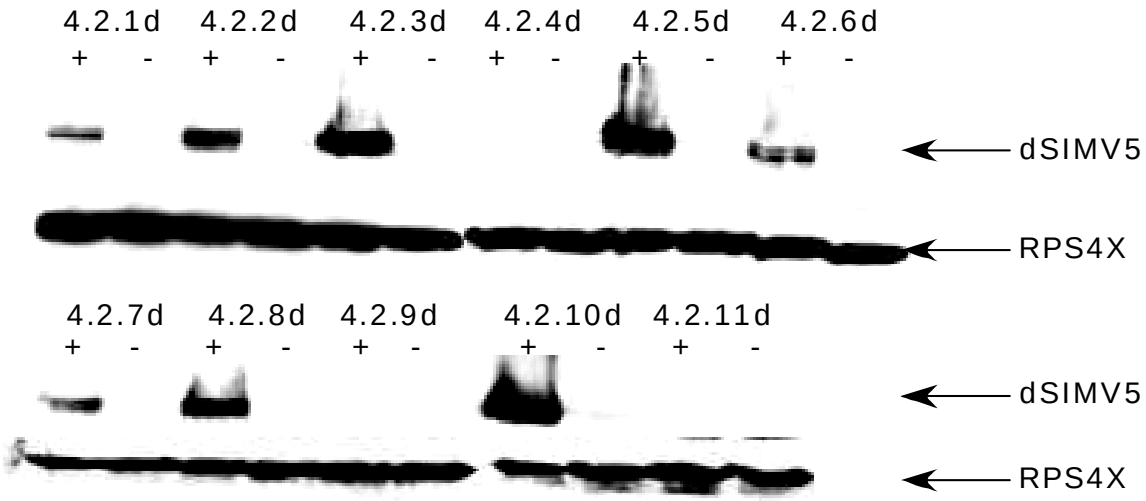
Figure 18. Inducible expression of SIM1HA in 293 4.2 cells.

(a) Western blotting of 293 4.2 hygromycin resistant clones stably transfected with pIND/SIM1HA using an antibody specific for the HA epitope and for ARNT2. Cells induced with 10 M ponasterone A for 48 hours (+) or uninduced (-).
(b) Luciferase reporter activity of the same clones. Induction is the ratio of induced/uninduced for 48 hours. Bars are mean +/- SEM.

SIM1 is a weak transcriptional activator when compared to dSIM. Additionally, transfection of ARNT2 and the 6X CME reporter in 293 cells results in substantial background activation over transfection of the reporter alone. SIM1 and dSIM have precisely the same amino acid sequence within their basic, DNA binding domains, and dSIM is a much stronger transcriptional activator as determined by the previous cotransfection assays (Fig. 14). Therefore, the 293 4.2 clone was transfected with pIND/dSIMV5 and eleven clones resistant to hygromycin were selected. Each clone was checked for inducibilty of dSIM expression by Western blotting and inducibilty of luciferase from the pGL3/6XCME reporter plasmid (Fig. 19).

Figure 19

(a)


4.2.1d
4.2.2d
4.2.3d
4.2.4d
4.2.5d
4.2.6d
+ - + - + - + - + - + -
dSIMV5
RPS4X
4.2.7d
4.2.8d
4.2.9d
4.2.10d
4.2.11d
+ - + - + - + - + -
dSIMV5
RPS4X

(b)

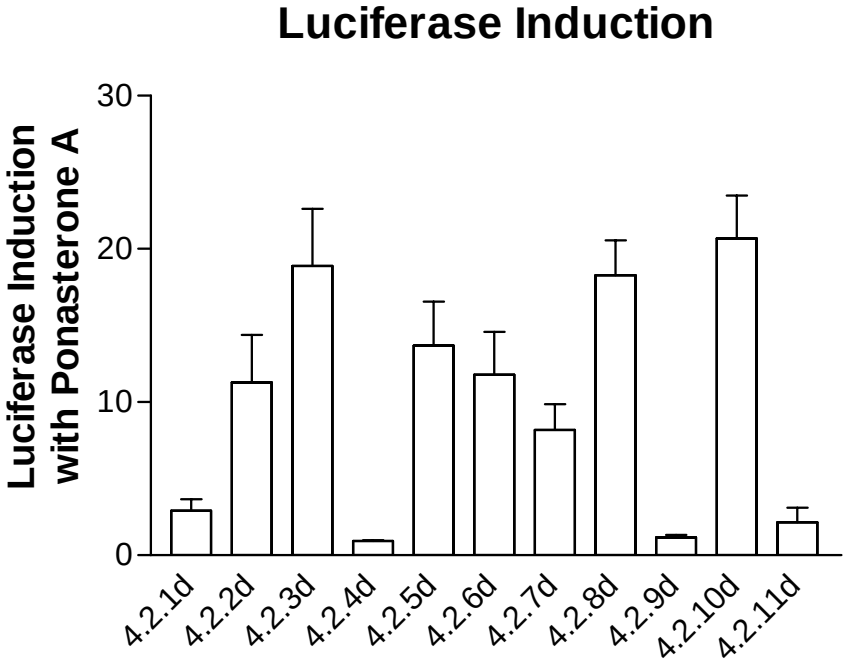

Luciferase Induction
Luciferase Induction with Ponasterone A
30
20
10
0
4.2.1d
4.2.2d
4.2.3d
4.2.4d
4.2.5d
4.2.6d
4.2.7d
4.2.8d
4.2.9d
4.2.10d
4.2.11d

Figure 19. Inducible expression of dSIM in 293 4.2 cells

(a) Western blotting of 293 4.2 hygromycin resistant clones stably transfected with pIND/dSIMV5 using an antibody specific for the V5 epitope and for RPS4X.
Cells induced with 10 μM ponasterone A for 48 hours (+) or uninduced (-).
(b)Luciferase reporter activity of the same clones. Induction is the ratio of induced/uninduced for 48 hours. Bars are mean +/- SEM.

Clones 4.2.3d, 4.2.5d and 4.2.10d had strong induction of dSIMV5 with ponasterone A as determined by Western blotting. Luciferase reporter transfection experiments indicated that 4.2.3d and 4.2.10d had the best ability to activate transcription from the 6XCME reporter construct. Both could induce Luciferase activity by approximately twenty-fold. Clone 4.2.3d was used for further experiments to identify transcriptional targets in mammalian cells.

Microarray profiling of 293 4.2.3d cDNA

To identify genes which are transcriptionally regulated by SIM, the 293 4.2.3d cell line was used in a cDNA microarray experiment. cDNA from cells of the 4.2.3d clone grown for 48 hours in the presence of ponasterone A was synthesized with dUTP labeled with the fluorescent label Cy3. cDNA from uninduced cells were synthesized with dUTP labeled with Cy5. Both of these labeled cDNAs were hybridized to a cDNA microarray spotted on a glass slide at the Microarray Core facility at UT Southwestern. The array used for this experiment contained 10,800 spots, each representing either a known human gene or a human Expressed Sequence Tagged Site (EST). This hybridization was performed twice, once as described above and once with the Cy3 and Cy5 dyes reversed.

There were 259 genes in which there was at least a two-fold change in one of the hybridizations between 293 4.2.10d cells either induced or uninduced. However, no gene was found to have greater than a two fold difference of expression in both experiments. Lowering the stringency of this experiment to a 1.4 fold change yielded twenty-four genes changed in both experiments (Table III).

Table III. cDNA microarray results

Genbank Accession	Gene Name	1st experiment	2nd experiment	Putative function
R66896	EST	3.81	1.89	?????
N53453	EST	3.67	1.67	?????
R95882	EST	3.31	1.82	?????
R98442	EST	3.29	1.5	?????
R98107	EST	2.85	2.15	?????
R98934	EST	2.74	1.41	?????
R98191	EST	2.73	1.87	?????
AA010352	Sjogren Antigen A2	2.73	1.6	
T69603	Complement component 1	2.62	1.6	
N59690	EST	2.58	1.62	Alu repeat
T69359	Mannose-binding lectin	2.57	1.79	
H48467	EST	2.47	1.54	?????
T97080	EST	2.39	1.7	
R02699	EST	2.28	1.62	Unc-5c
AA070226	Selenoprotein P	2.09	2.71	Antioxidant
R31395	EST	2.05	1.89	Oxysterol binding?
H48445	EST	2.04	1.68	?????
AA447393	KIAA0091	2.04	1.59	Site-1 protease
H93068		2.03	1.94	
T80232	Autotaxin	1.6	2.3	phosphodiesterase
N62620	Twik-1	-1.42	-2.08	K+ channel
W87741	EST	-2.05	-2.38	Proto-oncogene
R76553	EST	-2.15	-3.08	Metalloproteinase
H16637	V-CAM1	-2.44	-2.18	ECM

Discussion

Despite a high level of induction of the luciferase reporter, none of the genes examined in the microarray experiment were significantly induced with dSIM expression. Because of this result, no effort was made to corroborate any of the microarray results by independent methods such as Northern blotting or real-time RT-PCR. There are several possible reasons for this result. The pGL3/6XCME reporter has six binding sites for dSIM which might allow cooperative binding to this reporter not present in SIM1 binding sites in the human genome. SIM1 might normally require transcriptional coactivators not present in the 293 cell line. The microarray used for this experiment was originally designed for profiling the expression of cancer cells versus normal cells, and therefore, does not have genes which are likely to be involved in the normal function of SIM1, such as genes expressed in the hypothalamus that have a known function in feeding behavior. It would be interesting to use other arrays which are more biased for CNS expressed genes. In an attempt to overcome the possible need for transcriptional cofactors, the lab is currently making a fusion gene with mouse SIM1 and the transcriptional activation domain VP-16. A similar experiment has been performed by Sato and colleagues to identify transcriptional target of the Six5 transcription factor (Sato, Nakamura et al. 2002).

Chapter 5

Abnormal feeding behavior in response to dietary fat in *Sim1* mutant mice: a paradigm for gene x environment interaction in obesity

Introduction

The rise in prevalence of obesity over the past several decades is largely due to changes in environment, including an increasingly sedentary lifestyle and consumption of calorically dense foods rich in fat. Although this environment has enabled the obesity epidemic, most of the individual variance in body weight is nevertheless attributed to genetic factors (Stunkard 1991). Obesity is a polygenic trait, and there are major efforts underway to identify the underlying genes that contribute to obesity in the general population (Rankinen, Perusse et al. 2002). At the same time, studies of rare monogenic obesity mutations in humans and mice have elucidated novel pathways regulating energy homeostasis. To date, mutations in six human genes have been shown to cause severe, early-onset obesity : leptin (*LEP*); leptin receptor (*LEPR*); proprotein convertase, subtilisin/kexin-type, 1 (*PCSK1*); proopiomelanocortin (*POMC*); melanocortin 4 Receptor (*MC4R*); and single-minded homologue 1 (*SIM1*) (Rankinen, Perusse et al. 2002). Of these, only *SIM1* was identified by positional cloning without any prior knowledge of a mouse mutant phenotype or its potential physiologic function (Holder, Butte et al. 2000). One copy of *SIM1* was disrupted by a balanced translocation in a girl with early onset obesity, hyperphagia and accelerated linear growth as described in Chapter 3.

