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ELUCIDATING THE GENETIC BASIS OF SEVERE OBESITY: LEARNING FROM THE EXPERIMENTS OF NATURE

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NATIONAL UNIVERSITY OF SINGAPORE

2008

ELUCIDATING THE GENETIC BASIS OF SEVERE OBESITY: LEARNING FROM THE EXPERIMENTS OF NATURE

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Dedication

To Tsui Ling, Wen Wei and Sheng Hao

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Contents

Dedication……………………………………………………………………………

Acknowledgement………………………………………………………………….. i

Contents……………………………………………………………………………... v

Summary…………………………………………………………………………….. viii

List of tables………………………………………………………………………… xi

List of figures……………………………………………………………………….. xii

Chapter 1 Genetics of obesity and the weight regulation mechanism…………. 1

Obesity as a multifactorial trait…………………………………….….. 1

Monogenic obesity illuminates the molecular circuitry of energy homeostasis……………………………………………… 5

The leptin-melanocortin system…………………………………….. 6

The elusive satiety factor………………………………………….... 9

Leptin……………………………………………………………….. 10

Leptin deficiency…………………………………………………… 13

Leptin receptor deficiency………………………………………….. 16

Inspiration of the present study...….... 19

Chapter 2 Novel melanocortin 4 receptor gene mutations in severely Obese children………………………………………………………… 22

Summary……………………………………………………………. 22

Introduction…………………………………………………………. 23

Subjects and Methods……………………………………………… 25

Study subjects………………………………………………. 25

Metabolic/endocrine tests & body composition assessment… 26

DNA analysis……………………………………………… .. … 27

In vitro receptor function studies……………………………. 28

Statistical analysis…………………………………………… 30

Results……………………………………………………………….. 30

Impaired signaling properties of the two novel mutant Receptors…………………………………………………….. 35

Clinical characteristics of subjects with mutations………….. 35

Discussion…………………………………………………………… 42

Chapter 3 A POMC variant implicates β-MSH in the control of human energy balance……………………………………………….. 47

Summary…………………………………………………………….. 47

Introduction………………………………………………………….. 47

Methods……………………………………………………………… 51

Cohorts and human genetic studies…………………………. 51

Detection of mutations and genotyping……………………... 52

Nuclear magnetic resonance studies………………………… 56

Receptor activation studies………………………………….. 56

Competitive binding studies………………………………… 58

Physiological studies………………………………………... 59

Data analysis………………………………………………… 60

Results……………………………………………………………….. 60

Identification of missense mutations in POMC……………... 60

The novel mutation Tyr221Cys is linked with obesity or overweight status………………………………………… 61

Tyr221Cys β-MSH mutation alters three-dimensional

structure of β-MSH ………………………………………… 66

Tyr221Cys β-MSH mutation alters [Cys^5] β-MSH Signaling through MC4R…………………………………… 71

Clinical phenotype of subjects with Tyr221Cys mutation….. 74

A novel missense mutation His143Gln in α-MSH………….. 76

Discussion……………………………………………………… 81

Tyr221Cys mutation in β-MSH is associated with human early-onset obesity……………………….................81

Both α-MSH and β-MSH influence melanocortinergic tone in humans……………………………………………….. 82

Acknowledgement……………………………………………….……83

Chapter 4 Novel mutations of the POMC gene which affect POMC sorting to regulated secretory pathway……………………………......84

Summary…………………………………………………………84

Introduction……………………………………………….…………..85

Methods……………………………………………………….…………88

Subjects and human genetic studies…………………………..88

Construction of POMC wildtype, Cys28Phe, and Leu37Phe expression vectors…………………………….…88

Biochemical properties of POMC variants……….……….. 89

Results……………………………………………………………...91

Two novel mutations in N-terminus of POMC……………… 91

Mutant POMCs were less efficiently processed…………… 98

Discussion………………………………………………………103

Acknowledgement…………………………………………………… 106

Chapter 5 The role of melanocortin 3 receptor gene in childhood obesity………108

Summary……………………………………………………………108

Introduction…………………………………………………………109

Methods……………………………………………………………110

Study subjects and assessment………………………………………..110

DNA analysis……………………………………………………112

In vitro receptor function studies…………………………….. 113

Statistical analysis………………………………………………118

Results……………………………………………………………118

Common variants……………………………………………….. 120

Ile183Asn……………………………………………………….. 126

Ala70Thr………………………………………………………...129

Met134Ile……………………………………………………….. 129

Impaired signaling activities of mutant MC3Rs…………..…. 130

Discussion…………………………………………………………136

Chapter 6 Unraveling the biology of human weight regulation………………… 141

Related Publications by Candidate……………………………………………144

Bibliography…………………………………………………………….... 149

Summary

Background

Common obesity is a multifactorial trait, where an "obesogenic" environment of caloric abundance and ubiquitous automation, sedentary lifestyle, and genetic susceptibility interact to result in the obesity.

Aim

To investigate the role of three candidate genes in the pathogenesis of childhood obesity:

1. Pro-opiomelanocortin gene (*POMC*)
2. Melanocortin-4 receptor gene (*MC4R*)
3. melanocortin-3 receptor gene (*MC3R*)

Methods

More than 200 severely obese local children (Singapore) with percentage weight for height >150% were recruited to our Obesity Gene Study (OGS). *MC3R* and *MC4R* genes of this cohort were screened by direct sequencing. The *POMC* gene of more than 900 DNA samples from the Genetics of Obesity Study (GOOS) (Cambridge, UK) were screened using a combination of direct sequencing and denaturing high performance liquid chromatography (dHPLC).

Results

From 201 study subjects (OGS), three novel heterozygous *MC3R* mutations (Ile183Asn, Ala70Thr, and Met134Ile) were identified in three unrelated subjects. Compared to obese controls, the heterozygotes demonstrated higher leptin levels and adiposity, and less hunger. Family studies showed these mutations may be associated with childhood obesity. Two common variants Thr6Lys and Val81Ile in complete linkage disequilibrium were also

found. Obese subjects with the 6Lys/81Ile haplotype had significantly higher leptin levels, percentage body fat, and insulin sensitivity. The mutant and 6Lys/81Ile receptors demonstrated impaired signaling *in-vitro*.

Three *MC4R* mutations were identified in three subjects from 227 local obese children (OGS): c.631-634delCTCT, Tyr157Ser, and c.976delT. Signaling activities of the Tyr157Ser and c.976delT mutant receptors were impaired *in-vitro*.

In 538 Caucasian subjects with severe, early-onset obesity (GOOS), five probands were heterozygous for a rare missense variant in the region encoding β-MSH, Tyr221Cys. This frequency was significantly increased compared to the general UK Caucasian population, and the variant co-segregated with the obesity/overweight phenotype in affected family members. Obese children carrying the Tyr221Cys variant of β-MSH were hyperphagic and showed increased linear growth, reminiscent of MC4R deficiency. We also found a heterozygous *POMC* mutation His143Gln in one obese subject, which affected the core binding motif of α-MSH. However, the transmitting parent was not obese. Both mutant peptides demonstrated impaired binding and activation of the MC4R *in-vitro*. The results supported the role of β-MSH in human energy homeostasis. Compared to α-MSH, β-MSH may even be the more critical mediator of melanocortin signaling pathway in humans.

POMC screening of the GOOS cohort also revealed two heterozygous missense mutations, Cys28Phe and Leu37Phe, which resulted in substitution of highly conserved

residues of the sorting signal motif of POMC. Cys28Phe and Leu37Phe co-segregated with obesity/overweight in the families. *In-vitro* studies revealed less efficient sorting and processing of the two mutant POMC peptides, with less α-MSH production.

Conclusion

MC4R mutations resulted in an autosomal codominant form of obesity with variable expressivity. Heterozygous *MC3R* and *POMC* mutations did not result in autosomal dominant forms of obesity, but may contribute as predisposing factors to early-onset obesity, and exert an effect on the human phenotype.

List of Tables

Table		Page
2-1	Serial measurements of four mutation carriers	41
2-2	Comparison of HOMA, body fat and BMI between subjects with mutations and matched controls	46
3-1	POMC variants identified in obese cohort and control group	63
5-1	Additive effect of the 6K/81I variants on the adiposity, leptin levels and insulin resistance indices, in A) all subjects, and B) Chinese subjects	121
5-2	Comparison of each proband with a group of matched controls	131
5-3	Feeding behaviour scores of heterozygotes and obese controls	132

List of Figures

Figure		Page
1-1	The leptin-melanocortin system	8
2-1	Sequence chromatograms of	32-34
	A) Tyr157Ser; B) c.976delT; C) c.631-634delCTCT.	
2-2	Signaling properties of mutant MC4R Tyr157Ser & c.976delT	36
2-3	Pedigrees with A) Tyr157Ser; B) c.976delT; C) c.631-634delCTCT	39
2-4	A) genotypes of family with Tyr157Ser by PCR-digest;	40
	B) The three siblings with Tyr157Ser	
3-1	Structure of POMC and location of rare missense mutations	64
3-2	Sequence chromatogram of Tyr221Cys and residue change	65
3-3	Cosegregation of Tyr221Cys with obesity in families studied	67
3-4	Sequence alignment of β-MSH peptides of different species	68
3-5	Sequence alignment of ACTH and three forms of MSH	69
3-6	Chemical Shift Index values between wild-type and mutant β-MSH	70
3-7	[Cys^5] β-MSH binds to MC4R with lower affinity than β-MSH	72
3-8	[Cys^5] β-MSH has reduced ability to stimulate production of cAMP	73
3-9	Phenotypes of subjects with Tyr221Cys compared to controls	75
	A) food intake; B) height SDS; C) Fat free mass	
3-10	A) Sequence chromatogram of His43Gln mutation	77
	B) Cosegregation of His143Gln α-MSH mutation with obesity	78
3-11	[Gln^6] α-MSH binds to MC4R with lower affinity than α-MSH	79
3-12	[Gln^6] α-MSH has reduced ability to stimulate production of cAMP	80

Figure		Page
4-1	POMC processing by PC1 and PC2 into different peptides	87
4-2	A) Sequence chromatograms of Cys28Phe and Leu37Phe	92
	B) Co-segregation of C28F and L37F with overweight/obesity in families	93
4-3	Structure of POMC and location of mutations	95
4-4	Sequence alignment of N-terminus of POMC of different species	96
4-5	NT of POMC forms hairpin loop structure and carries sorting signal motif	97
4-6	Metabolic labeling and immunoprecipitation studies	99
4-7	A) Immunoassay revealed reduced αMSH production	101
	B) Western blot revealed less β-LPH and β-end	101
5-1	Sequence chromatograms of A) Ile183Asn; B) Ala70Thr; C) Met134Ile.	119
5-2	Functional properties of mutant MC3R *in vitro*	124
5-3	Pedigrees with A) Ile183Asn; B) Ala70Thr; C) Met134Ile	128
5-4	Dimerisation study: mutant *MC3R* transfected into cell lines stably expressing WT MC3R	134
5-5	Immunofluorescence staining of cells expressing MC3Rs	135

Chapter 1

Genetics of Obesity and the Weight Regulation Mechanism

Obesity as a multifactorial trait

Obesity is a global pandemic and a major health concern because of associated morbidities such as type 2 diabetes, hypertension, and coronary heart disease, and consequent premature mortality. The increasing obesity prevalence all over the world has been attributed to industrialisation and modernization which created an "obesogenic" environment that encourages sedentary lifestyle and increased calorie intake (Bell et al., 2005; French et al., 2001). This results in imbalance of energy intake and expenditure, and the net deposition of calories as fat. Although this trend of increasing body girth is very much driven by the "obesogenic" environment, it is facilitated by the individual's genetic susceptibility to excessive weight gain (Bouchard, 1991).

Obesity is a common but highly complex, multifactorial disorder of polygenic inheritance, which evolved from interaction between the modern "obesogenic" environment and the individual's genetic susceptibility to excessive weight gain. While it is well established that obesity runs in families, the familial clustering is not just due to a common lifestyle and shared environment. Studies in twins, adoptees, and families indicate that as much as 80 percent of the variance in the body mass index (BMI) is attributable to genetic factors. Relative risk of obesity among sibs was estimated to be 3 to 7 (Allison et al., 1996a), the concordance rate of obesity is higher between monozygotic twins than dizygotic twins (Allison et al., 1996b; Maes et al., 1997;

Stunkard et al., 1986a), and adoptees' weight is often closer to their biological parents than their adoptive parents (Stunkard et al., 1986b). These and several other comprehensive studies incorporating twins, adoptees and family data have estimated the heritability of BMI or body fat to be 25-40% (Bouchard et al., 1988; Stunkard et al., 1986b; Tambs et al., 1992; Vogler et al., 1995)

These studies, as well as numerous linkage and association studies, supported the role of genes in the pathogenesis of human obesity. However, obesity has a wide phenotypic variability, ranging from the mildly overweight to the morbidly obese, as well as the spectrum of early (childhood) to late (adult) onset. The relative contribution of the environment and genetic susceptibility towards the pathogenesis of obesity varied between different obese individuals, even within the same family, and may contribute to this phenotypic variability. The environment and a sedentary lifestyle may be the dominant contributing factor in the development of late onset obesity in an adult, while genetic factors may exert a greater influence in a young child who developed early onset obesity in the 'obesogenic' environment, and such notion is supported by the knowledge that the heritability of early-onset obesity may be considerably higher than that of adult-onset obesity (Stunkard et al., 1986b). This heterogeneity may even extend to the individual's response to weight-losing measures. Individuals where environmental factors are predominant may find it easier to lose weight compared to individuals where genetic factors predominate.

While family, twins and adoption studies as well as numerous linkage and association studies have provided considerable evidence which supported the genetic basis for human obesity, the current rapidly increasing prevalence of obesity is a relatively recent global event which occurred only in the last few decades. It is inconceivable that genetic mutations or major shifts in allelic frequencies of obesity-related genes are responsible for this, given the stable gene pool of the world's population in this short period of time (Flegal et al., 2002; Leibel, 2006). However, though the role of the obesity genes in this current epidemic is likely passive, its impact is highly significant, because individuals with these genes may be predisposed to severe or even morbid obesity when exposed to the modern "obesogenic" environment. Historically, mankind has faced prolonged periods of starvation and hardship, and was constantly required to gather or hunt for food. The ability to conserve energy in the form of adipose tissue would therefore confer a significant survival advantage, where the human body is enriched with genes which favour the storage of energy, and diminished energy expenditure (thrifty gene hypothesis), and therefore more likely to survive natural selection over the past centuries (Bell et al., 2005; Neel, 1962).