The PAS domain is conserved from prokaryotes to eukaryotes, and proteins with this domain often function in signaling pathways that sense the intracellular or extracellular environment (Gu, Hogenesch et al. 2000). A number of PAS proteins expressed in the CNS are critical for basic homeostatic responses, including Clock, BMAL1 and Period, which all respond to light to establish circadian rhythms (Lowrey and Takahashi 2000), and NPAS2, which has been proposed to sense redox potential in CNS neurons (Reick, Garcia et al. 2001).

There is growing interest in the role of transcription factors in the central control of feeding behavior. To date, mutations in four mouse genes encoding known or putative transcription factors, *Tubby* (Boggon, Shan et al. 1999), *Nhlh2* (Good, Porter et al. 1997), *Sf1* (Majdic, Young et al. 2002) and *Sim1* (Michaud, Boucher et al. 2001), have been shown to cause obesity. The molecular mechanisms by which mutations in these genes results in obesity has not been elucidated, largely because their transcriptional targets relevant to feeding behavior have not been identified. However, it is well known that feeding or fasting alters the expression of many neuropeptides. The mechanism of this regulation is unknown and likely involves transcription factors acting downstream of signaling pathways. Transcription is especially attractive as a mechanism for the long-term regulation of food intake and energy expenditure as has been demonstrated for other complex behaviors such as addiction (Nestler 2001).

In this chapter, I report a role for mammalian *Sim1* in energy balance, and in particular, the homeostatic feeding response to dietary fat content. *Sim1* deficient mice were generated by homologous recombination, and in this chapter I will show that heterozygotes become obese, like SW116. Obesity was principally due to hyperphagia. When shifted from

standard rodent chow to a high fat diet, these mice showed a dramatic and sustained increase in their excess energy intake, resulting in earlier onset and more severe obesity and associated insulin resistance. The interaction between *Sim1* dosage and dietary fat provides a model for gene-environment interactions of paramount importance for developing strategies to combat the current obesity epidemic.

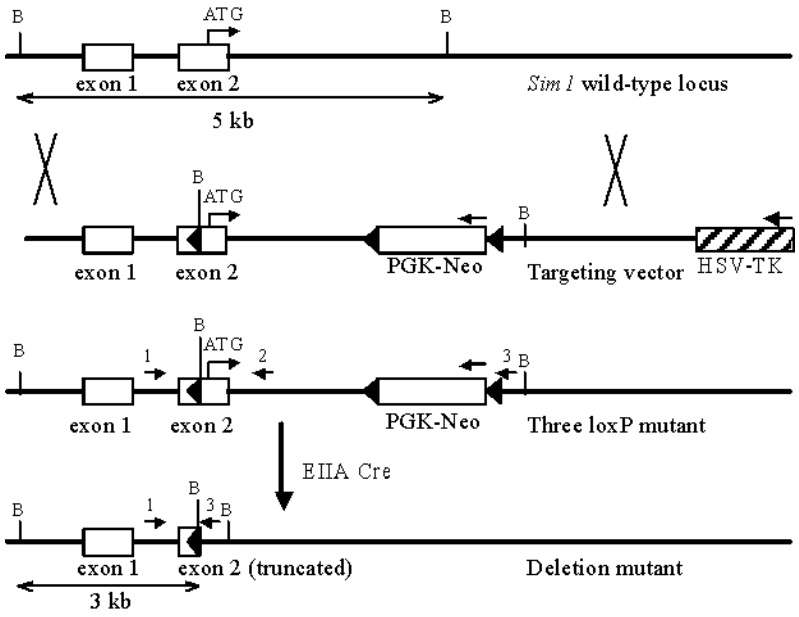
RESULTS

Targeted Inactivation of *Sim1*

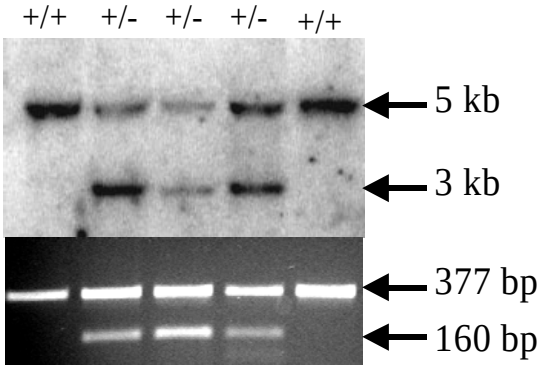
Mice carrying a null allele of *Sim1* were generated and provided by Ying-Hue Lee (Sinica Academy, Tiawan). The targeting strategy for generating *Sim1* heterozygous null mice (Fig. 20*a*) involved creating mice in which the second exon (encoding the translation intiation domain) was floxed by Lox P sites. Mice heterozygous for this allele were then crossed to transgenic mice with the Cre recombinase under control of the EIIa promoter which allows expression of Cre at the one cell embryo stage. This caused recombination and loss of the coding portion of the second exon of *Sim1* from this allele. This is a null allele as mice heterozygous for the deletion mutant allele display perinatal lethality as previously published (Michaud, Rosenquist et al. 1998).

Figure 20

(a)



(b)



(c)

Figure 20 *Sim1* heterozygous null mice

(a) Targeting strategy for generating *Sim1* heterozygous null mice (b) Correct targeting was determined by Southern blotting (upper panel) and PCR genotyping (lower panel). For both methods, the upper band represents the wild-type allele and the lower band is the null allele. (c) Six month old *Sim1* heterozygous null female (left) and wild-type littermate (right).

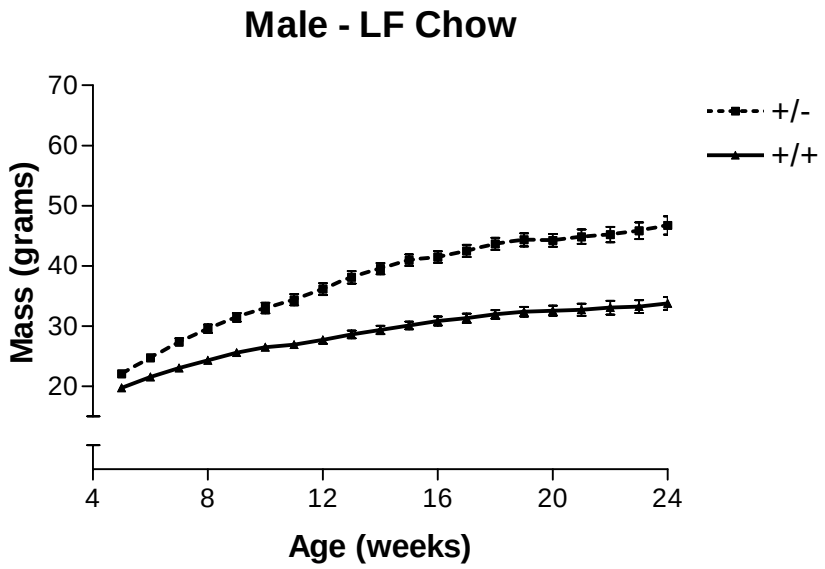
Increased body weight and length of *Sim1* +/- mice

Mice were weaned at three weeks of age onto either a chow containing 4% dietary fat (LF) or a chow containing 35% dietary fat (HF) and weighed weekly (Fig. 21 and 22). By eight weeks of age, heterozygous females were 13% heavier than controls (22.9±0.5 g vs. 20.2±0.4 g) on the LF diet (Fig. 22*a*) and 71% heavier than controls (38.1±1.7 g vs. 22.2±0.7 g) on the HF diet (Fig. 22*b*). Males were similarly affected: by eight weeks of age, heterozygotes were 22% heavier than controls (29.6±0.7 g vs. 24.3±0.4 g) on the LF diet (Fig. 21*a*) and 51% heavier than controls (46.9±1.5 g vs. 31.1±1.2 g) on the HF diet (Fig 21*b*). *Sim1* +/- mice of either sex continued to gain weight until at least six months of age on the LF diet (Fig. 21*a* and 22*a*) and four months of age on the HF diet (Fig. 21*b* and 22*b*).

Sim1 +/- mice were also longer than their wild-type littermates at six months of age (Fig. 23). Male heterozygotes were more than 0.5 cm longer than controls on either the LF or HF diet. Female heterozygotes were 0.5 cm or 0.4 cm longer than controls on the LF or HF diet, respectively.

Figure 21

(a)


Male - LF Chow
Mass (grams)
70
60
50
40
30
20
4
8
12
16
20
24
Age (weeks)
+/-
+/+

(b)

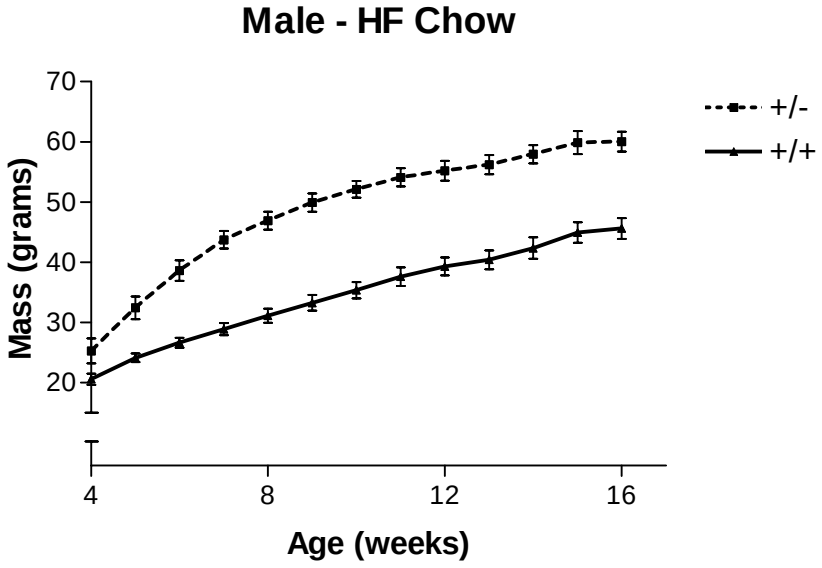
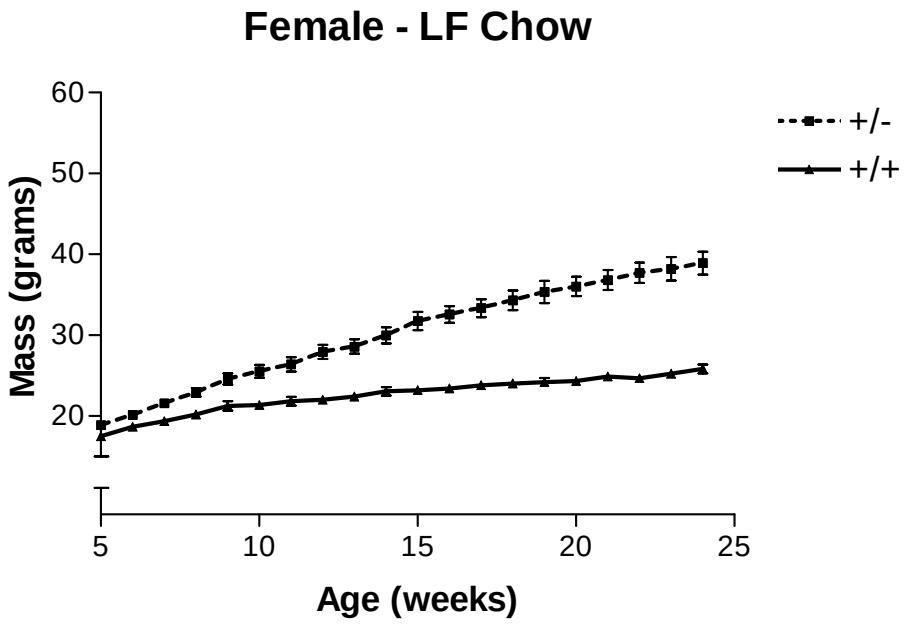

Male - HF Chow
Mass (grams)
70
60
50
40
30
20
4
8
12
16
Age (weeks)
+/-
+/+

Figure 21 Male *Sim1* deficient mice are obese.

Male *Sim1* deficient and wild-type mice were weaned at three weeks onto either a LF chow diet (a) or a HF diet (b). Weight was recorded weekly. (a) +/- n=15, +/+ n=15. (b) +/- n=9, +/+ n=11. Each point represents the mean +/- SEM.

Figure 22

(a)


Female - LF Chow
Mass (grams)
60
50
40
30
20
5
10
15
20
25
Age (weeks)
+/-
+/+

(b)

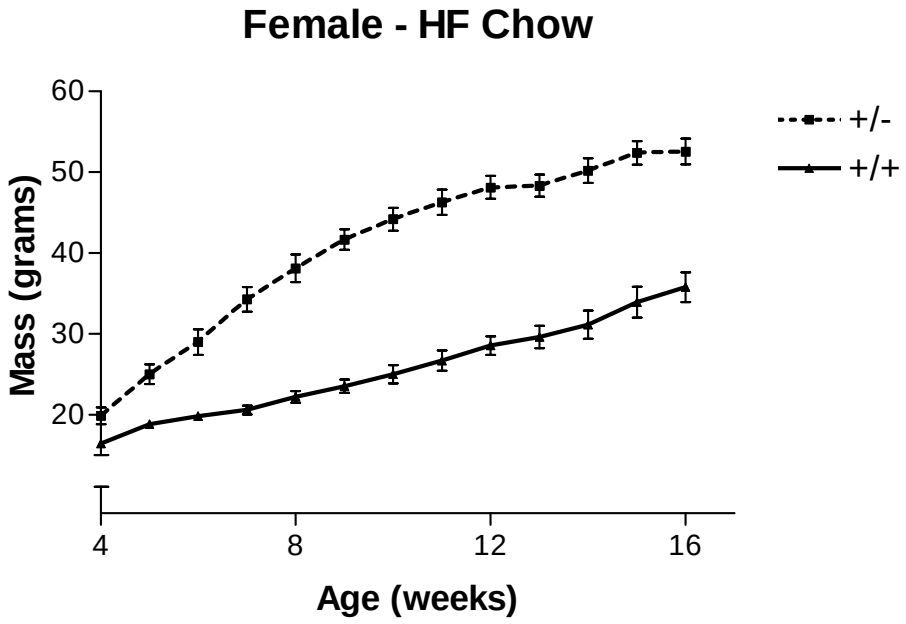
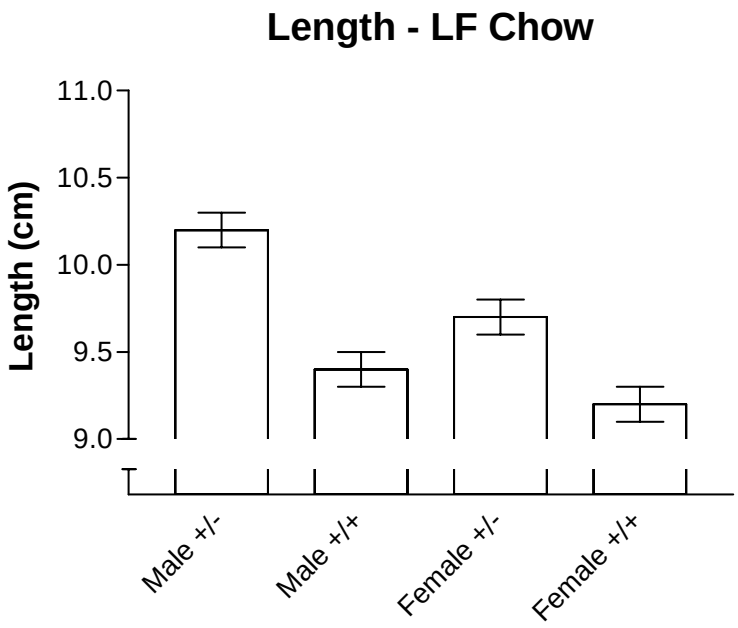

Female - HF Chow
Mass (grams)
60
50
40
30
20
4
8
12
16
Age (weeks)
+/-
+/+

Figure 22 Female *Sim1* deficient mice are obese.

Female *Sim1* deficient and wild-type mice were weaned at three weeks onto either a LF chow diet (a) or a HF diet (b). Weight was recorded weekly. (a) +/- n=14, +/+ n=14. (b) +/- n=8, +/+ n=11. Each point represents the mean +/- SEM.

Figure 23
(a)


Length - LF Chow
Length (cm)
11.0
10.5
10.0
9.5
9.0
Male +/-
Male +/+
Female +/-
Female +/+

(b)

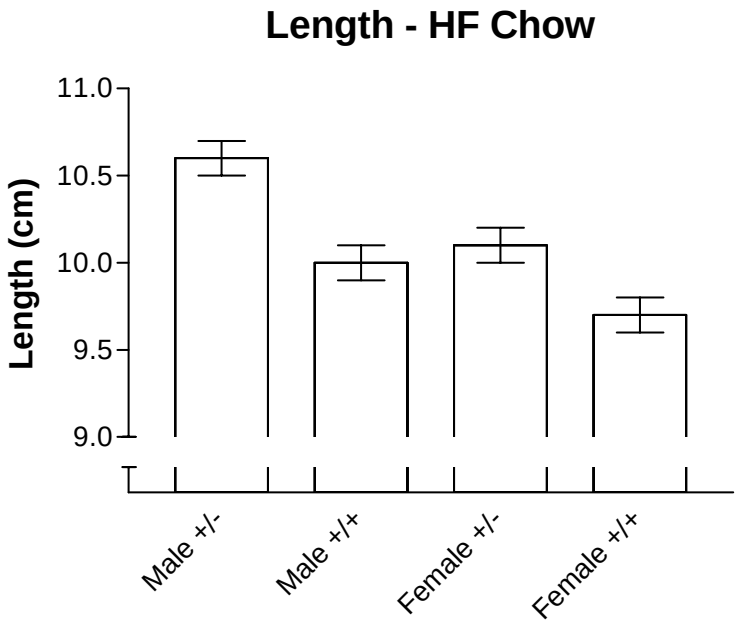

Length - HF Chow
Length (cm)
11.0
10.5
10.0
9.5
9.0
Male +/-
Male +/+
Female +/-
Female +/+

Figure 23 *Sim1* deficient mice are long.

Terminal length was measured in both male and female +/- and +/+ mice on the (a) LF diet (6 months old), Male +/- n=7, Male +/+ n=10, Female +/- n=9 and Female +/+ n=8 or (b) HF diet (4 months old), Male +/- n=9, Male +/+ n=11, Female +/- n=7 and Female +/+ n=11. All are $p<.01$ and each bar is the mean +/- SEM.

Increased fat and lean mass of *Sim1* +/- mice

Adiposity of six-month old *Sim1* +/- and +/+ littermates on the LF diet was measured by dual energy x-ray absorptiometry. *Sim1* +/- males had more than twice as much fat mass as +/+ littermates (13.2 ± 1.9 g vs. 5.1 ± 1.1 g), and +/- females had more than three times as much fat mass as +/+ littermates (14.8 ± 1.8 g vs. 3.9 ± 0.4 g) (Fig. 24).

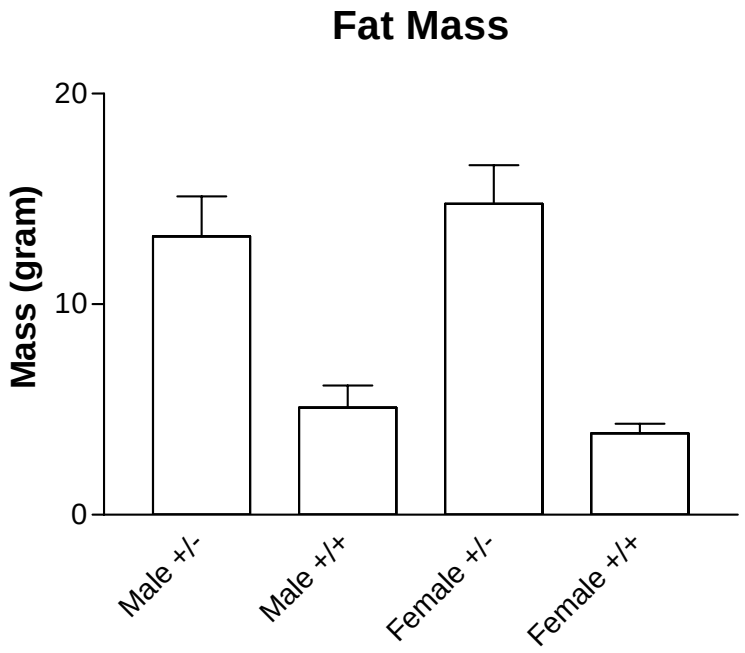


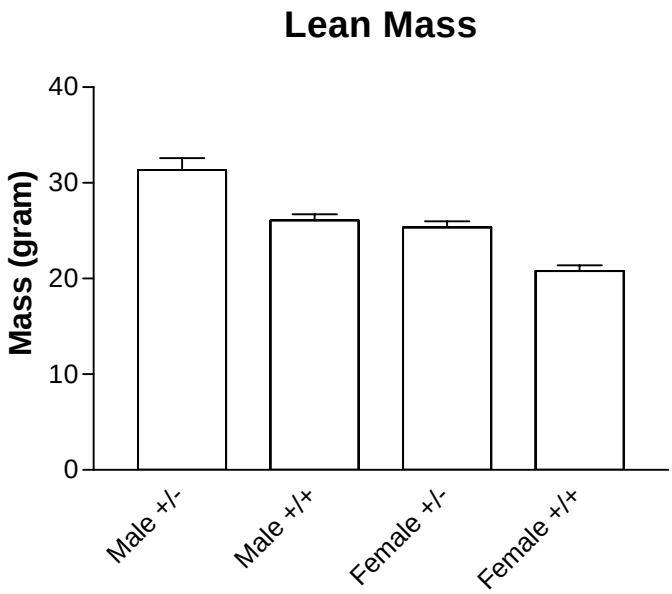
Figure 24 *Sim1* deficient mice have elevated fat mass

Dual Energy X-Ray Absorptiometry was used to measure lean mass and determine body fat percentage: male: +/- n=5, +/+ n=5, p<.01; female: +/- n=5, +/+ n=4, p<.01. Each bar is mean +/- SEM.