The human weight regulatory mechanism thus evolved, becoming more efficient in preventing weight loss, but relatively ineffective in preventing excessive weight gain. The modern "obesogenic" environment of industrialized countries developed over the past few decades in our bid to reduce work and improve efficiency and quality of life. The workforce became increasingly sedentary and reliant on machines and automation. Coupled with easy access to processed food, this led to reduction of energy expenditure

and increased caloric intake. While human ingenuity has succeeded in creating an environment of work efficiency and plenty, it has also inadvertently created a biology-environment mismatch, as the human weight regulation is unable to evolve fast enough to keep pace with the environmental change. This resulted in maladaptation of an otherwise sound and metabolically efficient physiological mechanism, with serious metabolic consequences. Consequently, the proportion of overweight people has risen steadily over the years. In particular, there is a pronounced increase in morbid obesity which cannot be explained by a mere shift in population mean (Flegal et al., 2002). The hypothesis is that the "obesogenic" environment has caused a subgroup of the population, who are genetically susceptible to severe weight gain, to become excessively obese (Friedman, 2003). These individuals may possess the 'thrifty genes' (obesity genes) which would otherwise be protective against starvation (and therefore confer selection advantage historically), but in the present day 'obesogenic' environment might develop severe obesity, such as high risk groups like the Pima Indians, Pacific Islanders, Afro-Americans and Hispanic-Americans (Cossrow and Falkner, 2004).

Obesity gene research has advanced rapidly over the past two decades, which provided revelation of the molecular mechanism of energy homeostasis in the process. Traditional methods employed to uncover these obesity genes include genome-wide scans which studied unrelated obese individuals, linkage studies which examined related pairs or families with obesity, and association studies which investigated association between obesity and polymorphic variants of candidate genes predicted to affect weight regulation. Unlike other multifactorial disorders, these approaches have not been as

promising for common obesity, because the obese phenotype is very heterogeneous, even within the same family. There is variable contribution from genetic, environmental and behavioural influences which differ for every obese individual, which confounded efforts to analyse this condition. While several syndromic forms of human obesity such as Prader-Willi syndrome and Bardet-Biedl syndrome have been genetically mapped and causative genes identified, their exact roles in the pathogenesis of obesity and the underlying molecular mechanisms have not been isolated yet (Boutin and Froguel, 2001).

Monogenic obesity illuminates the molecular circuitry of energy homeostasis

While the search for obesity genes has posed a major challenge, we have witnessed significant milestones in obesity gene research in the last decade, in the discovery of novel single gene defects which result in human obesity, namely leptin deficiency, leptin receptor deficiency, proopiomelanocortin (POMC) deficiency, prohormone convertase 1 deficiency (PC1), melanocortin 4 receptor deficiency, and tyrosine kinase B (TrkB) deficiency. These monogenic forms of human obesity resulted in deficiency of critical molecules and disruption of the leptin-melanocortin system which lead to the obese phenotype, and thus provide validation of the role of the leptin-melanocortin system in energy homeostasis, and unravel the molecular circuitry of human weight regulation.

Human energy homeostasis is regulated by a complex physiological system that requires the integration of several peripheral signals and central coordination in the brain to maintain a balance between food intake and energy expenditure. The hypothalamus functions as the central regulator in this system, in particular the arcuate nucleus which

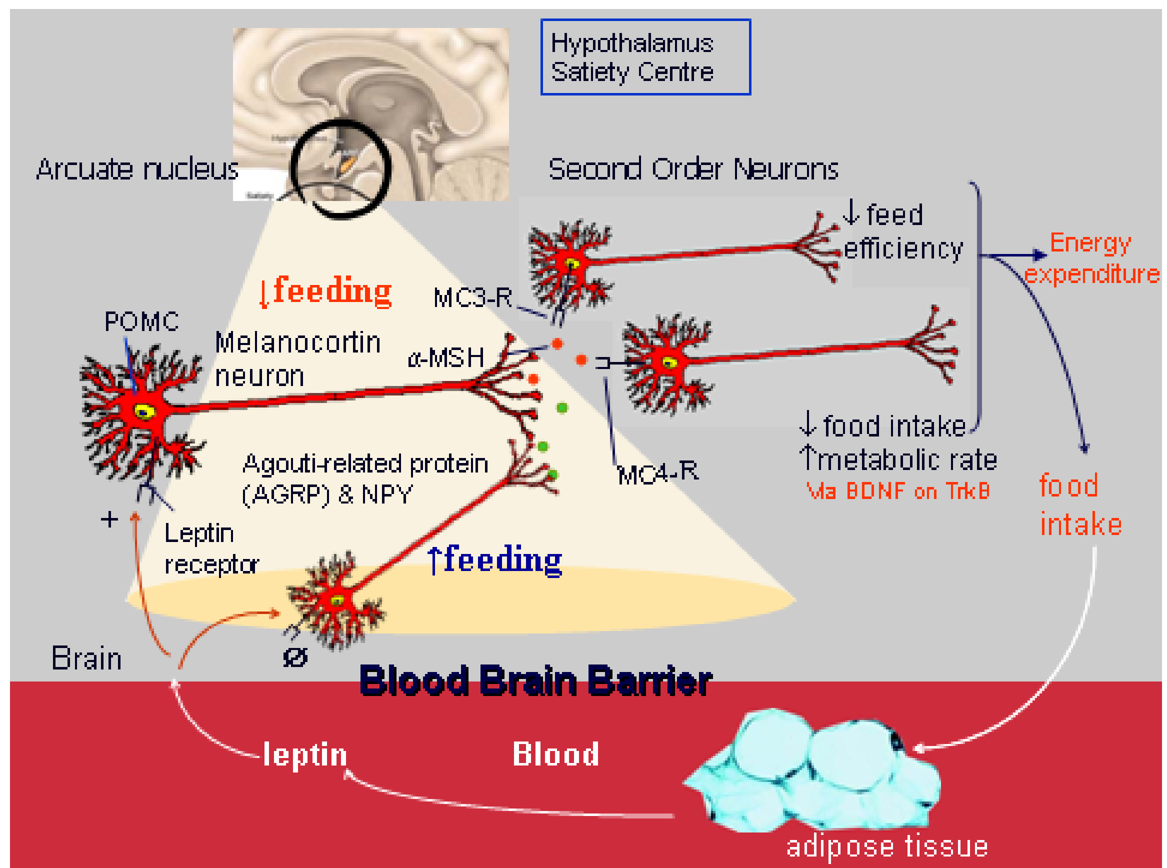
has an essential role. The monogenic forms of human obesity as well as studies of knockout mouse models validate the critical mediators of this weight regulation loop, by demonstrating that deficiencies of these molecules result in obesity unequivocally and also endorse the crucial role of the leptin-melanocortin pathway.

The Leptin-Melanocortin System

Various human and murine genetic studies have shed light on the biological weight regulation mechanism, akin to pieces of a jigsaw puzzle being put together which progressively unravel this integral system. Excess food intake is stored in adipose tissue. Adipose tissue secretes leptin in response to increased fat storage, which circulates as an afferent satiety signal and activates hypothalamic neurons expressing pro-opiomelanocortin (POMC) located in the arcuate nucleus, which innervates other hypothalamic regions known to regulate feeding behaviour (Cowley et al., 2001; Heisler et al., 2002; Saper et al., 2002). Pro-opiomelanocortin (POMC) is a polypeptide that undergoes tissue-specific post-translational processing, the products of which include the melanocortin peptides α, β, and γ-melanocyte-stimulating hormones (MSH) (Raffin-Sanson et al., 2003). One or more of the three melanocortin peptides is/are involved in the anorectic response by stimulating melanocortin-4 receptors (MC4R) on neurons downstream in the paraventricular nucleus (PVN) (Farooqi et al., 2003; Farooqi et al., 2000; Huszar et al., 1997; Schwartz et al., 2000; Vaisse et al., 2000), and melanocortin-3 receptors (MC3R) to reduce feed efficiency, which is the ability to convert food to fat stores (Butler et al., 2000; Chen et al., 2000; Feng et al., 2005; Lee et al., 2007b; Lee et al., 2002). The melanocortin system thus mediates the anorexigenic effects of leptin,

reducing food intake and increasing energy expenditure. MC3R is also located on POMC expressing neurons in the arcuate nucleus, and may form part of a feedback loop which negatively regulates the anorexic tone of the POMC expressing neurons (Jegou et al., 2000), where melanocortin peptides from activated POMC neurons negatively autoregulate further POMC expression through their inhibitory actions at the MC3R. Recent evidence suggests that the tyrosine kinase B receptor and the brain derived neurotrophic factor (Xu et al., 2003; Yeo et al., 2004) and nesfatin (Oh et al., 2006) are critical mediators downstream of MC4R. Leptin also inhibits neurons co-expressing the orexigenic neuropeptide Y and agouti-related peptide in the arcuate nucleus, which will otherwise promote feeding activity (Gropp et al., 2005). A schematic of this intricate leptin-melanocortin weight regulation system is illustrated in figure 1-1.

Figure 1-1. The leptin-melanocortin system. Leptin secreted by adipose tissue as satiety signal crosses the blood brain barrier to stimulate the melanocortin neurons in the arcuate nucleus of the hypothalamus, and upregulate production of POMC, which is broken down to neurotransmitter α-MSH and in turn stimulate MC4R and MC3R of neurons downstream to reduce food intake, increase metabolic rate, and decrease feed efficiency (i.e. stimulation of the anorexigenic pathway). Leptin also concomitantly inhibits the orexigenic pathway by exerting inhibition on the AGRP/NPY neurons in the arcuate nucleus.



The Elusive Satiety Factor

The regulation of energy balance has been the focus of discussion dating back to 1783, and the quest to understand the weight regulation mechanism started with the search for the satiety factor. The body's energy balance was postulated then to be controlled by a feedback loop, in which the amount of stored energy is sensed by the hypothalamus, which adjusts the food intake and energy expenditure to maintain a constant body weight. It is now established that the paraventricular nucleus (PVN), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), and arcuate nuclei (ARC) are the satiety control centers of the hypothalamus (Choi and Dallman, 1999; Cowley et al., 2001). Studies on rat models have shown that disruption of ARC, PVN and VMN produced increased food intake and obesity, and disruption of DMN produced decreased food intake.

The classical parabiotic (cross circulation) experiments by Hervey and other investigators demonstrated that there was a circulating satiety factor in the blood stream which regulates food intake (Coleman, 1973; Harris and Martin, 1984; Hervey, 1969):

a) overfeeding one of a pair of parabiotic mice (which were surgically joined with interchange of blood) reduced food intake and induced weight loss in the partner, apparently because of the transfer of a circulating hormone.

b) when an *ob/ob* mouse (obese mouse due to genetic defect and lacking the circulating satiety factor) was surgically joined to a normal animal, it ate less and gained less

weight. This occurred because the *ob/ob* phenotype which resulted from lack of the proposed satiety hormone was supplied by the normal animal in the parabiotic pair.

c) Mice homozygous for the *db* mutation were also obese. This *db/db* phenotype was due to a deficiency of the hypothalamic receptor for the purported satiety factor. When a normal mouse is paired with a *db/db* mouse, it rejected food and died of starvation, presumably due to an excess of the satiety factor from the *db/db* mouse.

Leptin

It is now established that the primary product of the *ob* gene is the satiety factor termed leptin, and the mice with the *ob* mutation (now designated Lep^{ob}) have a deficiency of leptin (due to a premature stop codon resulting in a truncated protein), while the mice with the *db* mutation (now designated $Lepr^{db}$) are deficient in the hypothalamic receptor for leptin (Leibel et al., 1997; Tartaglia et al., 1995; Zhang et al., 1994).

The discovery of leptin (Zhang et al., 1994) and the leptin receptor (Tartaglia et al., 1995) heralded a new era in obesity research. The protein, "leptin", is derived from the Greek word 'leptos', meaning thin. The etymology of the word "leptin" implies that its physiological role is primarily to suppress body fat, by decreasing food intake and increasing energy expenditure. Leptin is a 167 amino acid peptide made exclusively in adipose tissue in a wide range of animal species, including humans. The *ob* gene is located on the mouse chromosome 6, and the human homologue of the *ob* gene has been mapped to chromosome 7q31.3 (Friedman et al., 1991; Geffroy et al., 1995). Northern

blot or RT-PCR analysis of the messenger ribonucleic acid (mRNA) for the *ob* gene showed that it was expressed only in adipose tissue (Zhang et al., 1994).

Leptin is secreted by adipocytes as an afferent satiety signal, produced in proportion to the mass of adipose tissue, which acts as an endocrine organ. Both human and animal studies have demonstrated the close association between body fat, leptin mRNA, and the plasma leptin levels (Halaas et al., 1995; Levin et al., 1996; Weigle et al., 1995). Increase in fat storage will lead to increased leptin, which inhibits the satiety center in the hypothalamus, and also influences other neuroendocrine systems, including those related to puberty and fertility. There appears to be a lipostatic set point for weight regulation, in which the body will maintain a certain weight and body composition.

Leptin also has peripheral effects other than its central role in weight regulation. It stimulates oxidation of fatty acids in mitochondria and uptake of glucose in muscles, and prevents damaging accumulation of lipids in non-adipose tissues (lipotoxicity). Leptin stimulates 5'-AMP-activated protein kinase (AMPK) which increases fatty acid consumption and oxidation, and reduces fat storage. This reduces fat accumulation in muscle and liver cells, which in turn reduces insulin resistance (Friedman, 2002; Minokoshi et al., 2002).