Lean mass of both male and female +/- mice was also elevated by more than 20% compared to +/+ littermates (Fig. 25*a*), consistent with their increased length. The percent body fat of *Sim1* +/- mice of either sex was greater than +/+ littermates, with females more severely affected (37.1% vs. 15.6%) than males (29.3% vs. 16%) (Fig. 25*b*). Thus the increased weight of *Sim1* +/- mice was primarily due to increased body fat.

Figure 25

(a)


Lean Mass
Mass (gram)
40
30
20
10
0
Male +/-
Male +/+
Female +/-
Female +/+

(b)

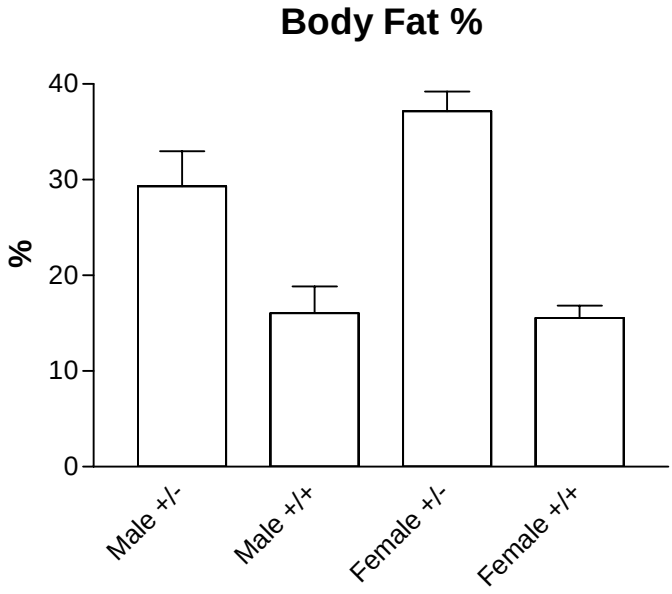

Body Fat %
%
40
30
20
10
0
Male +/-
Male +/+
Female +/-
Female +/+

Figure 25. *Sim1* deficient mice have greater lean mass and percentage of body fat.

Dual Energy X-Ray Absorptiometry was used to measure lean mass (a) and determine body fat percentage (b): male: +/- n=5, +/+ n=5, $p<.01$; female: +/- n=5, +/+ n=4, $p<.01$. Each bar is mean +/- SEM.

Mice with obesity due to leptin or leptin receptor deficiency have markedly increased bone mineral density (BMD) (Ducy, Amling et al. 2000). We therefore measured whole body BMD of the *Sim1* +/- mice but found no significant difference compared to +/+ littermates for either sex (Fig. 26).

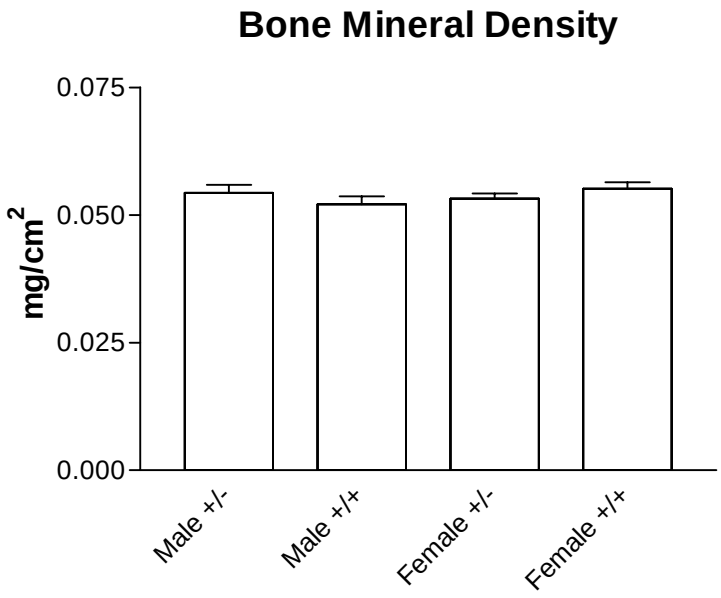


Figure 26 Bone Mineral Density of *Sim1* deficient mice is normal.

Dual Energy X-Ray Absorptiometry was used to measure bone mineral density (BMD): male: +/- n=5, +/+ n=5; female: +/- n=5, +/+ n=4 each bar is mean +/- SEM.

Altered feeding behavior of *Sim1* +/- mice

I measured daily food intake of mice on the LF chow diet over a five day period (Fig. 27 and 28, first data point). Both male and female *Sim1* +/- mice consumed 14% more food per day than +/+ littermates (male: 4.2 ± 0.1 g vs. 3.7 ± 0.1 g, female: 4.0 ± 0.1 g vs. 3.5 ± 0.1 g).

I then challenged these same mice with the HF diet for a seven day period. On the first day, there was an obvious difference between +/- and +/+ littermates. Male +/- mice had an insignificant increase in the mass of food consumed (5 ± 5%) while male +/+ littermates decreased their food intake (-16 ± 5%). Females had a similar response to elevated dietary fat: +/- mice had an insignificant increase in the mass of food consumed (4 ± 5%) compared with a decrease in food consumption by +/+ littermates (-18 ± 5%).

Food consumption was summed over the final five days of the HF diet, after daily food consumption stabilized (Fig 27 and 28, 1st shaded area). In contrast to the small increase in the mass of food consumed on the LF diet, female +/- mice consumed 68% more food than the female +/+ littermates (16.9 ± 0.6 g vs. 10.0 ± 0.5 g). Males had a similar response to the HF diet: +/- mice consumed 46% more food than +/+ littermates (18.3 ± 0.5 g vs. 12.5 ± 0.5 g). Thus on a per-gram basis there was a 3 to 4-fold increase on the HF versus the LF diet in the percent difference in food intake by +/- versus +/+ animals. Taking into account the higher caloric content of the HF diet, male +/- mice consumed +57% and females +49% more energy on the HF diet than on the LF diet. By contrast, +/+ mice consumed only +20% (males) or +21% (females) more energy on the HF diet than on the LF diet.

The mice were then switched back to the LF diet. Food intake decreased substantially, with no significant difference between +/- and +/+ mice. This drop in food consumption was presumably due to decreased palatability. Food consumption gradually returned to normal over the next week. When the mice were rechallenged with the HF diet for one week, the same abnormal feeding response was observed in +/- mice of either sex.

Figure 27

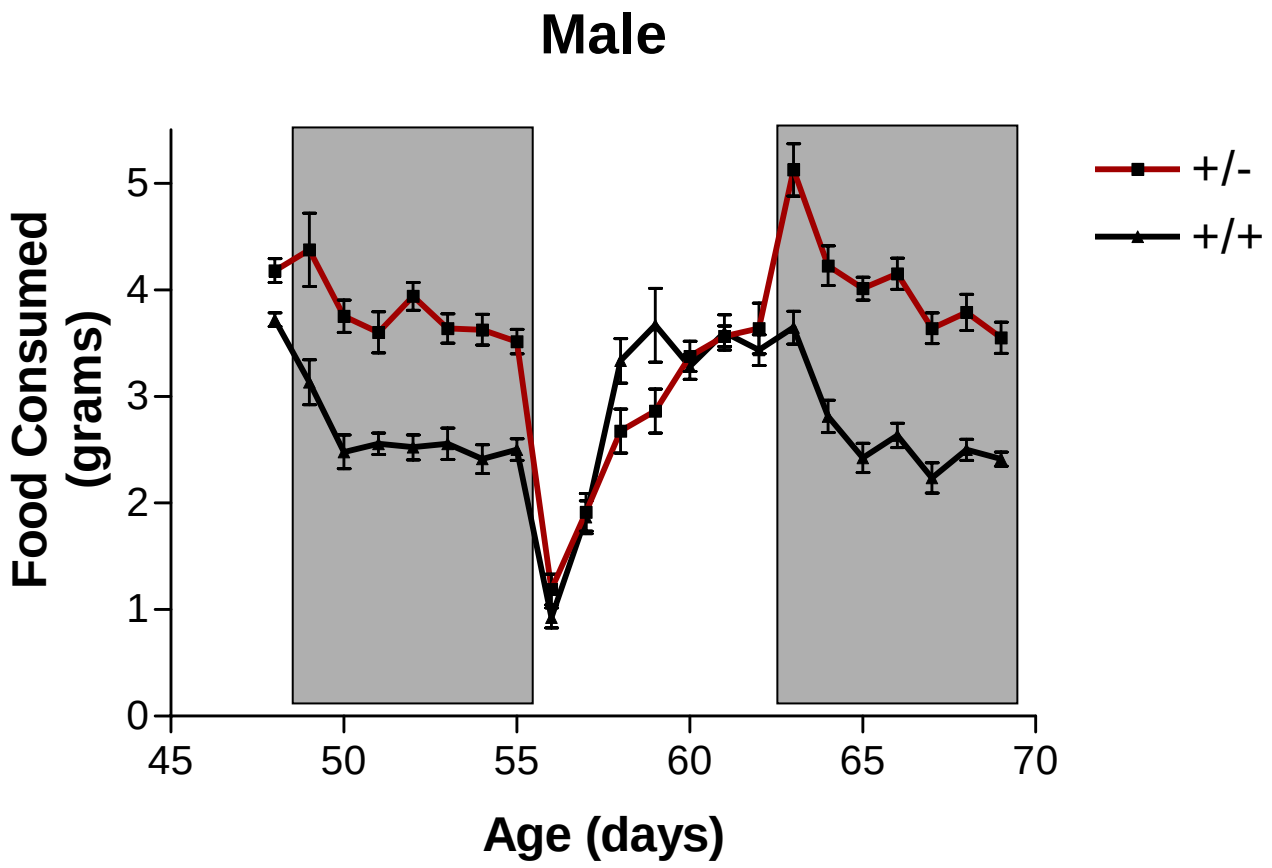


Figure 27 Male *Sim1* deficient mice are hyperphagic.

Food intake of *Sim1* +/- and +/+ mice was measure on a daily basis. The first data point represents the daily average of five days on the LF diet. The shaded regions represent food intake on the HF diet. +/- n=8, +/+ n=9. All points are mean +/- SEM.

Figure 28

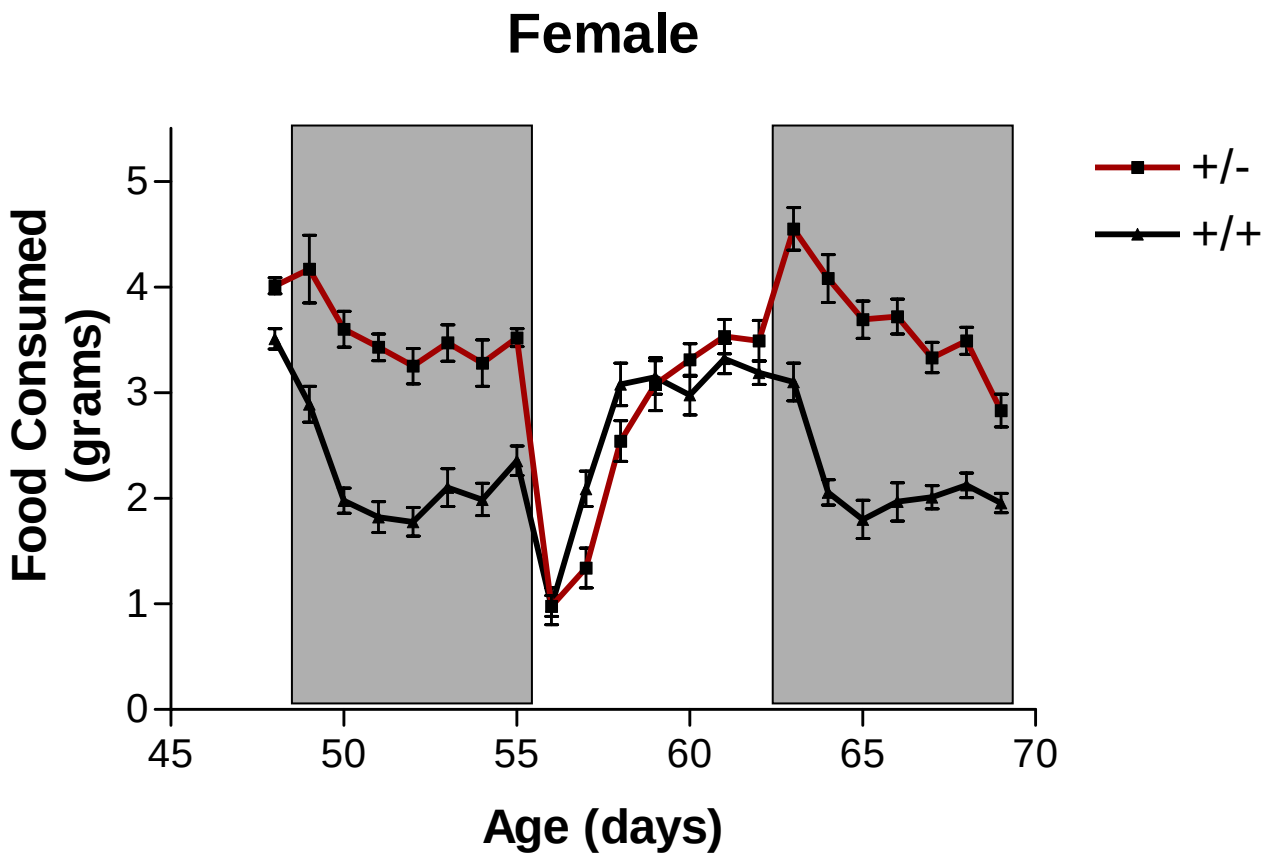


Figure 28 Female *Sim1* deficient mice are hyperphagic.

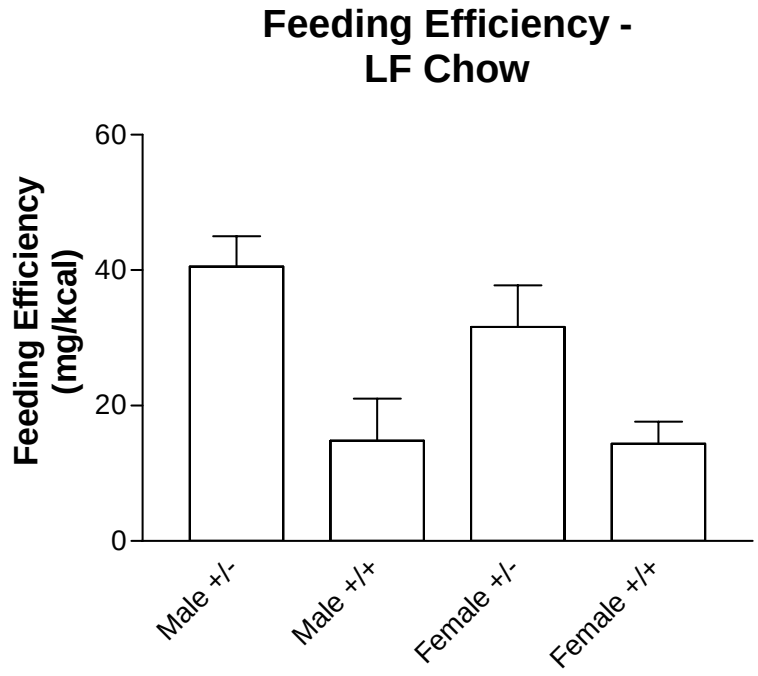
Food intake of *Sim1* +/- and +/+ mice was measure on a daily basis. The first data point represents the daily average of five days on the LF diet. The shaded regions represent food intake on the HF diet. +/- n=10, +/+ n=9. All points are mean +/- SEM.

Increased feeding efficiency and normal activity of *Sim1* +/- mice

Feeding efficiency was increased in mice fed either the LF or HF diet (Fig. 29). Male +/- mice had 2.5-fold or 1.9-fold greater feeding efficiency than male +/+ littermates on the LF or HF diet, respectively. Female +/- mice had twofold or threefold greater feeding efficiency than female +/+ littermates on the LF or HF diet, respectively. *Sim1* +/- mice of either sex showed modestly decreased wheel-running activity on both the LF and HF diets (Fig. 30); the differences were not statistically significant.

Figure 29

(a)


Feeding Efficiency -
LF Chow
Feeding Efficiency
(mg/kcal)
60
40
20
0
Male +/-
Male +/+
Female +/-
Female +/+

(b)

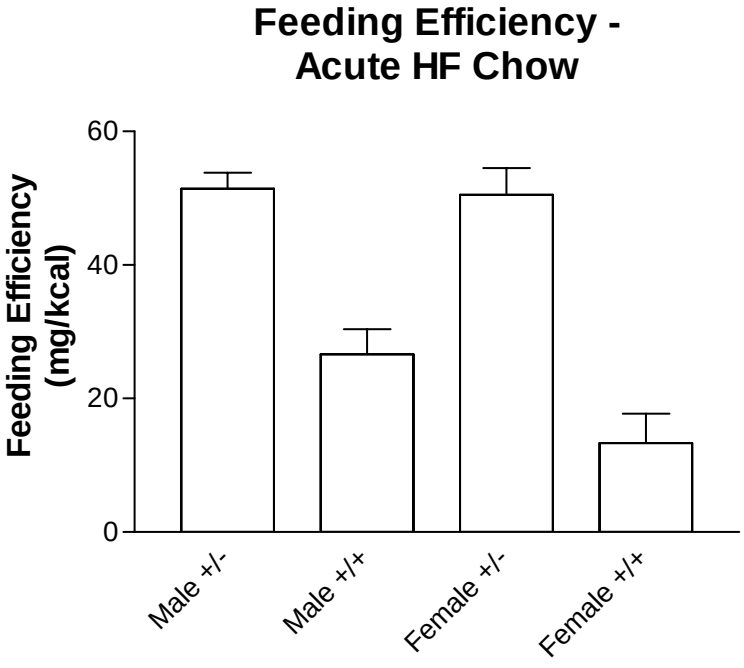
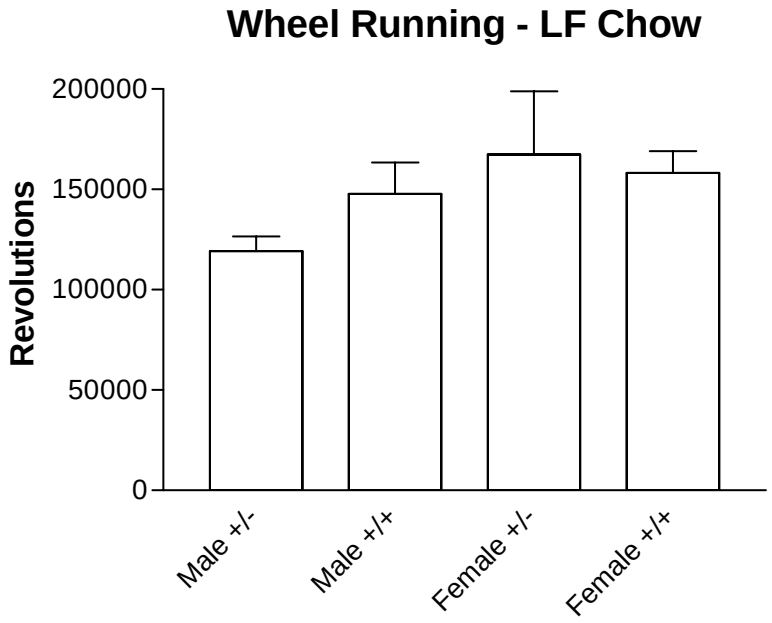

Feeding Efficiency -
Acute HF Chow
Feeding Efficiency
(mg/kcal)
60
40
20
0
Male +/-
Male +/+
Female +/-
Female +/+

Figure 29 *Sim1* deficient mice have altered feeding efficiency.

(a) Male +/-, n=9; male +/+, n=6, p=.01; female +/-, n=7; female +/+, n=10, p=.02 (b) Male +/-, n=8; male +/+, n=9, p<.001; female +/-, n=10; female +/+, n=9, p<.001. Bars are mean +/- SEM.

Figure 30

(a)


Wheel Running - LF Chow
Revolutions
200000
150000
100000
50000
0
Male +/-
Male +/+
Female +/-
Female +/+

(b)

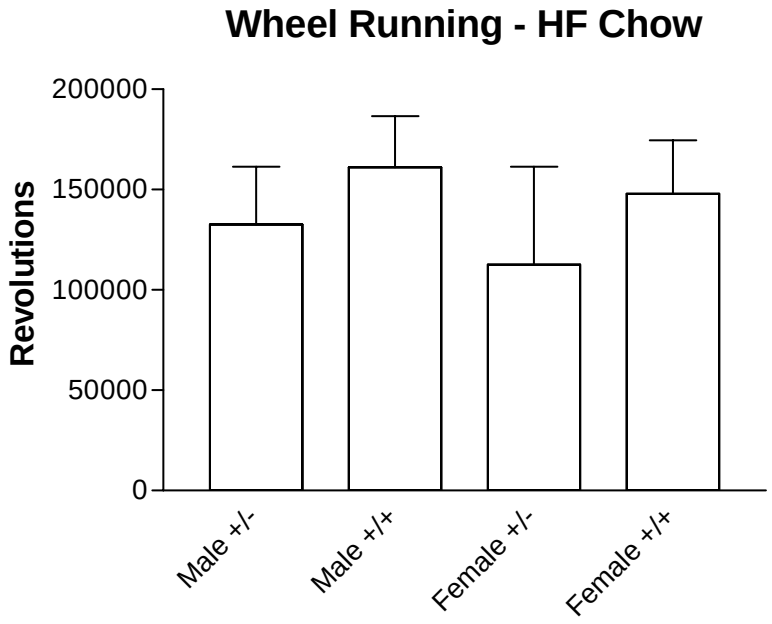

Wheel Running - HF Chow
Revolutions
200000
150000
100000
50000
0
Male +/-
Male +/+
Female +/-
Female +/+

Figure 30 Activity of *Sim1* deficient mice.