In the *ob/ob* (leptin deficient) mice, systemic or intra-cerebroventricular administration of leptin reduces food intake and increases energy expenditure, resulting in reduced body fat. It also increases activity of the sympathetic nervous system,

decreases insulin, reduces glucose and improves insulin-sensitive glucose disposal (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Schwartz et al., 1996a). Supraphysiological doses of leptin have similar effects in non-obese animals (Collins et al., 1996). In order to promote weight loss in normal lean mice or obese adults, leptin must be administered in doses that raise serum leptin concentrations by 20 to 30 times the normal level for a given fat mass (Campfield et al., 1995; Heymsfield et al., 1999). It is therefore proposed that the principal function of leptin is to maintain the body fat mass, and the system may be more adept at preventing weight loss than weight gain, by signaling to the brain that calorie intake and the amount of energy stored as fat are sufficient (Rosenbaum and Leibel, 1999). In such a model, the depletion of fat mass during starvation leads to reduced leptin synthesis per fat cell. The reduced action of leptin on the hypothalamus initiates compensatory changes in energy intake and output, which favours a return to the usual body weight. The threshold concentration of leptin below which these changes occur, is highly individualized, and influenced by genetic and developmental factors. Any further elevation of serum leptin concentration above the threshold will have little effect on energy homeostasis, except for supraphysiological levels such as leptin administration.

The majority (>90%) of people with common obesity were hyperleptinemic, and do not have low leptin levels or mutations of the leptin gene (Considine et al., 1995; Maffei et al., 1996). On the contrary, there is strong positive correlation between serum leptin concentrations and the percentage body fat, body mass index, and basal insulin concentrations (Considine et al., 1996b; Maffei et al., 1995), and leptin levels are reduced

in obese individuals who lose weight (Halaas et al., 1995). This led to the hypothesis of resistance to the action of leptin in these individuals, so that the increase in adipose tissue mass is maintained. As the vast majority of obese individuals do not have defective leptin receptors and therefore not contributory to the hyperleptinemia observed (Considine et al., 1996a; Gotoda et al., 1997), the relative insensitivity of the hypothalamic satiety centre to leptin action may be due to abnormal carriage of leptin across the blood brain barrier (Caro et al., 1996; Schwartz et al., 1996b), or more intriguingly, defective mediators in the pathway distal to the leptin receptor. This hypothesis is obviously shared by many, given the myriad of research in quest of genetic defects downstream to the leptin receptors.

Leptin deficiency

Leptin deficiency from disruption of both leptin genes result in severe obesity in mice and humans. The *ob/ob* (leptin deficient) mouse is characterized by obesity, hyperphagia, hyperglycemia (Schwartz et al., 1996a), hyperinsulinemia due to insulin resistance (Bray and York, 1971), hypothermia (Trayhurn et al., 1977), impaired hypothalamic-pituitary-thyroid axis (Ohtake et al., 1977), and hypogonadotropic hypogonadism causing infertility (Swerdloff et al., 1976), and leptin replacement reverses these endocrine and metabolic defects (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995).

Human congenital leptin deficiency is rare. Five children from three consanguineous Pakistani families were reported to be homozygous for a frameshift *Lep* mutation comprising of a single G deletion affecting codon 133 (Δ133G), which led to 14

aberrant amino acids, followed by a premature stop codon (Farooqi et al., 2002; Farooqi and O'Rahilly, 2004; Gibson et al., 2004; Montague et al., 1997). The mutant leptin was not secreted, but accumulated intracellularly as a consequence of misfolding and aggregation, and was subsequently degraded by the proteasome (Rau et al., 1999). There were also three related Turkish subjects homozygous for a missense mutation Arg105Trp due to a C to G transition, which resulted in impaired processing and secretion of leptin (Strobel et al., 1998).

These individuals had undetectable or very low leptin levels, and exhibit extreme early onset obesity. Their birth weights were within normal limits, but rapidly become obese by 3 to 4 months of age, with marked hyperphagia and were constantly hungry. They had high percentage body fat of 54 to 57%, and linear growth was not stunted with IGF-I levels within normal range. Bone age was advanced by about 2 years, but bone mineral density (BMD) was appropriate for age and gender. There was no evidence of impairment in basal or total energy expenditure, and body temperature was normal, unlike the ob/ob mice, whose oxygen consumption, energy expenditure, and body temperature were low (Montague et al., 1997). Thus, leptin may be less central to the regulation of energy expenditure in humans than in mice. Another difference in humans with either leptin or leptin receptor mutations was the consistently normal glucocorticoid concentrations, in contrast to the marked excess in ob/ob mice. The human cases with leptin deficiency had hypogonadotropic hypogonadism, with delayed puberty or primary amenorrhoea, yet had normal responses to short human chorionic gonadotropin stimulation test (test of gonadal function) and gonadotropin releasing hormone

stimulation test (test of pituitary function), which were suggestive of a hypothalamic defect.

Three leptin deficiency children homozygous for the Δ133G mutation received up to 50 months of recombinant human methionyl leptin (R-metHuLeptin) replacement, administered as subcutaneous injections once daily, in escalating doses if weight gain was documented over two successive 2 month periods, to achieve 10, 20, 50, 100 and 150% of predicted leptin concentration based on height and weight (Farooqi et al., 2002). There was dramatic weight loss which started within 2 weeks of initiation, and sustained through the trial period. Refractory periods of weight gain did occur but were overcome with increases in leptin doses. Fat mass represented 98% of the weight loss, with reciprocal increase in lean mass, and there was reduced food intake up to 84% during *ad libitum* meal tests. It was so successful that a morbidly obese boy weighing 42 kg at 3 years of age achieved normal weight after 2 years of therapy, weighing 32 kg (75th percentile on local weight chart) after 48 months of treatment. There was no discernible change in basal and total energy expenditure. An 11-year-old prepubertal girl with congenital leptin deficiency who received leptin replacement began to manifest pubertal progress subsequently, with gradual increase in pulsatility of gonadotropins, and achieved regular menstrual cycles by 12.1 years. The other 2 younger children (<6 years) remained appropriately prepubertal during leptin replacement. Congenital leptin deficiency caused hypogonadism and pubertal failure, but leptin replacement permitted puberty to progress appropriately in the 11 year old girl but yet did not cause precocious puberty in the other two younger children. The authors proposed that leptin may act as a

permissive metabolic gate which allowed progression of appropriately timed pubertal development. These reports of human leptin deficiency and the effects of leptin replacement provided the first evidence of the importance of leptin in energy regulation as well as endocrine functions in humans.

Family studies showed that leptin deficiency was inherited in an autosomal recessive fashion. However, though heterozygotes for the mutant *Lep* gene were not morbidly obese, 76% of them were obese with BMI more than 30 kg/m^2. The blood leptin levels of these heterozygotes were lower than matched controls, with poor correlation between body fat mass and leptin levels, and their body fat percentage exceeded the predicted body fat percentage(Farooqi et al., 2001). This observation demonstrated that haploinsufficiency of one *Lep* gene may result in increased adiposity, lower leptin, and higher likelihood of obesity, but not to the extent of an intermediate phenotype typical of an autosomal co-dominant condition. The study of this group of individuals who are partially deficient in leptin also showed that differences in circulating leptin levels, within the range found in normal humans, can directly influence adiposity.

Leptin Receptor Deficiency

The leptin receptor is a member of the cytokine family of receptors with several splice variants. The long leptin receptor isoform is considered the principal signaling isoform. The intracellular signaling system for leptin appears to involve activation of the JAK-STAT system, with Stat-3 being the phosphorylated intermediate. The human *LEPR* gene has been mapped to chromosome 1p31. Leptin receptors are distributed widely,

including brain and many peripheral tissues (lungs, kidneys, muscle, and adipose tissue), suggesting that this peptide may provide a wide range of tissues with information about fat stores. If obesity in humans were due to leptin receptor mutations, then one would expect a much higher leptin concentration than predicted, based on fat mass, but this is not the case (Rosenbaum et al., 1996). Considine et al examined expression of the *LEPR* gene in the hypothalamic tissue from 7 lean and 8 obese humans obtained shortly after autopsy (Considine et al., 1996a). Using RT-PCR, there was no difference in the amount of *LEPR* mRNA between the lean and obese subjects. A Gln23Arg polymorphism due to A-to-G substitution at nucleotide 668 of the *LEPR* cDNA was detected, where 11 subjects were heterozygous and 3 were homozygous. The occurrence of the polymorphic allele(s) did not correlate with the body mass index in the patients studied. The results suggested that leptin resistance observed in obese humans is unlikely to be due to a defect in the leptin receptor. Gotoda et al determined the entire coding sequence of the human leptin receptor cDNA from peripheral blood lymphocytes of 22 morbidly obese patients (Gotoda et al., 1997). Five common DNA sequence variants were found to be distributed throughout the coding sequence at codons 109, 223, 343, 656, and 1019, with one rare silent mutation at codon 986, as well as a novel alternatively spliced form of transcript. None of the five common variants, including three that predict amino acid changes, were null mutations causing morbid obesity, because homozygotes for the variant sequences were also found in lean subjects. Furthermore, the frequency of each variant allele and the distribution of genotypes and haplotypes were similar in 190 obese and 132 lean white British males selected from a population-based epidemiologic survey. The results

suggested that these are polymorphisms, and that mutations in the leptin receptor gene are not a common cause of human obesity.

The diabetes (*db/db*) mouse had abnormal splicing of the long (hypothalamic) leptin receptors(Lee et al., 1996), and had features similar to that of the *ob/ob* (leptin deficient) mouse. There was early onset morbid obesity with hyperphagia and reduced energy expenditure, infertility secondary to hypogonadotropic hypogonadism, diabetes with dyslipidemia, hypercortisolism, and decreased growth hormone production with stunted linear growth (Chua et al., 1996). The first human *LEPR* mutation was discovered in a consanguineous family of Kabylian origin (northern Algeria) where three siblings had morbid obesity since early childhood (Clement et al., 1998). This *LEPR* gene mutation resulted from G to A substitution at the +1 position of intron 16 (one base after exon 16), which led to exon skipping and loss of transmembrane and cytoplasmic domains. The truncated protein might be secreted as a leptin binding protein (like the short isoform) which trapped serum leptin in bound form and prolonged its half-life, contributing to the very high leptin levels observed. The homozygous mutation caused severe obesity and pituitary dysfunction. The affected sisters had normal birth weight but rapidly gained weight in the first few months of life. Manifestations included bizarre eating behaviour, fighting for food, impulsivity and stubbornness, labile emotions and social dysfunction, reminiscent of the Prader-Willi Syndrome. However, they were not mentally retarded, and had resting metabolic rates similar to that predicted, with normal core temperature. Growth hormone and thyrotropin levels were low, with the presence of

hypogonadotropic hypogonadism. These findings supported leptin and its receptor as important physiological regulators of several endocrine functions.

Farooqi et al screened the *LEPR* genes of 300 subjects with severe early onset obesity and hyperphagia, inclusive of 90 probands from consanguineous families, and reported seven homozygotes and one compound heterozygote for nonsense or missense *LEPR* mutations which resulted in impaired receptor signaling (Farooqi et al., 2007). Affected subjects also had delayed puberty due to hypogonadotropic hypogonadism. Unexpectedly, serum leptin levels were within the range predicted by the elevated fat mass in these subjects. Thus serum leptin levels cannot be used as a marker for leptin receptor deficiency. The clinical features of leptin receptor-deficient subjects were less severe than those with congenital leptin deficiency.

Inspiration of the present study

The discovery of leptin and its receptor heralded a new era in obesity research, as it inspired an unprecedented surge of research activities leading to an explosion of new knowledge about the intricate molecular mechanism of weight regulation. These research efforts further established leptin as the key long term regulator of the biological weight regulation mechanism and the hormonal link between adipocyte and the brain. The melanocortin system downstream of leptin became the focus of research efforts in the past decade. The phenotypic similarity between murine and human forms of leptin and leptin receptor deficiencies demonstrated the high conservation of weight regulation mechanism across species, and supported the applicability of knockout mouse models

deficient in candidate genes or molecules predicted to participate in energy regulation in the study of human energy homeostasis. The flurry of research activities which generated knockout obese mouse models also saw a corresponding rise in reports of human obesity due to single gene defects affecting the melanocortin system, with striking resemblance to the murine forms. These monogenic forms of human and murine obesity validate the melanocortin system and its key molecules as an integral part of the weight regulation mechanism, as deficiencies of these critical molecules due to the genetic defects lead to unequivocal obesity as the predominant phenotypic feature. The subsequent chapters give a detailed account of our contribution to this field of obesity research, not only in the discovery of novel *MC4R* mutations causing monogenic human obesity, but also the role of genetic variants of *POMC* and *MC3R* in the pathogenesis of common obesity.

The research work on *MC3R* and *MC4R* genes were performed in Singapore using a local cohort of severely obesity children. The research on *POMC* gene was performed in Cambridge, United Kingdom, in the laboratory of Professor Steve O'Reilly and Dr Sadaf Farooqi, using the DNA biobank of their Genetics of Obesity Study (GOOS) cohort. The NMR study decribed in chapter 3 was performed by Dr Glenn Millhauser and his team from the University of California, Santa Cruz, California. Also in the same chapter, genotyping of the Tyr221Cys variant in UK cohort of the EPIC-Norfolk study using Taqman was performed by Matthew Sims and team from the MRC Epidemiology Unit, Cambridge, UK. Genotyping of Tyr221Cys variant in the French obese cohort using FRET based assay was performed by David Meyre and team from the Institute of Biology, Pasteur Institute, Lille, France and Section of Genomic Medicine and Genome

Centre, Imperial College London, UK. The metabolic labelling and western blot studies of Chapter 4 were performed by Assoc Professor John WM Creemers from the University of Leuven, Belgium. The α-MSH immunoassay described in chapter 4 was performed by Dr Rob Oliver and Professor Anne White from the University of Manchester, UK.

Chapter 2

Novel Melanocortin 4 Receptor Gene Mutations in Severely Obese Children

Summary

Melanocortin 4 receptor (MC4R) deficiency is the commonest monogenic form of obesity. The significance of *MC4R* mutations in Asian obese populations has not been adequately examined. The objective of this study is to determine the role of *MC4R* mutations in severely obese Asian children. We screened 227 obese local children and adolescents for *MC4R* gene mutations by polymerase chain reaction (PCR) and direct sequencing. We identified three mutations in three subjects: 4 bp deletion from nucleotides 631-634 (c.631-634delCTCT), Tyr157Ser (c.470A>C), and 1 bp deletion at nucleotide 976 (c.976delT) (1.32% of study subjects). The latter two mutations are novel. The Tyr157Ser mutation was not found in 188 non-obese controls using restriction enzyme digest analysis. *In vitro* transient transfection studies supported the pathogenic role of both novel mutations Tyr157Ser and c.976delT, where the signalling activities of the mutant receptors were impaired. Heterozygous *MC4R* mutations were associated with early onset severe obesity, and homozygosity of the *MC4R* mutation Tyr157Ser resulted in morbid obesity. *MC4R* mutations result in an autosomal codominant form of obesity with variable expressivity. MC4R deficiency is not as common among the obese children in this study compared to other populations. Family studies revealed that adults heterozygous for the mutations were less obese compared to the children. We hypothesise that this may be due to amelioration of phenotype severity with age, genetic anticipation, or difference in exposure to modifying factors at critical stages of childhood such as the environment.