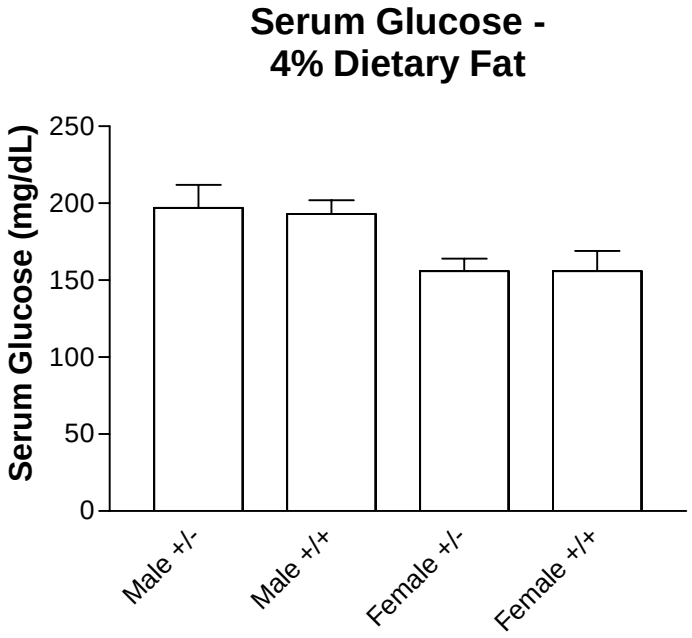
Activity of *Sim1* deficient and wild-type littermates was determined by voluntary wheel running. Data was collected over a five day period on both the LF(a) and HF(b) diets. Male +/- n=4, male +/+ n=4, female +/- n=5 and female +/+ n=5.

Elevated serum insulin and leptin levels in *Sim1* +/- mice

Serum glucose levels of six month old *Sim1* +/- or +/+ mice on the LF diet were not significantly different (Fig. 31). Female but not male +/- mice weaned onto the HF diet had slightly, yet significantly, elevated serum glucose levels compared with +/+ littermates (185 ± 10 mg/dL vs. 162 ± 7 mg/dL; Fig 31*b*). *Sim1* +/- mice had elevated serum insulin levels on the LF diet compared with +/+ littermates (Fig 32*a*). Weaning onto the HF chow diet increased the serum insulin levels of both +/- and +/+ mice; they did not differ significantly (Fig. 32*b*). Serum leptin levels were elevated in *Sim1* +/- mice on the LF diet compared to +/+ littermates (Fig. 33*a*). On the HF diet, there was no difference between *Sim1* +/- males and +/+ males (Fig. 33*b*). There was a tendency of elevated serum Leptin for the female +/- mice versus *Sim1* +/+.

Figure 31

(a)


Serum Glucose -
4% Dietary Fat
Serum Glucose (mg/dL)
250
200
150
100
50
0
Male +/-
Male +/+
Female +/-
Female +/+

(b)

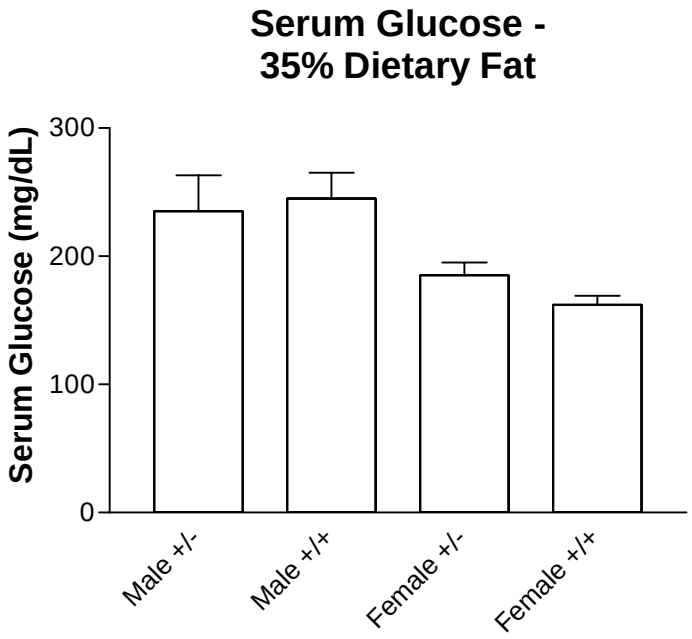
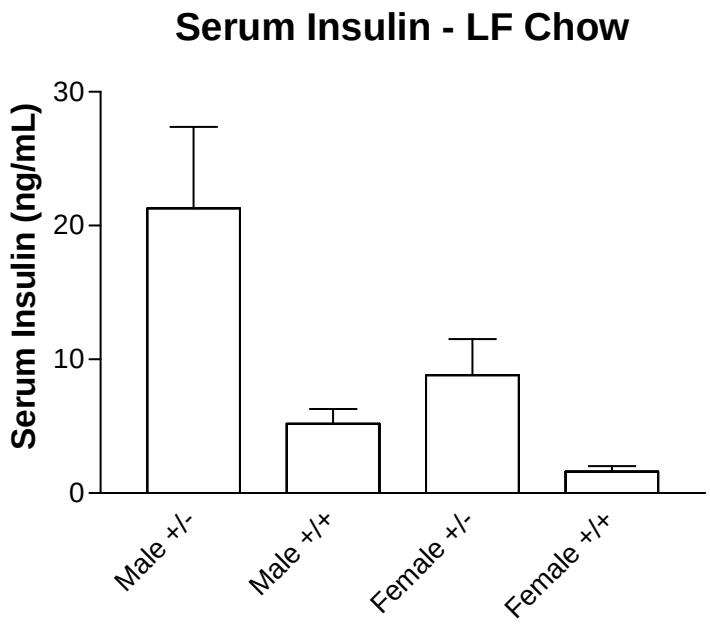

Serum Glucose -
35% Dietary Fat
Serum Glucose (mg/dL)
300
200
100
0
Male +/-
Male +/+
Female +/-
Female +/+

Figure 31. Serum glucose in *Sim1* deficient mice.

Serum glucose was determined in male and female mice fed either (a) LF chow (six months old), male +/- n=8, male +/+ n=12, female +/- n=8, female +/+ n=10 or (b) HF chow (four months old) male +/- n=8, male +/+ n=10, female +/- n=8, female +/+ n=10, $p<.05$. Bars are the mean +/- SEM.

Figure 32

(a)


Serum Insulin - LF Chow
Serum Insulin (ng/mL)
30
20
10
0
Male +/-
Male +/+
Female +/-
Female +/+

(b)

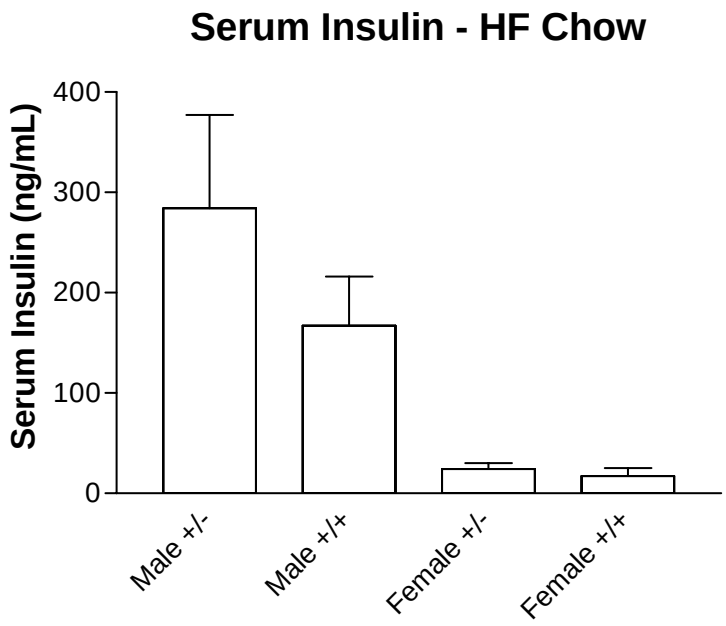
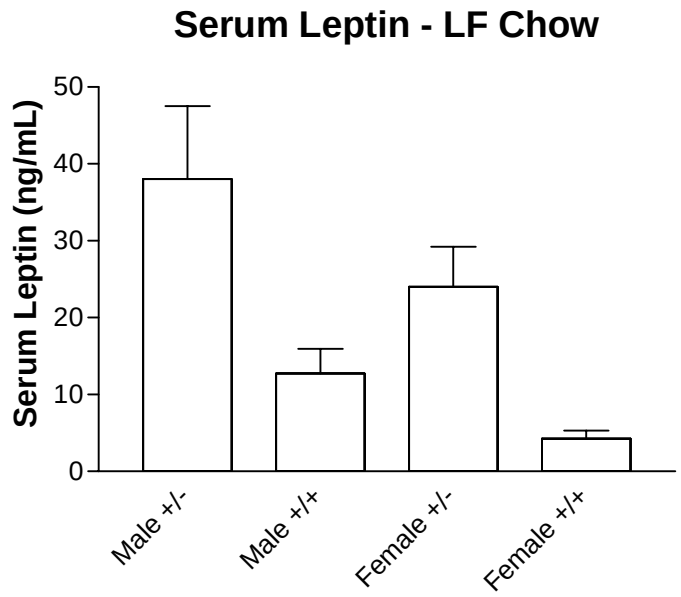

Serum Insulin - HF Chow
Serum Insulin (ng/mL)
400
300
200
100
0
Male +/-
Male +/+
Female +/-
Female +/+

Figure 32. Serum insulin is elevated in *Sim1* deficient mice.

Serum insulin was determined in male and female mice fed either (a) LF chow (six months old), male +/- n=10, male +/+ n=10, p<.01, female +/- n=8, female +/+ n=10, p<.01 or (b) HF chow (four months old) male +/- n=9, male +/+ n=10, female +/- n=8, female +/+ n=10. Bars are the mean +/- SEM.

Figure 33

(a)


Serum Leptin - LF Chow
Serum Leptin (ng/mL)
50
40
30
20
10
0
Male +/-
Male +/+
Female +/-
Female +/+

(b)

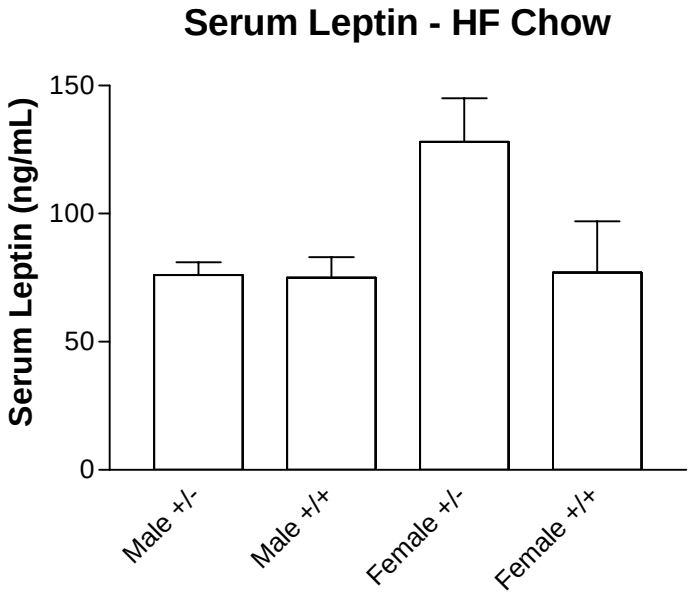

Serum Leptin - HF Chow
Serum Leptin (ng/mL)
150
100
50
0
Male +/-
Male +/+
Female +/-
Female +/+

Figure 33. Serum leptin is ELEVATED in *Sim1* deficient mice.

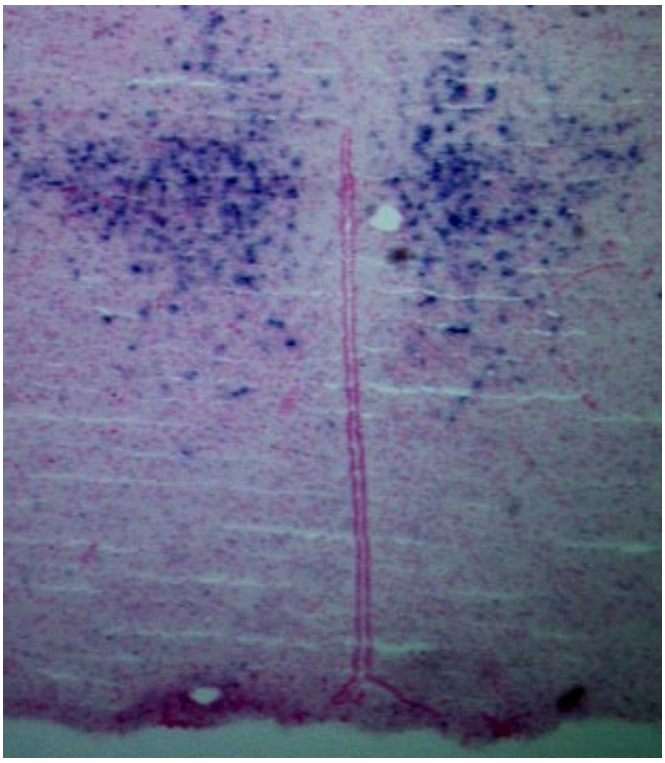
Serum leptin was determined in male and female mice fed either (a) LF chow (six months old), male +/- n=8, male +/+ n=12, $p<.01$, female +/- n=6, female +/+ n=8, $p<.001$ or (b) HF chow (four months old) male +/- n=9, male +/+ n=9, female +/- n=8, female +/+ n=10. Bars are the mean +/- SEM.

Expression of *Sim1* in the hypothalamus

Michaud and colleagues reported that in newborn mice, *Sim1* expression in the hypothalami of mice is limited to the PVH and SON (Michaud, Rosenquist et al. 1998). Subsequent reports suggested that *Sim1* was also expressed in other regions of the hypothalamus in adult mice . In collaboration with Ralph DiLeone (Psychiatry, UT Southwestern), the expression pattern of *Sim1* in the mouse hypothalamus was investigated by RNA *in situ* hybridization (Fig. 34). In addition to the PVH and SON, *Sim1* expression was identified in the hypothalamus within the lateral hypothalamic area (LHA) as well as the dorsal medial hypothalamus (DMH). As discussed in Chapter 1, both of these nuclei have been implicated in regulation of proper weight regulation and feeding behavior.

Figure 34

(a)



(b)

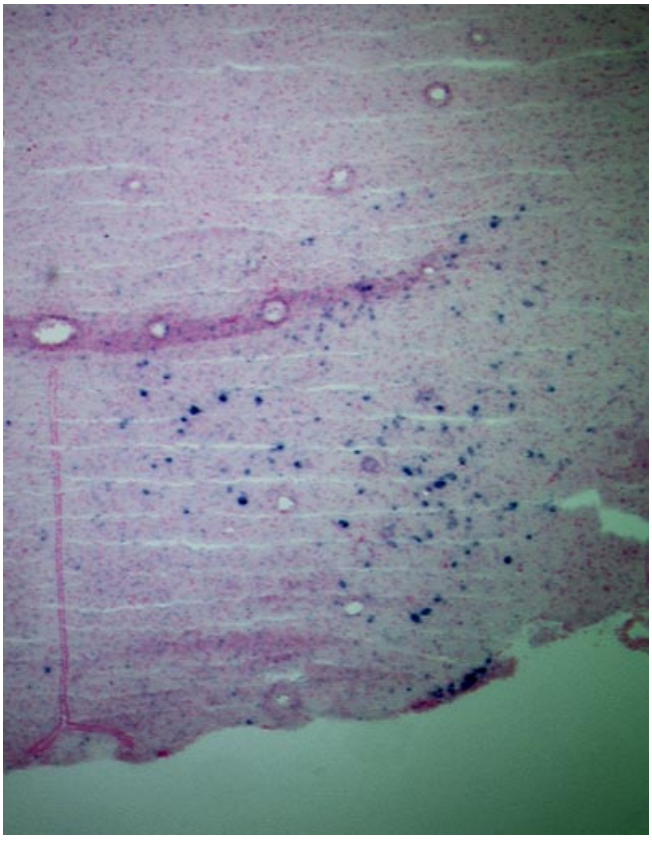


Figure 34. *Sim1* expression in the adult mouse DMH and LHA

Sim1 expression by RNA *in situ* hybridization in the (a) DMH and (b) LHA. Cells stained blue express *Sim1*.

Discussion

Sim1 haploinsufficiency causes obesity in mice. The phenotype initially appeared to be milder than that we observed in SW116, whose growth curve was comparable to those of humans with other monogenic forms of obesity. For example, the girl became obese during infancy, whereas obesity was not evident in *Sim1* +/- mice fed a standard rodent chow diet until around the time of sexual maturity (~5-6 weeks of age). However, weaning the mutant mice on a high-fat diet significantly enhanced the obesity phenotype. I surmise that high dietary fat content also played a role in our patient's obesity, although her food intake could not be accurately measured. In addition to the diet effect, there was also a gender difference in the hyperphagia of *Sim1*-deficient mice, with females more severely affected than males. The gender of our patient with a heterozygous *SIM1* mutation may have been a factor in the severity of her phenotype, although this cannot be examined unless other humans with *SIM1* mutations are identified.

Although mutations in many genes have been shown to cause obesity in mice, interactions of these genes with the environment have only recently been intensively studied. *Mc4r*-deficient mice are also exquisitely sensitive to an acute increase in dietary fat (Butler, Marks et al. 2001). Homozygotes significantly increase their caloric intake when acutely provided a diet with moderately elevated fat, while wild-type controls remain isocaloric. By contrast, leptin deficient mice respond normally to an acute increase in dietary fat (Butler, Marks et al. 2001). *Sim1* +/- mice similarly have a dramatic rise in caloric consumption when switched from the LF to HF chow diet compared with wild-type littermates. When examining the mass of food consumed, wild-type mice decreased the mass of food they consumed

substantially when switched from the LF chow to the HF chow. *Sim1* +/- mice decreased the mass of food they consumed only slightly compared with wild-type littermates; therefore, *Sim1* deficient mice appear to be somewhat resistant to the normal homeostatic response to elevated dietary fat.

Sim1 and *Mc4r* deficient mice are the only mice reported with obesity, increased linear growth and altered response to elevated dietary fat (Table III). By contrast, leptin-deficient mice show stunted linear growth and a normal response to dietary fat. Leptin deficient mice also show increased bone mineral density (BMD). Our patient with a heterozygous *SIM1* mutation had an elevated bone mineral density at age 67 months (t-score +2.3 S.D., age matched), while the *Sim1* +/- mice had normal BMD. Interestingly, a similar species difference may be true for MC4R signaling defects: patients with *MC4R* mutations generally have elevated BMD (Farooqi, Yeo et al. 2000), while BMD is normal in A^y mice (Ducy, Amling et al. 2000), which are deficient in central melanocortin signaling.

There are some differences between the mouse *Sim1* and *Mc4r* mutant phenotypes. Metabolic rate was significantly lower in *Mc4r* deficient mice compared to controls, as reflected by their markedly increased feeding efficiency (Butler, Marks et al. 2001). By contrast, feeding efficiency of *Sim1* +/- mice was only modestly increased. Furthermore, the metabolic abnormality of *Mc4r*-deficient mice but not *Sim1*-deficient mice was exacerbated by an acute increase in dietary fat (Butler, Marks et al. 2001). *Mc4r* -/- mice on a moderate fat diet showed decreased wheel running activity versus controls (Butler, Marks et al. 2001), whereas *Sim1* +/- mice on either a HF or LF diet did not show significant decreases in activity although there was a trend for decreased activity.

Table IV. Comparison of *Sim1* deficient mice with *Mc4r* and leptin deficient mice

	Sim1	*Mc4r*	leptin
Obesity	Moderate	Moderate	Severe
Linear growth	Long	Long	Short
Feeding	Hyperphagic	Hyperphagic	Hyperphagic
Energy expenditure	??????	Hypometabolic	Hypometabolic
Insulin	Elevated	Elevated	Elevated
Glucose	Normal	Elevated	Elevated
Acute high dietary fat food intake	Abnormal	Abnormal	Normal
Bone Mineral Content	Normal	Normal	Elevated

The similar growth and feeding behavior phenotypes of *Sim1* and *Mc4r* mutant mice strengthens the notion that these two genes may act within the same hypothalamic pathway(s) (Holder, Butte et al. 2000). Whether there is a molecular interaction between the two genes is not known. *Mc4r* mRNA levels in the hypothalami of *Sim1* +/- mice appear to be normal (unpublished data), suggesting that *Mc4r* is not a direct *Sim1* transcriptional target. In P1 mice, *Sim1* is expressed in the paraventricular (PVH) and supraoptic nuclei (SON) of the hypothalamus. The PVH has been implicated in regulation of feeding by lesioning studies. Interestingly, rats with hyperphagic obesity due to electrolytic PVH lesions showed the same diet and gender effects as *Sim1* +/- mice (Kirchgessner and Sclafani 1988). However, Sim1 may act outside the PVH to influence feeding behavior. We detected *Sim1* expression in adult mice in the PVH as well as the lateral (LHA) and dorsomedial hypothalamus (DMH) and the amygdala, brain regions that are also implicated in feeding behavior. The PVH, SON, DMH

and LHA all express *Mc4r* (Mountjoy, Mortrud et al. 1994). Whether *Sim1* and *Mc4r* are coexpressed in neurons is unknown and warrants further study.