Introduction

The human melanocortin 4 receptor (MC4R) is a 332 amino acid protein encoded by a single exon localised on chromosome 18q22 (Gantz et al., 1993b; Sundaramurthy et al., 1998). The MC4R is a seven transmembrane G-protein coupled receptor highly expressed in hypothalamic nuclei which regulate energy homeostasis (Mountjoy et al., 1994; Mountjoy and Wild, 1998). MC4R is modulated by the endogenous agonist α-melanocyte stimulating hormone (MSH) and antagonist agouti-related protein, and signals through activation of adenylate cyclase (Schwartz et al., 2000). Mice with inactivation of both copies of the *MC4R* genes produced an obesity syndrome with hyperphagia associated with pathological lack of satiety, hyperinsulinaemia with hyperglycaemia, and increased linear growth, but unlike leptin deficient mice, had normal reproductive function (Huszar et al., 1997). Heterozygotes had an intermediate weight between the homozygotes and wild-type mice, and females were more affected than males. *MC4R* knockout mice continue to increase feeding on a high fat diet, but do not increase thermogenesis. Interestingly, the MC4R knockout mouse exhibits normal feeding and returns to previous weight in response to food restriction. Thus MC4R does not appear to be required for normal feeding or metabolic response to fasting. However, MC4R is required for normal response to high fat diet by maintaining satiety, and increasing thermogenesis and metabolic rate.

MC4R deficiency resulting from disruption of one or both *MC4R* alleles represents the commonest monogenic form of human obesity (Farooqi et al., 2003; Vaisse et al., 2000). Obese individuals with MC4R deficiency displayed a common, non-

syndromic form of obesity and were not characterized by any peculiar phenotypic abnormalities. The subjects with *MC4R* mutations were obese from an early age, but with increase in both fat and lean masses, were excessively hungry from 6-8 months of age with persistent food-seeking behaviour, and become distressed if food was not provided. They had higher food intake when compared to obese controls when assessed with ad-libitum meals. There was increased growth velocity in childhood, where those with *MC4R* mutations were taller than matched obese controls, and the bone age exceeded the chronological age by 1 to 4.9 years. Pubertal onset and secondary sexual characteristics were normal. They also had significantly higher insulin levels compared to matched controls, but the majority were not diabetic. The proportions of type 2 diabetic or glucose intolerant subjects, triglyceride levels, and leptin levels were not statistically different between both mutated and non-mutated obese groups. The affected subjects did not have any developmental, intellectual or behavioural problems, and there were no dysmorphic features.

In-vitro function of mutant MC4-receptors correlated with the severity of the clinical phenotype, indicating that weight regulation is sensitive to amount of functional MC4 receptors. Subjects with inactivating (null) MC4R mutations were heavier, taller, and more hyperphagic than those with partially active *MC4R* mutations (Farooqi et al., 2003).

Most family studies revealed autosomal co-dominant pattern of inheritance. Homozygotes for *MC4R* mutations exhibited a more severe phenotype than heterozygotes, where they were heavier and taller. Transmission of the mutations in the affected families

indicated variable penetrance and expressivity that is not related to the functional severity of the mutations *in-vitro*. Family studies of heterozygous probands demonstrated co-segregation of mutation with early onset obesity with 100% penetrance, while that of homozygous probands demonstrated early onset obesity only in 68% of heterozygous family members. There is variable age of onset of obesity as well as its severity, even for the same mutation within the same family. The reason for this variability in penetrance and expressivity is yet to be fully elucidated.

Human MC4R deficiency was reported to affect 4 and 5.8 % of severely obese French and British populations respectively (Farooqi et al., 2003; Vaisse et al., 2000). However, studies elsewhere reported low incidence of *MC4R* mutations in their respective obese populations (Adams et al., 2007; Hinney et al., 2003; Jacobson et al., 2002; Larsen et al., 2005; Miraglia Del Giudice et al., 2002; Ohshiro et al., 1999; Rong et al., 2006; Wang et al., 2006). The prevalence and spectrum of MC4R mutations in the obese Asian populations has not been well studied. Therefore, we embarked on a study to determine the role of *MC4R* mutations in Singapore's obese paediatric population (Lee et al., 2007a).

Subjects and Methods

Study Subjects

The *MC4R* gene was analysed in 227 unrelated children and adolescents with early onset severe obesity (149 boys and 78 girls; 116 Chinese, 82 Malays, 22 Indians, 7 others). The mean (standard deviation) age was 10.9 (3.3) years, BMI 32.1 (5.5) kg/m^2, WFH 170 (22)

%, and all subjects developed obesity before the age of 10 years. These children were recruited mainly from the nationwide school health clinic, a primary healthcare service and facility which routinely screens schoolchildren. Consecutive patients who met the inclusion criteria were invited to participate in the study. The inclusion criteria were severe obesity with body weight at least 140% of ideal weight for height (WFH) based on local chart, and onset of obesity before 6 years of age. We recruited young children who develop severe early onset obesity in an attempt to increase the likelihood of identifying children who are genetically predisposed to obesity, as we hypothesized that genetic factors rather than the environment play a predominant pathogenic role in these individuals. As a local BMI chart for children was not available at the time of the study, WFH was used as an acceptable alternative measure of obesity (Mei et al., 2002). This research was approved by the Research and Ethics Committee of the National University Hospital, and informed written consent was obtained from all subjects and parents.

Metabolic / Endocrine Tests and Body Composition Assessment

Blood samples were obtained from the obese subjects in the fasted state for biochemical profiling, including venous glucose for oral glucose tolerance test (OGTT), fasting serum C-peptide (CP) and insulin (Ins) levels. Insulin resistance was calculated using the homeostasis model assessment (HOMA) (Matthews et al., 1985). Bone mineral density of the second to fourth lumbar spine and body fat composition were assessed using Dual Energy X-ray Absorptiometry (DEXA) (Norland DEXA model XR-36, Fort Atkinson, Wisconsin, USA; coefficient of variation is 1%).

DNA analysis

Genomic DNA extracted from peripheral leukocytes was analysed. The single exon of *MC4R* gene was amplified by PCR using the following primers: Forward primer 5'-GAGAACAAGAAAGCAAAGAGCAG-3', and reverse primer 5'-TACCCTACACGGAAGAGAAAGC-3'. Amplification was carried out for 35 cycles. Each cycle consisted of a 45 seconds denaturing step at 95°C, a 45 seconds annealing step at 55°C, and a 1 minute extension step at 72°C. Prior to the 35 cycles, the sample was kept at 95°C for 3 minutes for denaturation. After the 35 cycles, there was a final elongation phase at 72°C for 5 minutes. The size of the amplicon was 1374 bp. The amplicons were directly sequenced in 2 fragments using the following sequencing primers: 5'-TGGAGGAAATAACTGAGACG-3' and 5'-TGTCATCATCTGCCTCATC-3'. Novel sequence variants were confirmed by reverse sequencing. Heterozygous frameshift mutations were also reconfirmed by cloning each of the two alleles from each affected subject into pDrive vectors followed by direct sequencing (see "PCR and Cloning" below). Sequencing was performed using BigDye v2.0 Terminator Cycle Sequencing Kit (PE Applied Biosystems) and analysed on the ABI Prism 3100 Genetic Analyzer.

A potentially novel missense *MC4R* mutation Tyr157Ser was found during the screening process. 188 genomic DNA samples from 99 healthy children with normal height and weight [mean age (SD) = 7.1 (4.6) years; mean body mass index (BMI) (SD) = 16.8 (3.4) kg/m^2; 63 males & 36 females; 61 Chinese, 20 Malays, 18 Indians] and 89 DNA samples from healthy adults (BMI < 25 kg/m^2; 30 males, 59 females; 27 Chinese, 30 Malays, 32 Indians) were analysed as normal controls. Restriction enzyme *Rsa* I was used

to detect *MC4R* mutation Tyr157Ser in 188 control samples and the family members of the proband. Using primers which amplified the wild-type *MC4R* gene between nucleotide positions 811 and 1120, the resultant PCR product had two *Rsa* I recognition sites at nucleotide positions 834 and 861, yielding three fragments with *Rsa* I digest, one of which is 259 bp. Using the same primers to amplify the mutant *MC4R* gene Tyr157Ser, there is only one *Rsa* I recognition site at nucleotide position 861 on the mutant amplicon, producing only two fragments, one of which is 286 bp. Electrophoresis using polyacrylamide gel was able to differentiate the 259 bp and 286 bp fragments, thus identifying the mutant from wild-type alleles. Primers for PCR-digest technique: forward primer 5'-CTGCTTTCAATTGCAGTGGA-3', reverse primer 5'-ATGGTCAAGGTAATCGCTCC-3'. PCR conditions: denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 54°C for 45 seconds, elongation at 72°C for 45 seconds, followed by final elongation at 72°C for 5 minutes.

In vitro Receptor Function Studies

PCR and Cloning

Wild-type and mutant *MC4R* were directly amplified from DNA of normal and affected subjects. The forward and reverse primers contain *Kpn* I and *Xho* I restriction sites respectively: MC4R-F (5'GGTACCATGGTGAACTCCACCCACCG3'), MC4R-R (5'CTCGAGTTAATATCTGCTAGACAAGT3'). The amplicons were cloned into the pDrive UA vector (Qiagen, GambH, Hilden, Germany) and the sequences verified by direct sequencing.

The *MC4R* gene was excised with *Kpn* I and *Xho* I restriction enzyme and subcloned into the pBudCE4 mammalian dual expression vector (Invitrogen Corp) under the control of EF-1α promoter. The synthetic cDNA sequence encoding *Renilla* luciferase enzyme was excised from the phRG-TK vector (Promega Corp, Wisconsin, USA) with *Hind* III and *Bam*H I, and subcloned into the above pBudCE4/MC4R construct under the control of the CMV promoter. All constructs were sequenced for verification.

HEK 293 cells were maintained in DMEM (Sigma) supplemented with 10% FBS, 100 U/mL penicillin G, 100 mcg/mL streptomycin sulfate and 250 ng/mL amphotericin B (Sigma). Cells were incubated at 37^{0}C in humidified air containing 5% CO_2.

Transient Transfection Study

Transient transfections were performed using Effectene reagent (Qiagen GambH, Hilden, Germany) according to the manufacturer's recommendation. The cAMP responsive firefly luciferase vector, pCRE-Luc (Stratagene, California, USA) was transiently co-transfected in HEK 293 cells with the construct expressing the wild-type or mutant MC4 receptors and *Renilla* luciferase (pBudCE4/*Renilla*/MC4R) in a 9:1 ratio. After 24-hour incubation, transfected cells were washed once with 1x PBS and fresh media were added with serial dilutions of α-MSH ranging from 1 pM to 10 uM, and the generation of cAMP was assayed through the activation of co-transfected pCRE-Luc. The data was expressed as ratio of firefly luciferase activity to *Renilla* luciferase activity (relative light

units) to normalize for transfection efficiency and amount of MC4R produced. Cells expressing only pCRE-Luc and pBudCE4/*Renilla* construct were used as negative controls. Each dilution was performed in triplicates, and incubated for a further 24-hour period prior to cell lysis and luciferase readings.

Both firefly and *Renilla* luciferase activities were determined using the Dual Luciferase Reporter Assay System (Promega Corp, Wisconsin, USA) in a TD-20/20 (Turner Designs) luminometer as recommended by the manufacturer.

Statistical analysis

Graph fit curves and EC_{50} were obtained and performed using GraphPad Prism 4 for Windows (version 4.02 GraphPad software, San Diego, California, USA), and curves were fitted using the logistical equation.

Results

From the 227 obese subjects studied, we identified three mutations in three unrelated subjects: a missense mutation Tyr157Ser (c.470A>C) in homozygosity; a heterozygous mutation with T deletion at nucleotide position 976 (c.976delT); a heterozygous mutation with 4 bp deletion of nucleotides 631-634 (c.631-634delCTCT); the former two mutations are novel. A single nucleotide change from A to C at nucleotide position 470 resulted in the substitution of hydrophobic tyrosine by hydrophilic serine at codon 157 (Tyr157Ser) (figure 2-1A), located at the second intracytoplasmic loop of the transmembrane domain. A deletion of T nucleotide at position 976 of the coding region led to a frameshift which

resulted in readthrough of the stop codon, followed by an out-of-frame stop codon situated 63 nucleotides downstream at nucleotide position 1062. While the wild-type MC4R protein sequence ended at residue 332, the resultant mutant receptor is predicted to be a longer polypeptide chain with 28 aberrant amino acid residues from codon 326, an extra 21 amino acids longer than the wild-type MC4R (Figure 2-1B). The third *MC4R* mutation c.631-634delCTCT involved four base pairs deletion (CTCT) at nucleotide position 631-634, or at codon 211 (figure 2-1C). This mutation results in a frameshift that introduces five aberrant amino acids culminating in a premature stop codon (Met-Ser-Thr-Cys-Ser-X) in the region encoding the fifth transmembrane domain, thus leading to a truncated protein which would likely result in a non-functional receptor.

Figure 2-1A. Sequencing of the *MC4R* gene in a proband revealed A to C transition in homozygosity at nucleotide position 470 (arrow), leading to the substitution of tyrosine by serine at codon position 157.

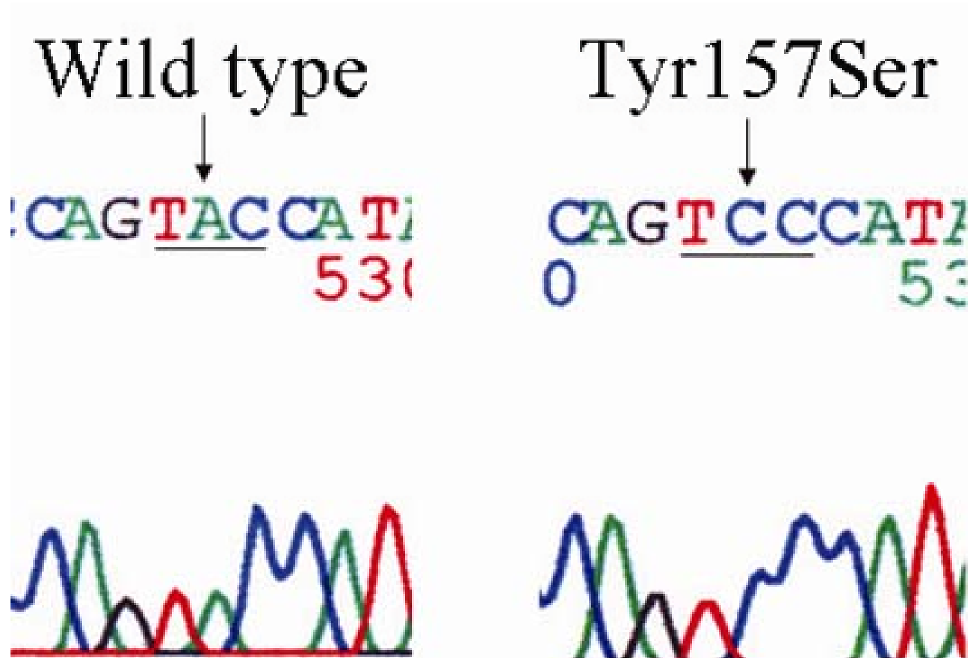
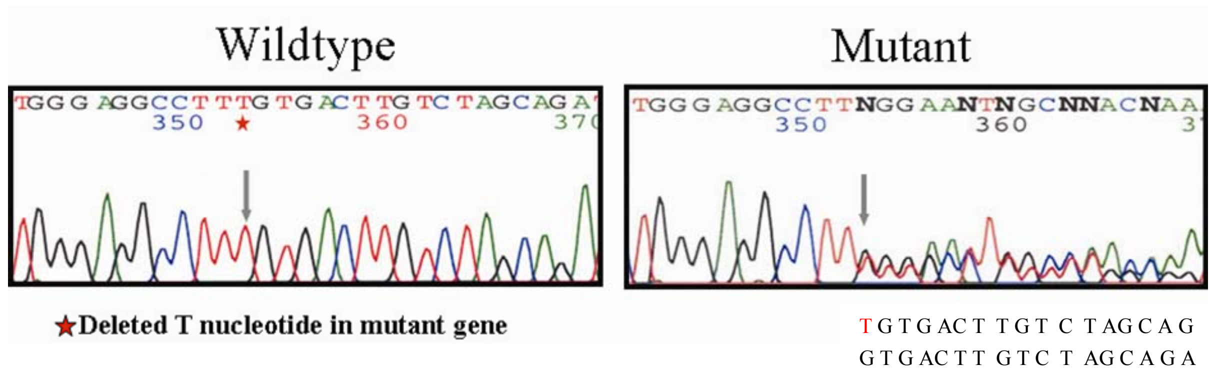


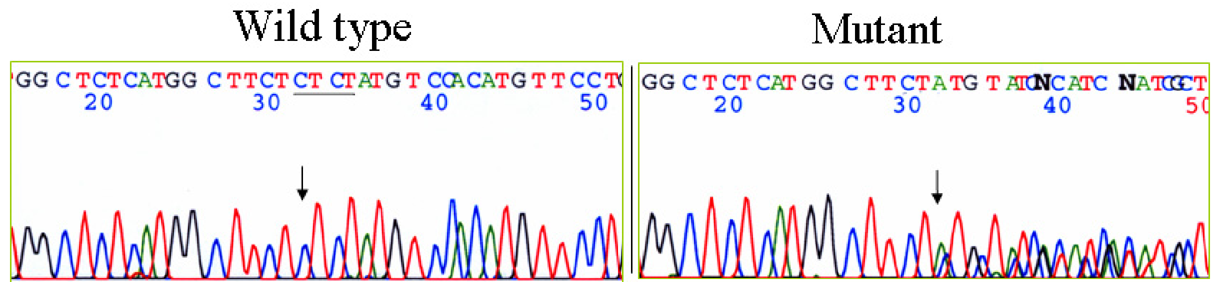
Figure 2-1B. A deletion of T nucleotide at position 976 of the coding region (marked by a star sign in wild-type *MC4R* sequence) resulted in the heterozygous frameshift mutation found in the second proband; the arrows indicate the starting point of the deletion., this single base pair deletion resulted in 28 aberrant amino acid residues from residue 326 onwards (in italics and underlined), 21 residues more than the wild-type sequence.



residue	324	325	326	327	328	329	330	331	332	333	334	335	336	337
Wild-type	Gly	Leu	Cys	Asp	Leu	Ser	Ser	Arg	Tyr	X				
mutant	Gly	Leu	***Val***	***Thr***	***Cys***	***Leu***	***Ala***	***Asp***	***Ile***	***Lys***	***Trp***	***Gly***	***Gln***	***Ser***

residue	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	
mutant	***Thr***	***Gln***	***Tyr***	***Arg***	***Asn***	***Met***	***His***	***Lys***	***Arg***	***Leu***	***Phe***	***His***	***Ser***	***Tyr***	***Pro***	***Thr***	***X***

Figure 2-1C. The *MC4R* sequence of the third proband revealed deletion of 4 base pairs at nucleotide position 631 to 634 in heterozygosity (affecting codon 211). The 4 base pairs deleted were CTCT (as underlined in the wild-type sequence), and the arrows indicate the starting point of the deletion. This frameshift mutation resulted in 5 aberrant amino acid residues from residue 211 onwards, followed by a premature termination.



residue	206	207	208	209	210	211	212	213	214	215	216	217	218	219…….
Wild-type	Ala	Leu	Met	Ala	Ser	Leu	Tyr	Val	His	Met	Phe	Leu	Met	Ala …….
mutant	Ala	Leu	Met	Ala	Ser	***Met***	***Ser***	***Thr***	***Cys***	***Ser***	***X***			

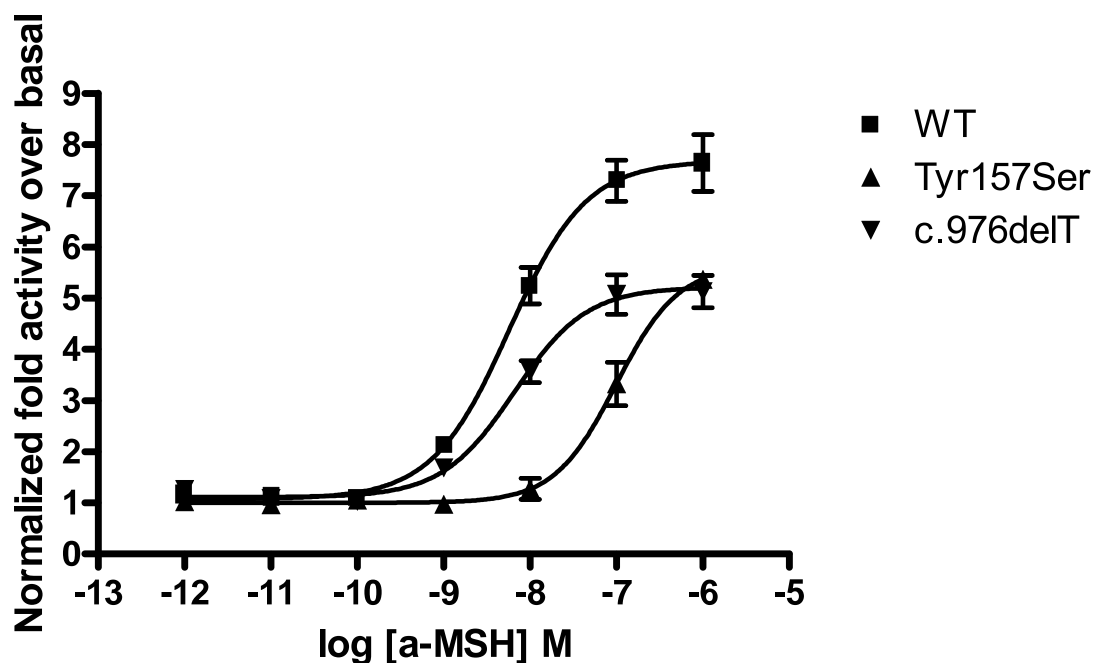
Impaired signaling properties of the two novel mutant receptors (Tyr157Ser and c.976delT)

The two mutant MC4 receptors resulting from the mutations Tyr157Ser and c.976delT demonstrated significantly reduced signaling activity compared to wild-type MC4 receptors (Figure 2-2). The c.631-634delCTCT mutation was described previously (Farooqi et al., 2000; Hinney et al., 1999; Yeo et al., 1998), and receptor function studies had shown that this mutant receptor was non-functional (Farooqi et al., 2000).

Clinical characteristics of subjects with mutations

Tyr157Ser The novel *MC4R* mutation Tyr157Ser was found in homozygosity in a 9-year-old Indian boy with consanguineous parents (first cousins) (Figure 2-3A). His parents, younger sister and younger brother were heterozygotes (Figures 2-3A and 2-4). This mutation Tyr157Ser was not found in 188 DNA samples from non-obese individuals (normal controls) (Figure 2-4A). The genotype and phenotype of the family members are described in figure 2-3A. All the heterozygous family members were obese, but the homozygous proband was more severely affected, demonstrating an additive effect. The proband homozygous for the mutation was afflicted with more obesity-related complications compared to the other heterozygous family members, with severe bilateral genu varus (Figure 2-4B), moderate obstructive sleep apnoea, higher insulin resistance and eventually diabetes mellitus. Weighing 3.25 kg at birth, the proband was progressively overweight since 6 months of age, gaining weight at about 10 kg per year, and was 80.5 kg by 6 years of age. When he presented at 8.8 years old, he weighed 92.4 kg with body mass index (BMI) of 44 kg/m^2 and percentage ideal weight for height of 231%.

Figure 2-2. Constructs with wild-type and mutant *MC4R* were co-transfected with pCRE-Luc into HEK293 cells and stimulated with varying amounts of α-MSH. Y axis represents the fold increase over basal Relative Light Units (RLU), which was the ratio of luminescence generated by Firefly luciferase (from pCRE-Luc) to Renilla luciferase (from pBudCE4/Renilla/*MC4R* construct); the data was expressed as such to normalise for transfection efficiency and amount of MC4R produced. This represented the amount of cyclic AMP generated with increasing concentration of the ligand α-MSH (represented by x-axis). Each data point represented the mean and standard error range of 3 independent experiments performed in triplicates. Compared to the wild-type, there was statistically significant reduction in signaling activity of the Tyr157Ser and c.976delT mutant MC4 receptors. Maximum fold activity: wild-type 7.677 (95% C.I. 7.117 - 8.237), Tyr157Ser 5.633 (95% C.I. 4.695 to 6.571), c.976delT 5.214 (95 % C.I. 4.824 to 5.604);
EC_{50}: wild-type 5.747 $x10^{-9}$ (95% C.I. 3.66 $x10^{-9}$ - 9.024 $x10^{-9}$), Tyr157Ser 9.954 $x10^{-8}$ (95% C.I. 6.167 $x10^{-8}$ - 1.607 $x10^{-7}$), c.976delT 6.581 $x10^{-9}$ (95% C.I. 4.077 $x10^{-9}$ - 1.062 $x10^{-8}$)



He was tall for his age with height standard deviation score (SDS) of +2.8, and elevated fasting insulin level 25 mU/L, but he did not have glucose intolerance then. His 24 hours urinary cortisol levels were normal, diurnal rhythm of his cortisol secretion was intact, and was suppressible with low dose dexamethasone suppression test. He used to swim, but had stopped as he was limited by the genu varus. His estimated caloric intake was 2379 kcal/day, with 22% from fats, 18% from proteins and 60% from carbohydrates. At 11.5 years of age, he was in early puberty (Tanner stage 2) and weighed 130 kg with BMI of 51.9 kg/m^2 and WFH 260%. At 11 years 8 months, he was diagnosed to have type 2 diabetes mellitus confirmed with two separate oral glucose tolerance tests and an elevated glycated haemoglobin level of 7.4%. His diabetes control was poor and, coupled with poor dietary control, required insulin shortly after diagnosis. Currently at 13 years old, his diabetes control continued to be poor with HbA1c levels ranging from 9% to 13.5%.

His father had high cholesterol and triglyceride levels despite lower adiposity and insulin resistance. The proband and his father were relatively inactive, and did not engage in regular physical activity. His mother had gestational diabetes during all her pregnancies, but did not develop diabetes mellitus post-partum. She was heaviest at 21 years of age weighing 107 kg, but had since lost some weight (currently 96 kg) through dieting and exercise. His father's heaviest weight recorded was 90.1 kg.

c.976delT

The proband is a Chinese boy who was born at term with birth weight of 3.1 kg, and was noted to be overweight since infancy. When he presented at 7.9 years, he was

severely obese with borderline hyperlipidaemia, but no glucose intolerance (Figure 2-3B and Table 2-1). At 12.8 years of age, his WFH increased to 189% and BMI was 37 kg/m^2. His father is heterozygous for the mutation and was obese as a child, but not as severely obese as an adult (Figure 2-3B). The mutation co-segregated with the obese phenotype in this family.