The phenotype of an independent mouse *Sim1* null mutation has been previously reported (Michaud, Boucher et al. 2001). Heterozygotes developed hyperphagic obesity, with normal activity and energy expenditure. The effects of dietary changes on the phenotype were not examined. Homozygotes died shortly after birth (Michaud, Rosenquist et al. 1998). They lacked all PVH neurons, as judged by the absence of CRH, TRH, oxytocin, vasopressin, or somatostatin immunostaining. Heterozygotes were reported to have a 24% decrease in overall PVH cellularity compared to controls, with no specific neuronal subtype affected; the +/- phenotype was attributed to this PVH hypocellularity.

However, there are reasons to doubt that diminution in PVH cells is the sole cause of obesity in these mice. First, even complete bilateral PVH lesions in weanling female rats did not result in hyperphagia or increased linear growth, suggesting that the newborn hypothalamus has functional plasticity and can compensate for congenital lesions with regard to energy balance (Bernardis 1984). Second, mutations in necdin, a Prader-Willi syndrome candidate gene, reduced the number of neurons of the PVH without causing hyperphagia or obesity (Muscatelli, Abrous et al. 2000). Third, as noted previously, we detected *Sim1* expression in regions of the brain outside the PVH that also influence feeding behavior. Selective inactivation of *Sim1* using a cre-loxP system (Tsien, Chen et al. 1996) might tease apart its function in different hypothalamic and brain regions. Regardless of the precise mechanism of obesity in *Sim1* +/- mice, these animals provide an excellent model for human

hyperphagic obesity and gene x environment interactions that influence the propensity toward obesity in response to a high fat diet.

Chapter 6

Conclusions and Future Directions

SIM1 is a novel obesity gene

The combined evidence from the human subject with a mutation in *SIM1* and the mice engineered to be deficient for *Sim1* strongly indicates that *SIM1* is a novel obesity gene. Both the human and mouse phenotypes suggest that the obesity is primarily due to hyperphagia and not metabolic deficiency. This obesity is due to a dominant haploinsufficieny in contrast to most monogenic obesities which are due to recessive loss of function mutations such as leptin and the leptin receptor. In fact, only mutations in *MC4R* and *SIM1* are known dominant causes of obesity. This suggests that changes in dosages in these genes, at least a fifty percent decrease, can result in dramatic changes in weight, such as the early-onset obesity observed in humans with *SIM1* or *MC4R* mutations. Common polymorphisms either in the coding region of these genes that cause subtle differences in molecular activity or in the promoters that subtly change expression levels could be responsible, in part, for elevated susceptibility to common obesity. It would be of interest to screen a large population of individuals with common obesity for association with a *SIM1* polymorphism.

In addition to obesity, *Sim1* deficient mice are significantly longer than wild-type mice which parallels the elevated linear growth of SW116. The mechanism of this increase in linear growth is not clear. Not all mice with early-onset obesity are longer than wild-type mice. Leptin deficient mice are significantly shorter while *Mc4r* deficient mice are longer

than wild-type littermates. A^y mice are also significantly longer than wild-type mice, and it has been reported that there is a transient increase in IGF-1 levels in these mice at four weeks of age (Wolff, Kodell et al. 1999). It has been proposed that this transient elevation causes the increased length of A^y mice. IGF-1 expression is regulated by the pulsatile release of growth hormone from the pituitary, and IGF-1 is released primarily by the liver. Growth hormone release is regulated by growth hormone releasing hormone from the hypothalamus. How deficiency of MC4R causes changes in this pathway and whether similar changes occur in *Sim1* deficient mice is unknown.

Sim1 and Diet Induced Obesity

The recent rise in the prevalence of obesity in the United States and other developed countries has been due in large part to change in amount and composition of food consumed. Caloric-dense food rich in triglycerides have become a much larger proportion of the diet of most in the United States possibly due to easy access of fast foods. Because of this, mouse models of diet-induced obesity are potentially particularly useful in examining the physiology and genetics of hyperphagia and obesity.

One mouse model of diet-induced obesity is the C57bl/6J inbred strain (Lin, Thomas et al. 2000). These mice display a significant rise in mass and adiposity when chronically place on a high fat diet. The obesity is due in large part to hyperphagia as displayed by elevated caloric intake. Recently, the molecular consequences of an acute shift to a high fat diet were examined in C57bl/6J mice (Ziotopoulou, Mantzoros et al. 2000). When mice of this strain were shifted to a high fat diet for two days, no significant change in body weight

was observed. However, careful examination of the expression of transcripts encoding both orexigenic and anorexigenic peptides revealed changes that are an apparent attempt to remain isocaloric. *Npy* expression decreased to 30% of normal and *Agrp* levels fell to 50% of that of mice chronically fed a low fat diet. Both Npy and Agrp are orexigenic neuropeptides that are largely coexpressed in the same neurons of the arcuate nucleus. The mechanism of this transcriptional control in response to an acute elevation in dietary fat is unknown.

It would be interesting to examine the expression of *Npy* and *Agrp* in *Sim1* deficient mice acutely fed a high fat diet. As demonstrated in chapter 5, *Sim1* deficient mice fail to adequately suppress the mass of food they consume when acutely switched to the high fat diet. Whether this is because of a lack of normal suppression of *Npy* or *Agrp* expression is not known and deserves further investigation. The resistance of *Sim1* deficient mice could also be due to inability of these mice to properly respond to changes in Npy or Agrp concentrations. This seems a more likely explanation as both *Npy* and *Agrp* are expressed in the arcuate nucleus were *Sim1* is not expressed. Receptors for both Npy (Npy1r and Npy5r) and Agrp (Mc4r) are expressed in the PVH where *Sim1* is highly expressed. Either changes of expression of these receptors or downstream signaling components could possibly explain the resistance of *Sim1* deficient mice to elevated dietary fat. Indeed as discussed earlier, *Mc4r* deficient mice have a similar syndrome of resistance to elevated dietary fat.

It is not clear what the signal from the periphery to the hypothalumus is that causes the changes in neuropeptide expression mentioned above. It has been demonstrated that leptin levels are slightly elevated two days after an acute shift to a high fat diet (Ziotopoulou, Mantzoros et al. 2000). Elevated leptin levels would be consistent with a decrease in

expression of *Npy* and *Agrp* as intracerbroventricular injection of leptin causes reduced expression of *Npy* and *Agrp* (Korner, Savontaus et al. 2001). However, *ob/ob* mice deficient in leptin respond normally to elevated dietary fat (Butler, Marks et al. 2001). This suggests that leptin is not necessary for the normal acute homeostatic response to dietary fat.

Future Directions

One important question not answered by this work is the mechanism by which *SIM1* deficiency results in hyperphagia and obesity. No transcriptional targets of SIM1 have been identified. Chapter 4 outlines one approach for identifying genes whose transcription are regulated by SIM1. Other approaches might also address this problem.

Tissue from *Sim1* deficient mice could be used in cDNA microarray or subtractive hybridization experiments. Dissected hypothalami from *Sim1* heterozygous deficient mice and wild-type littermates might be used as a source of RNA for these expression profiling experiments. Heterozygous null mice would be preferred over homozygous null mice due to the incorrect development of the paraventricular hypothalamus of null mice. However, direct targets of Sim1 might only have a fifty percent reduction of expression within the neurons which coexpress the target gene and Sim1. Even in the context of dissected hypothalami, neurons might express the target gene and not Sim1. This would cause significant background expression which would make interpreting microarray or subtractive hybridization experiments difficult. Another option is to examine expression in kidney from *Sim1* null mice versus wild-type littermates. Although *Sim1* null mice die within twenty-four hours of birth, there is not an obvious defect in kidney structure in newborn mice. This is in

contrast to the brain of *Sim1* null mice which lack the neurons of the PVH and SON. The kidney is also a simpler structure than the brain with fewer unique cell types potentially simplifying problems with background expression. Although the kidney is an attractive option as a source of tissue for expression profiling, Sim1 target genes in the kidney could potentially be different than target genes in the hypothalamus. Nevertheless, cDNA microarray or subtractive hybridization experiments could complement cell culture experiments for identifying Sim1 transcriptional targets.

Another option for searching for target genes would be to create mice that over-express *Sim1* in the correct tissue specific pattern as endogenous expression. This might best be done using BAC transgenesis as no promoter has been characterized with precisely the same expression pattern as *Sim1*. A BAC transgene with sufficient sequence 5' to the first exon of *Sim1* and 3' to the untranslated region might express the transgene in the correct nuclei of the hypothalamus (Heintz 2001). Use of BAC transgenes has previously been demonstrated to be useful for precise overexpression in mice (Yang, Wynder et al. 1999). With integration of multiple copies of the transgene, *Sim1* expression might be greatly elevated and more dramatically change the expression of target genes in the hypothalamus compared with wild-type littermates. It would also be of interest to study the physiology of mice which overexpress *Sim1* with respect to whole body mass, feeding behavior and linear growth which are all significantly altered in *Sim1* deficient mice. Over-expression of *Sim1* could result in a converse phenotype to the deficient mice including decreased mass and length compared with wild-type littermates and depressed food consumption.

The biochemistry of Sim1 has not been studied in detail and could yield insight into its function in feeding behavior and weight regulation. Many PAS proteins have ligands which are critical to their function. For mammalian PAS proteins, this is best exemplified by the Aryl hydrocarbon receptor (Ahr). The Ahr binds cyclic hydrocarbons in the liver such as the toxin dioxin to activate the transcription of detoxification enzymes (Gu, Hogenesch et al. 2000). Within the CNS, NPAS2 has been shown to bind lactate to regulate its transcriptional activity (Rutter, Reick et al. 2001). Whether Sim1 binds a ligand to regulate its transcriptional activity is not known. Although it behaves as a transcriptional activator in cell culture without a specific activator added, the ligand could be a metabolic factor, such as lactate for NPAS2, which is present in the cell culture media or manufactured within the cell in sufficient quantities to allow Sim1 to act as a transcriptional activator in this assay.

Mammalian PAS proteins have been shown to be regulated by phosphorylation to control its activity. The PER2 (Period) protein is phosphorylated by casein kinase I ε and humans with mutations in a CKIε phosphorylation site of PER2 have a sleep disturbance known as Famalial Advanced Sleep Phase Syndrome (Toh, Jones et al. 2001). These individuals have an advanced circadian rhythm such that they tend to begin sleeping earlier than unaffected individuals and wake earlier. Whether SIM1 is phosphorylated in some physiologic circumstances for regulation of its functions has not been studied in detail.

Further studies of SIM1's biochemistry and identification of genes whose expression are regulated by SIM1 as outlined in this chapter could clarify its role in feeding behavior and body weight regulation.

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