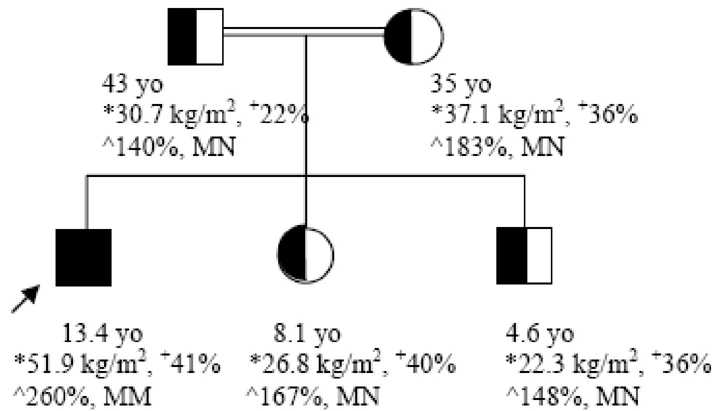
c.631-634delCTCT

An obese 16-year-old Malay female subject was heterozygous for this mutation (Table 2-1 and figure 2-3C). She weighed 73 kg with WFH 162% and %BF 44.1% when she presented with type 2 diabetes mellitus and hypercholesterolemia. She was born at term with birth weight of 2.58 kg. She was obese before 10 years of age and presented at 16 years of age with polydipsia and polyuria. Her fasting glucose was elevated at 17.2 mmol/L and HbA1c was 10.3%. Only the proband and her mother were genotyped (Figure 2-3C). Her father also had type 2 diabetes and weighed 100 kg. As her mother did not carry the mutant allele, we inferred that the mutant allele was inherited from her father. At 22 years of age, she weighed 80 kg with WFH still at 160%. Her diabetes control had been poor due to poor compliance. Her recent HbA1c was 8.3% on a combination of metformin, glipizide and repaglinide. She recently developed diabetic nephropathy with significant albuminuria (1484 mg/day) and was treated with enalapril.

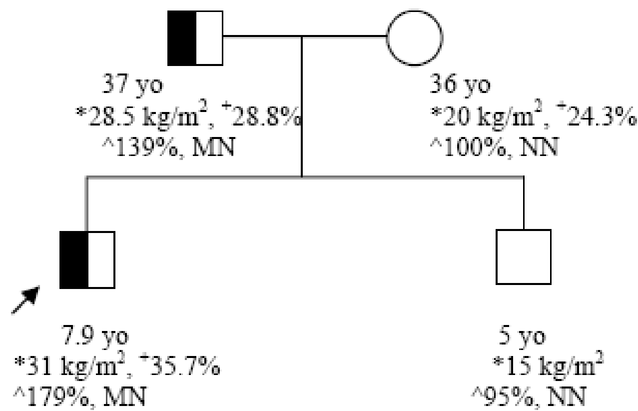
Figure 2-3. Family pedigrees with *MC4R* mutations. The age (in years), body mass index (*), percentage body fat (+), percentage ideal weight for height (^), and genotypes are listed. N represents the normal *MC4R* allele, and M represents the mutant *MC4R* allele

TCT.

A. Family with Tyr157Ser



B. Family with c.976delT



C. Family with c.631-634delCTCT

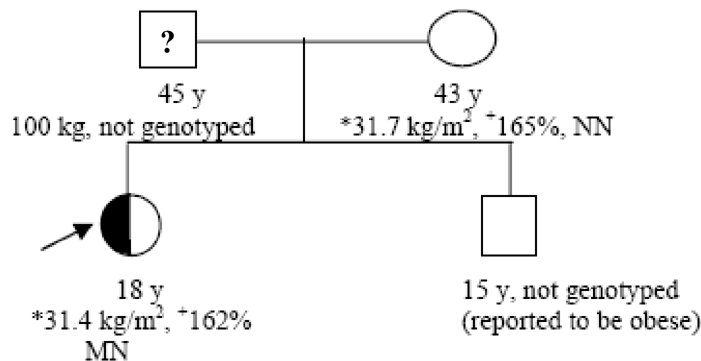
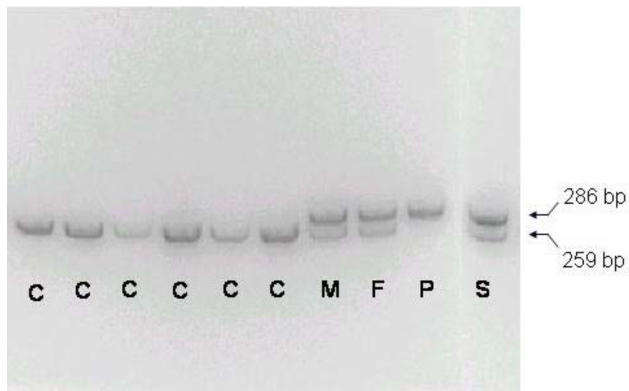


Figure 2-4 A. PCR-digest method to screen for Tyr157Ser mutation. Control samples (C) with wild-type alleles would yield 259 bp fragment, while that of the proband (P) who was homozygous for the Tyr157Ser mutation would produce 286 bp fragment; those of the family members heterozygous for the mutation would produce both fragments (F=father, M=mother, S=sister). The younger brother was subsequently genotyped by direct sequencing and confirmed to be heterozygous for this mutation. **B**. The homozygous proband (13 years old) with his younger sister (8 years old) and younger brother (4.5 years old), who were both heterozygous, demonstrating the additive effect of this mutation on the severity of obesity.

A.



B.

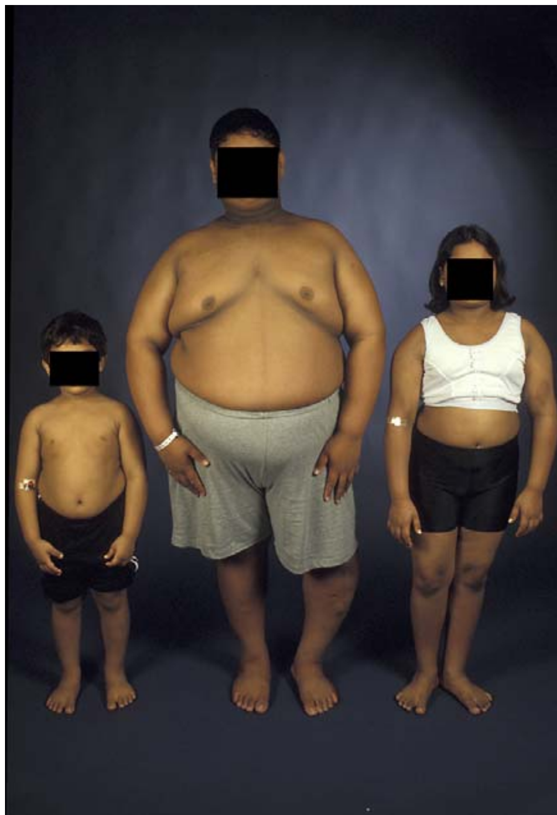


Table 2-1. The serial anthropometric measurements, insulin, C-peptide, HOMA, bone density and percentage body fat (when available) of four young *MC4R* mutation carriers.

	Obese subjects without mutation (n=224)	Proband Y157S MM		Sister Y157S MN		Proband TΔ MN		Proband CTCTΔ MN	
Age (years)	10.9 ± 3.3 (2.0-19.0)	8.8	11.5	3.5	8.1	7.9	12.8	16.3	22
BMI (kg/m^2)	32.1 ± 5.5 (22.8-53.9)	44.0	51.9	21.0	26.8	31.0	37	31.4	32
WFH (%)	170 ± 22 (128-273)	231	260	140	167	177	189	162	160
Height standard deviation score	1.44 ± 1.15 (-1.59-5.89)	2.84	1.9	1.38	1.07	2.10	1.8	-0.05	0.02
Fasting glucose (mmol/L)	4.8 ± 1.8 (3-16.3)	4.4	5.6	4.3	4.0	4.4	-	10.5	-
Fasting Insulin (mU/L)	36.6 ± 45.3 (2.5-539.0)	25.4	77.3	7.7	17.2	27.1	-	37.4	-
Fasting C-Peptide (pmol/L) (NR:298 – 1324)	1324 ± 806 (312-5602)	2296	2265	-	693	1096	-	1750	-
Insulin resistance by Homeostasis Model Assessment (HOMA)	7.67 ± 9.61 (0.57 – 110.20)	4.97	19.24	1.47	3.06	5.39	-	17.45	-
Percentage body fat by DEXA (%)	40.4 ± 5.0 (26 – 54)	48.4	41	53	40	39.0	-	40	-
Lumbar spine bone density (g/cm^2) / z score	0.77 ± 0.12 (0.2–1.36) / 0.64 ± 0.64 (-0.55–3.41)	0.71 / 1.36	0.99 / 1.63	0.56 / 2.31	0.68 / 1.2	0.61/0.20	-	-	-

Values expressed as mean ± SD (range); NR= normal range

N represents the normal *MC4R* allele, and M represents the mutant *MC4R* allele.

TΔ = c.976delT

CTCTΔ = c.631-634delCTCT

Discussion

The missense mutation Tyr157Ser is a rare variant not found in our control samples, and it resulted in substitution of tyrosine residue by serine at codon 157, which is located at the second intracytoplasmic loop of the transmembrane domain. The transmembrane domain directly interacts with G proteins and controls cyclic adenosine monophosphate (cAMP) production, and thus we postulate that this mutation may interfere with G-protein interaction, leading to reduced cAMP generation. Tyrosine at codon 157 is a highly conserved residue present in the MC4R sequence of many other species, from teleost fish through to mammals (accession numbers: Zebrafish NP_775385, Chicken NP_001026685, Mouse NP_058673, Rat NP_037231, Human NP_005903). The c.976delT mutation resulted in an abnormal MC4R protein with aberrant amino acid residues from codon 326, located in the intracellular domain. The c.631-634delCTCT mutation led to a frameshift that introduces five aberrant amino acids culminating in a premature stop codon in the region encoding the fifth transmembrane domain, thus resulting in a truncated protein, if the mRNA is stable enough for translation in vivo. These three mutations resulted in mutant MC4 receptor proteins which had reduced signaling activity *in vitro*. The pathogenic role of these mutations are further supported by the family studies which demonstrated cosegregation with the obese phenotype, and also an additive effect whereby the homozygosity of the Tyr157Ser mutation resulted in a more severe obese phenotype compared to the heterozygotes. We recognized that more family members should be studied to draw firm conclusions about the mode of inheritance, but unfortunately, we were not successful in recruiting them for extended family studies.

The prevalence of pathogenic *MC4R* mutations reported in various obese populations varied widely, ranging from 0.5 to 5.8% (Farooqi et al., 2003; Hinney et al., 2003; Jacobson et al., 2002; Larsen et al., 2005; Miraglia Del Giudice et al., 2002; Ohshiro et al., 1999; Rong et al., 2006; Vaisse et al., 2000; Wang et al., 2006). One possible explanation is differences within the study cohort in terms of ethnicity, age of onset, and severity of obesity. A British study of about 500 subjects with early onset severe obesity reported the highest prevalence of 5.8% (Farooqi et al., 2003), while a French study of adults with severe obesity where a third were obese since childhood, yielded a prevalence of 4% (Vaisse et al., 2000). However, a German cohort of extremely obese children found only 1.9% (Hinney et al., 2003), and an Italian study of 208 children with severe early onset obesity found only one patient with a pathogenic *MC4R* mutation (<0.5%) (Miraglia Del Giudice et al., 2002). The significance of *MC4R* mutations in Asian obese populations has not been adequately examined. A small Japanese study of 50 obese adults revealed no pathogenic mutations (Ohshiro et al., 1999), while two studies involving Chinese adult and paediatric subjects identified a few mutations of uncertain significance in less than 1.5% of the cohort (Rong et al., 2006; Wang et al., 2006). Despite recruiting only children and adolescents with early onset severe obesity similar to those of other studies (Farooqi et al., 2003; Vaisse et al., 2000), our study found only three mutations in three subjects (1.3%). Taken together with the results of other studies, it is unlikely that the higher prevalence reported in some cohort studies were due to selection of study subjects with early onset severe obesity, but rather that *MC4R* mutations are truly more prevalent in northern European obese populations (Farooqi et al.,

2003; Vaisse et al., 2000). We recognized that the ethnic heterogeneity of our study population would be inadequate to estimate the true frequency of *MC4R* mutations for the various ethnic groups (Malays and Indians especially) and limit the comparision to other studies of homogeneous populations. Nevertheless, our study gave an estimate of the low prevalence of *MC4R* mutations in this part of the world. Our Chinese subgroup was fairly large (n=116), and the low prevalence (0.9%) was consistent with that reported in other studies of Chinese subjects (Rong et al., 2006; Wang et al., 2006).

Though there were a few common mutations described across populations, such as the c.631-634delCTCT mutation in our Malay subject which was previously reported in English and German Caucasian subjects (Hinney et al., 1999; Yeo et al., 1998), a significant number of novel and 'exclusive' or 'private' mutations were reported in different obese populations from various parts of the world, and these mutations were not frequent even within these populations (Farooqi et al., 2003; Hinney et al., 2003; Hinney et al., 1999; Jacobson et al., 2002; Larsen et al., 2005; Miraglia Del Giudice et al., 2002; Ohshiro et al., 1999; Rong et al., 2006; Vaisse et al., 2000; Wang et al., 2006). One possible reason for this relative lack of founder effect is that the pathogenicity of *MC4R* mutations in obesity may be a fairly recent event in human evolution.

Our family studies revealed variable phenotype and it appeared that the children and younger subjects were more severely affected compared to the adults / parents. At least two large studies have shown incomplete penetrance and variable expressivity in their cohort of *MC4R* mutation carriers (Hinney et al., 2006; Vaisse et al., 2000). Heterozygous

mc4r$^{+/-}$ mice also display a broad variability in phenotype with an adult weight ranging from that of wild-type to that of homozygous *mc4r*$^{-/-}$ mice (Huszar et al., 1997). This is not surprising, considering that the obese phenotype is also dependent on the environment, behaviour, and other modifier genes. The current environment may be more 'obesogenic' compared to that of the previous generation, and exposure to disease modifying factors such as the environment at critical stages of childhood may determine the eventual phenotype. A second possible reason is genetic anticipation, where the next generation is more affected. Another possibility is amelioration of phenotype as the young mutation carriers become adults. Young British subjects with *MC4R* mutations were described to have early onset hyperphagic obesity, higher lean body mass, increased linear growth with higher height SDS scores, hyperinsulinaemia and higher bone mineral density compared to matched obese controls, and amelioration of hyperphagia and degree of hyperinsulinaemia observed in adulthood (Farooqi et al., 2003). Our follow up observation of several young *MC4R* mutation carriers revealed increasing WFH during childhood and the early pubertal years serially, and perhaps even insulin levels and insulin resistance indices were rising, but their percentage body fat, and height standard deviation scores were decreasing (Table 2-1). The trend in bone mineral density changes was not discernible. Our young subjects with heterozygous *MC4R* mutations did not demonstrate a clear increase in insulin resistance compared to obese matched controls, except for proband with c.631-634delCTCT who presented with glucose intolerance (table 2-2) (unable to find matched controls of proband homozygous for Tyr157Ser). Unfortunately, the small number of mutation carriers in our cohort precluded any meaningful interpretation. Large studies such as those in the UK, France and Germany with high numbers of *MC4R* mutation carriers will be able to provide

valuable insights on the natural history of the *MC4R* mutant phenotype with follow up studies of their young subjects as they mature into adulthood.

Table 2-2. HOMA, percentage body fat, and BMI of three subjects heterozygous for *MC4R* mutations compared to obese controls matched for age (± 1.5 years), gender, ethnicity, and WFH (± 10%).

	Sister Y157S MN	Obese Controls* (n=4)	Proband TΔ MN	Obese Controls* (n=5)	Proband CTCTΔ MN	Obese Controls* (n=3)
Age (years)	8.1	8.4 (0.8)	7.9	8.7 (0.5)	16.3	16.3 (0.5)
BMI (kg/m^2)	26.8	27.4 (2.4)	31.0	31.3 (0.8)	31.4	33.4 (2.4)
WFH (%)	167	170 (4)	177	175 (4)	162	159 (8)
Insulin resistance by Homeostasis Model Assessment (HOMA) (NR <2)	3.06	8.97 (4.5)	5.39	5.06 (0.71)	17.45	6.85 (4.4)
Percentage body fat by DEXA (%)	40	47.2 (1.4)	39.0	41.6 (1.2)	40.0	39.1 (1.4)

Values expressed as mean (SEM)

N represents the normal *MC4R* allele, and M represents the mutant *MC4R* allele.

TΔ = c.976delT

CTCTΔ = c.631-634delCTCT

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Chapter 3

A POMC variant implicates β-melanocyte-stimulating hormone in the control of human energy balance

Summary

The MC4R plays a critical role in the control of energy balance. Of its two pro-POMC-derived ligands, α- and β-MSH, the majority of attention has focused on α-MSH, partly reflecting the absence of β-MSH in rodents. We screened the POMC gene in 538 patients with severe, early-onset obesity and identified five unrelated probands who were heterozygous for a rare missense variant in the region encoding β-MSH, Tyr221Cys. This frequency was significantly increased ($p < 0.001$) compared to the general UK Caucasian population and the variant cosegregated with obesity/overweight in affected family members. Compared to wild-type β-MSH, the variant peptide was impaired in its ability to bind to and activate signaling from the MC4R. Obese children carrying the Tyr221Cys variant were hyperphagic and showed increased linear growth, both of which are features of MC4R deficiency. These studies support a role for β-MSH in the control of human energy homeostasis.

Introduction

The pro-opiomelanocortin (*POMC*) gene is expressed in neurons originating in the arcuate nucleus of the hypothalamus and in the nucleus tractus solitarius (NTS) of the caudal medulla, as well as in the corticotrophs of the anterior pituitary and in the skin and lymphoid system (Hadley and Haskell-Luevano, 1999). The human *POMC* gene is

located on chromosome 2p23.3 and spans 7665 kb. It comprises of 3 exons, and though all three exons are transcribed, only part of the mRNA is translated. Exon 1 only contains untranslated sequences, part of exon 2 codes for signaling peptide and the first few amino acid residues of the N-terminal peptide (NT), and exon 3 codes for most of the translated mRNA, namely the C-terminal of NT, joining peptide (JP), adrenocorticotropic hormone (ACTH) and β-lipotropic hormone (β-LPH) (figure 3-1). The signaling peptide is necessary for the translocation of the nascent protein through the membrane of the rough endoplasmic reticulcum (RER), after which it is rapidly cleaved. The POMC protein (accession no. NP_000930) is then engaged in the secretory pathway and ready for processing by the prohormone convertases 1 & 2 (PC1 & PC2). POMC undergoes extensive and tissue-specific posttranslational processing by proprotein convertases (PCs) to yield a range of biologically active peptides (Pritchard et al., 2002; Raffin-Sanson et al., 2003; Seidah and Chretien, 1999).

Pituitary corticotrophs express prohormone convertase 1 (PC1), but not PC2, which cleaves 4 specific dibasic Lys-Arg cleavage sites and results in the production of NT, JP, ACTH, and β-LPH. In contrast, the expression of PC2 (in addition to PC1) within the hypothalamus results in additional processing and cleavage of all dibasic Lys-Arg and Arg-Arg sites of the precursor polypeptide, leading to the production of α-, β-, and γ-MSH (the melanocortins) but not ACTH. The melanocortins mediate their effects through a family of five related G protein-coupled receptors, two of which, MC3R and MC4R, are highly expressed within the central nervous system (Gantz and Fong, 2003).

Genetic defects impairing the synthesis and processing of POMC and in the receptors for its constituent melanocortin peptides have clearly established that the melanocortin system plays a critical role in energy homeostasis in rodents and humans (Cone, 2005).

POMC mutations have helped to shed light on the role of the melanocortin system in energy homeostasis (Coll et al., 2004). Mice (Challis et al., 2004; Yaswen et al., 1999) and humans (Farooqi et al., 2006; Krude et al., 1998; Krude et al., 2003) genetically lacking POMC-derived peptides are severely obese, and were associated with severe glucocorticoid deficiency due to concomitant absence of ACTH. Importantly, heterozygous null POMC mutations in mice (Challis et al., 2004) and humans (Krude et al., 2003) predispose to obesity but do not necessarily result in a severe or intermediate phenotype, and do not exhibit cortisol deficiency. The heterozygous *Pomc*$^{+/-}$ mice had similar phenotype to wildtype mice on standard chow (Challis et al., 2004; Yaswen et al., 1999), but developed obesity when put on a high-fat diet (Challis et al., 2004), exhibiting an intermediate obese phenotype between wild-type and *Pomc*$^{-/-}$ mice. Heterozygous parents of obese children homozygous for *POMC* null mutations had high normal to high BMI (Krude et al., 2003), and other family members heterozygous for *POMC* null allele were more overweight than those with wildtype alleles (Farooqi et al., 2006). Heterozygous partially inactivating *POMC* mutations Arg236Gly were also found more frequently in obese children (Challis et al., 2002). Thus it can be inferred that a single functional copy of the *POMC* gene may not be sufficient for maintaining normal energy

homeostasis under certain conditions such as in an "obesogenic" environment, and haploinsufficiency can interact with dietary factors to increase body weight.

There is still uncertainty regarding the relative importance of particular POMC-derived melanocortin ligands in the control of energy balance. *In vitro* studies established that α- and β-MSH both bind to the MC4R with high affinity and with similar IC_{50} values, while γ-MSH preferentially binds to MC3R with a 50-fold higher affinity than to MC4R, which it binds to poorly (Abbott et al., 2000). Some studies showed that β-MSH has higher affinity for MC4R in stably expressing cell lines (Schioth et al., 1996) and in rat hypothalamic homogenates (Harrold et al., 2003). Intracerebroventricular (icv) administration of β-MSH in rats induced early growth response factor-1 expression in hypothalamic nuclei (Millington et al., 2001). Several in vivo studies have demonstrated that rodents given a single icv injection of α-MSH display a dose-dependent reduction in food intake (Poggioli et al., 1986). In direct comparisons of icv administration of melanocortin peptides, β-MSH has been shown to reduce food intake at equimolar doses (Abbott et al., 2000). Despite this, attention has been principally focused on α-MSH as the probable endogenous ligand in rodents (Mountjoy and Wong, 1997), and presumed to be the de facto ligand in the human melanocortin system as well. This is largely because rodents (the most common experimental species) lack the proximal dibasic site that is necessary for the proteolytic cleavage event that produces β-MSH in humans (mouse accession number P01193, rat accession number AAA41903). Thus the role for β-MSH in the control of energy balance has been largely overlooked.

Common obesity is a polygenic/multifactorial trait, and genetic variations affecting molecules of the melanocortin pathway may be contributory genetic factors. The region of human chromosome 2 containing the POMC gene has also been identified by several studies as a susceptibility locus for common human obesity (Comuzzie et al., 1997; Hager et al., 1998; Hixson et al., 1999). In order to determine whether mutations within the melanocortin peptides might predispose to obesity, we screened the coding regions of the POMC gene for mutations in 538 UK Caucasian subjects with severe early-onset obesity in the absence of obvious glucocorticoid deficiency.

Methods

Cohorts and Human Genetic Studies

538 UK Caucasian subjects with severe early-onset obesity were screened using a combination of direct nucleotide sequencing and denaturing high-performance liquid chromatography (Transgenomic WAVE DNA fragment analysis). Subjects were recruited as part of the Genetics of Obesity Study (GOOS) cohort (O'Rahilly et al., 2003), and mutations in leptin, leptin receptor, and *MC4R* genes had been excluded by direct nucleotide sequencing. These 538 subjects were selected from more than 960 subjects initially screened based on ethnicity, such that only Caucasian subjects were used for this study. The mean BMI SDS of this group was 3.8 ± 0.9, mean age of onset < 5 years. BMI SDS was calculated using age- and gender-specific UK population data (Cole et al., 1995). All studies were approved by the local ethics committee and were conducted in

accordance with Declaration of Helsinki principles. Each subject, or the parent in the case of children below 16 years of age, provided written informed consent. Subjects with mutations of the *POMC* gene and their relatives were invited to the Wellcome Trust Clinical Research Facility at the Addenbrooke's Hospital, Cambridge, United Kingdom, for further assessment.

Three hundred randomly selected non-obese adult controls from the MRC Ely Study (Wareham et al., 2000) were screened using the same techniques to compare the allelic frequency and facilitate interpretation of the potential pathogenicity of the mutations found.

Detection of mutations and genotyping

Genomic DNA was isolated from whole blood lymphocytes. The coding region of *POMC* gene, located in part of exon 2 and in exon 3, is screened by a combination of denaturing high-performance liquid chromatography (DHPLC) (Transgenomic WAVE^{TM} DNA fragment analysis system, Nebraska, USA) and direct sequencing. Exon 2 is analysed by DHPLC following PCR (c.-164T to c.132+55G). Exon 3 is analysed as three overlapping segments, where the first segment (5' end to c.348G) is analysed by direct sequencing following amplification of the whole exon. The middle (c.303G to c.685G) and third (c.535G to c.804+56T) (3' end) segments of exon 3 were amplified by PCR and analysed by DHPLC. The first segment was analysed by direct sequencing in view of the increased number of genetic variants in that region as reported in the literature. All

abnormal waveforms detected by DHPLC are re-analysed by PCR and direct sequencing to identify the genetic variants responsible. Sequencing was performed using BigDye® v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, Carlifornia, USA) and analysed on the ABI 3100 Avant Genetic Analyser. Sequences were assembled and examined using Sequencher software (Gene Codes, Ann Arbor, Michigan, USA). Prior to the mutation screening proper, a preliminary screen of *POMC* gene of 100 DNA samples by direct sequencing found several variants, and the samples with these variants were subjected to our DHPLC method which yielded abnormal waveforms, and this support our mutation detection method.

The PCR was performed using BioTaq (Bioline, London, United Kingdom), or AmpliTaq Gold (Applied Biosystems, Carlifornia, USA) with proofreading *Pfu* DNA polymerase (Promega) for PCR products for DHPLC (ratio 9:1). Primers for exon 2 PCR: forward 5'-TTC TCA GCA TTG CTG CTG TCC-3', reverse 5'-CCA GCT CCA GTC CCA TCT AAT-3'; PCR conditions: 1 mM $MgCl_2$ (final concentration), 95^0C 10 min (hot start), followed by 35 cycles of 95^oC x 30 s, 63.9^oC x 1 min, 72^oC x 30 s, then followed by 72^oC x 7 min final extension. Primers for exon 3 PCR: forward 5'-CCT AGG CGC AGT GAC GGG CG-3', reverse 5'-AGC AGG GCA GGG GAG AGC AAG-3'; PCR conditions: 1 mM $MgCl_2$, 95^0C 2 min, followed by 35 cycles of 95^oC x 30 s, 69.5^oC x 1 min, 72^oC x 1 min, then followed by 72^oC x 7 min final extension. Primers for amplification of middle segment of exon 3: forward 5'-GCA GAA GCG CGA GGA CGT CTC-3', reverse 5'-CCC AGC GGA AGT GCT CCA TCC-3'; PCR conditions: 1.5

mM $MgCl_2$, 95^0C 10 min (hot start), followed by 35 cycles of 95^oC x 30 s, 66.8^oC x 1 min, 72^oC x 30 s, then followed by 72^oC x 7 min final extension. Primers for amplification of third segment of exon 3: forward 5'-GAG CTG ACT GGC CAG CGA CT-3', reverse 5'-AGG GGC TTT GGG GTC GAC CTC-3'; PCR conditions: 1 mM $MgCl_2$ (final concentration), 95^0C 10 min (hot start), followed by 35 cycles of 95^oC x 30 s, 63.4^oC x 1 min, 72^oC x 30 s, then followed by 72^oC x 7 min final extension. Sequencing primers for exon 2: forward 5'-TGC TGC TGT CCT TAA AAT GCC-3', reverse 5'-CCA GCT CCA GTC CCA TCT AAT-3'. Forward sequencing primers for exon 3: 5'-AGG CGC AGT GAC GGG CGC GGC-3', 5'-TCT CAG CGG GCG AAG ACT GC-3', 5'-GAG CTG ACT GGC CAG CGA CT-3'. Reverse sequencing primers for exon 3: 5'-AGT AGG AGC GCT TGC CCT CG-3', 5'-CTG TAG GGG CCC TCG TCC TTC-3', 5'-TTT GGG GTC GAC CTC CTG GG-3'.

Denaturing high-performance liquid chromatography (DHPLC) analysis: Unpurified PCR products at 3:1 ratio with a wild-type reference before subjected to a 3 min, 95 °C denaturing step followed by gradual reannealing from 95–65 °C over 30 min. 5 µL of each mixture was loaded onto a DNASep column (Transgenomic, Omaha, Neb., USA) and the amplicons were eluted in 0.1 M triethylammonium acetate, pH 7, with a linear acetonitrile gradient at a flow rate of 0.9 ml/min[2]. Heteroduplex mismatches were recognized by the appearance of aberrant patterns in the elution profiles under appropriate temperature conditions, which were calculated by the WAVEMaker software

ver 3.4.4 of the Wave Nucleic Acid Fragment Analysis system HSM device (Transgenomic, Omaha, USA).

Genotyping of the Tyr221Cys variant was performed in 4849 subjects from a UK Caucasian population-based cohort, the EPIC-Norfolk study (mean [SD] BMI 25.9 [3.7] kg/m^2, age 57 [9.3] years) (Khaw et al., 2001). This was performed by our collaborators Dr Matthew Sims, Dr Manj Sandhu, and Dr Nicholas J Wareham from the MRC Epidemiology Unit, Cambridge, United Kingdom. 4846 of the control subjects (55% women) did not carry this *POMC* variant, and only 3 (all women) were heterozygous for this variant. These subjects were screened using a custom-made assay based on TaqMan chemistry (Applied Biosystems, Warrington, United Kingdom). The assay conditions were 95 ^{o}C for 10 mins, then 40 cycles of 92 ^{o}C for 15sec, and 54 ^{o}C for 1 min on 10ng primer-extension preamplified DNA. The probe and primer information: Probe 1 Tm 65.4 ^{o}C, GC 76.9%, Length 13 bp, VIC fluorescence, AGGGCCCCTgCAG; Probe 2 Tm 66 ^{o}C, GC 71.4%, Length 14 bp, FAM fluorescence, AGGGCCCCTaCAGG. Left primer CCGACCTGGAGCACAGC, right primer TTCTCGGAGGTCATGAAACC.

Genotyping of the Tyr221Cys variant was also performed in two groups of French obese subjects: 597 morbidly obese adults (BMI 47.5 ±7.53), 535 obese children (BMI sds = 4.37 ± 1.31) and also in 723 lean controls (BMI 23.29 ± 2.74). These subjects were genotyped using a FRET (Fluorescence Resonance Energy Transfer) based assay using

the LightTyper technology (Roche, Meylan, France and Mannheim, Germany) as previously reported (Meyre et al., 2005). Probes for LightTyper were synthesized by TIB Molbiol Syntheselabor Germany. This was performed by Dr Vincent Vatin, Dr David Meyre, and Prof Philippe Froguel from the Institute of Biology, Pasteur Institute, Lille, France and Section of Genomic Medicine and Genome Centre, Imperial College London, UK.

Nuclear Magnetic Resonance (NMR) studies

The NMR studies were performed by Darren A. Thompson, Michael E. Madonna, and Glenn L. Millhauser from the Department of Chemistry and Biochemistry, University of California, Santa Cruz, California. Two-dimensional ^{1}H NMR (2D-NOESY and 2D-TOCSY) performed on peptides corresponding to the β-MSH segment of POMC was used to assign chemical shifts in both wild-type and a mutant with a Tyr221Ser mutation. Ser was used instead of Cys since, at NMR concentrations, free -SH groups tend to form disulfides. For flexible peptides, a negative CSI indicates a turn or helical structure and a positive CSI indicates the presence of extended, β sheet conformers (Mielke and Krishnan, 2004; Wishart et al., 1992).

Receptor activation studies

The mutant [Gln^{6}] α-MSH and [Cys^{5}] ß-MSH peptides were synthesized (Bachem, St Helens, Merseyside, UK) and determined to be >96% pure by HPLC. Mass spectrometry confirmed the composition of the peptides. Wild-type human α-MSH (Bachem) and β-

MSH (Bachem) were >98% pure by HPLC. Wild-type and mutant α-MSHs were supplied as trifluoroacetate salts and N-acetylated. Appropriate dilutions of wild-type α-MSH and β-MSH and mutant peptides were prepared in their respective diluents as recommended by manufacturer. Wild type α-MSH and β-MSH were dissolved in 0.05% acetic acid, mutant [Gln^6] α-MSH in water, and mutant [Cys^5] ß-MSH in 0.1% trifluoroacetic acid to maintain low pH and reduce dimerisation risk. Wild-type *MC4R* was amplified directly from control DNA using primers MC4Rforward (5'-AATAACTGAGACGACTCCCTGAC-3') and MC4Rrev*Eco* (5'-CGCTTAAGTTAATATCTGCTAGACAAGTCAC-3'). MC4Rforward flanks an endogenous *Eco*RI site, whereas MC4Rrev*Eco* contains a terminal *Eco*RI restriction site, thus facilitating cloning of the PCR products into the mammalian expression vector pcDNA3 (Invitrogen Corp., San Diego, California, USA). PCR was performed using Expand DNA polymerase (Roche Diagnostics Corp., Indianapolis, Indiana, USA), according to manufacturer's protocols. Cloned product was then sequenced.

HEK293 cells were transiently transfected with wildtype MC4R and cotransfected with a cAMP-dependent luciferase reporter construct. cAMP/Luciferase reporter assays were performed as described previously (Yeo et al., 2003). HEK293 cells were maintained in DMEM (Life Technologies, Rockville, Maryland, USA) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL Fungizone (Life Technologies). Cells were incubated at 37°C in humidified air containing 5% CO_2, and transfections were carried out using Fugene reagent (Roche

Diagnostics Corp) according to manufacturer's protocols. Cells were generally at 70–80% confluence on the day of transfection. The cAMP responsive luciferase construct (pLUC) was used here as described previously (Persani et al., 1993): 300 ng of wild-type *MC4R* expression vector was transfected with 60 ng pLUC reporter construct and 20 ng of internal control plasmid, pRL-CMV (Promega Corp., Madison, Wisconsin, USA), which constitutively expresses *Renilla* luciferase. After transfection, cells were serum starved for 8 hours. Varying concentrations of wild-type and mutant MSH peptides were then added to the cells and allowed to incubate for 16 hours. The cells were lysed, and the two different luciferases were sequentially activated and quenched using the Dual-Luciferase Reporter Assay System (Promega) and the resulting luminescence detected using an AutoLumat LB953 luminometer (EG&G Berthold, Baqd Wildbad, Germany) according to manufacturers' protocols. Mock transfection of HEK293 cells using pCDNA3 vectors without *MC4R* inserts were performed and used as controls.

Competitive binding studies

Whole HEK293 cells stably expressing wild-type MC4R were exposed to tracer amounts of [^{125}I]NDP-MSH, and the ability of increasing concentrations of β-MSH or [Cys^5] β-MSH to inhibit radioligand binding was measured as described previously (Yeo et al., 2003). All wild-type and mutant peptides were obtained from Bachem as described previously. *MC4R* inserts with sticky *Eco*RI ends were cut from the pCDNA3/MC4R construct described above, and cloned into pIRESpuro3 vector (BD Bioscience Clontech, NJ, USA), which confer resistance to puromycin. Transfection was carried out using Fugene reagent (Roche Diagnostics Corp) according to manufacturer's protocols, and the

transfected HEK293 cells were then selected with puromycin at 3 mcg/ml of medium over four weeks. Stable expression of MC4R by the cells were confirmed with reverse transcription PCR, and a dose response in cAMP production to increasing concentration of stimulatory wild-type α-MSH as described above. Cells were harvested washed once in binding buffer [minimal essential salts, 25 mM HEPES pH 7.0 (Invitrogen Life technologies), 0.2% BSA] and distributed in 96-well plates. Appropriate dilutions of α-MSH, β-MSH, and mutant peptides were prepared in binding buffer. Cells were incubated at room temperature for 2 h with 0.05 ml of binding buffer containing 0.0037 Mbq of [^{125}1]NDP-MSH ([Nle^4, D-Phe^7]-α-melanocyte stimulating hormone (Amersham, UK) and the appropriate concentration of study peptide. Non-specific binding was determined in the presence of 10 µM α -MSH. The cells were then washed once with 0.1 ml ice cold binding buffer, once with 0.1 ml ice cold PBS, and resuspended in 0.1 ml Microscint 20 (Perkin Elmer / Packard). Radioactivity was counted using a Packard Topcount Microplate Scintillation Counter according to the manufacturer's instructions.

Physiological studies

Measurements of food intake and body composition were undertaken after appropriate informed consent and using protocols previously described (Farooqi et al., 2003). Height was measured to the nearest 0.5 cm using a portable free-standing stadiometer (Leicester Height Measure, London, United Kingdom). Weight was measured in the fasting state on a digital electronic balance accurate to the nearest 100 g (Fereday & Sons 824/890, London, United Kingdom). Body composition was measured using whole-body dual-energy x-ray absorptiometry (DXA) to determine bone mineralization, total lean mass,

and total fat mass (DPX software; Lunar Corp., Madison, Wisconsin, USA). A semiquantitative assessment of ingestive behavior was undertaken in children under the age of 18 years. An ad libitum meal of fixed size and content (18 MJ) was provided at breakfast after an overnight fast. The contents were covertly weighed before and after consumption and total energy intake and nutrient composition calculated using standard tables (Farooqi et al., 2003). In addition, energy intake was expressed per kilogram of lean body weight as a means of comparing intake between subjects of different age and body size.

Data analysis

Graph fit curves and EC_{50}/IC_{50} were obtained and performed using GraphPad Prism 4 for Windows (ver 4.02 GraphPad software, San Diego, California, USA), and curves were fitted using the logistical equation. For transient tranfection studies, data is expressed as fold induction of luciferase activity, and each point represents the mean and standard error range of 10 independent experiments performed in quadruplicate. For ligand binding studies, data is expressed as a percentage of the maximum counts of [^{125}I]NDP-MSH binding to wild-type MC4R for each respective ligand. Each point represents the mean and standard error range of four independent experiments in triplicates.

Results

Identification of missense mutations in POMC

We identified a number of sequence variants in POMC in severely obese children (Table 3-1). In addition to a number of synonymous SNPs and a common 9 bp insertion that has

been reported previously (Challis et al., 2002; Echwald et al., 1999; Miraglia del Giudice et al., 2001), we identified seven rare nonsynonymous SNPs. Three of these missense mutations directly affect regions of the POMC gene that encode melanocortin peptides (Figure 3-1). Arg236Gly was identified in three patients but also two controls. This mutation disrupts a di-basic cleavage site between β-MSH and β-endorphin (β-end), resulting in a β-MSH/ β-endorphin fusion protein that binds to MC4R but has reduced ability to activate the receptor (Challis et al., 2002). Previous studies showed that this mutation was present in both obese subjects and controls but not highly prevalent, and while it is not a highly penetrant cause of inherited obesity, this mutation may increase the risk of obesity (Challis et al., 2002; Echwald et al., 1999). Novel heterozygous missense mutations were found in the *POMC* region which codes for β-MSH (Tyr221Cys, five subjects) and α-MSH (His143Gln, one subject). Other mutations were rare and/or occurred in regions of the POMC pro-peptide of uncertain function. Our further studies concentrated on the α- and β-MSH variants.

The Novel mutation Tyr221Cys is linked with obesity or overweight status

A novel heterozygous missense mutation Tyr221Cys in β-MSH coding region was found in five unrelated UK Caucasian probands (Table 3-1) (figure 3-2). This variant was also found in 1/300 non-obese UK Caucasian adults. To establish the frequency of this allele in an unselected UK Caucasian population, we genotyped a random subset of 5000 subjects recruited to the EPIC-Norfolk study. 4,852 subjects were successfully genotyped, and we identified 3 heterozygotes, all of whom were non-obese. Thus, Tyr221Cys is

found at significantly higher frequency in a population of UK Caucasians subjects with early-onset obesity than in unselected UK Caucasian controls (5/538 versus 4/5152, Chi-Square 22.4, $p < 0.001$). i.e. Tyr221Cys in β-MSH is more common in obese subjects than in controls. The Tyr221Cys variant was not found in 1132 obese French Caucasian subjects (597 morbidly obese adults [BMI 47.5 ± 7.53] and 535 obese children [BMI sds = 4.37 ± 1.31]), nor in 723 lean French Caucasian subjects (BMI 23.29 ± 2.74). These observations suggest that Tyr221Cys is likely to be a rare variant in Caucasian populations.

Table 3-1. Identification of variants in *POMC*. Synonymous and nonsynonymous variants in severely obese children and controls.

POMC variants	Obese children (n = 538)	Controls (n = 300)
Nonsynonymous SNPs & insertion		
Leu37Phe	1	0
9 bp insertion (c.297-298insAGCAGCGGC)	51 (9.48%)	28 (9.33%)
Pro132Ala	1	0
His143Gln	1	1 (0.33%)
Ala195Thr	1	0
Glu214Gly	8 (1.49%)	2 (0.67%)
Tyr221Cys	5 (0.93%)	1 (0.33%)
Arg236Gly	3 (0.56%)	2 (0.67%)
Synonymous SNPs		
Cys6Cys	5	0
Ser94Ser	4	0
Ala195Ala	4 (0.74%)	1 (0.33%)
Leu116Leu	8 (1.49%)	7 (2.33%)

Figure 3-1. Structure of POMC and location of rare missense mutations.

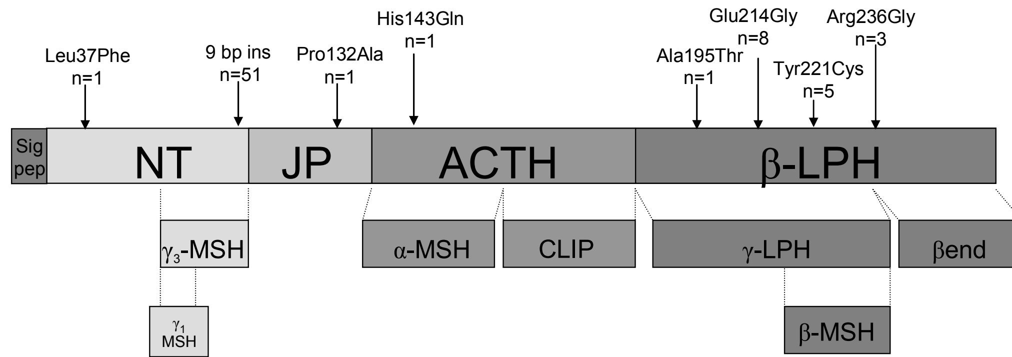


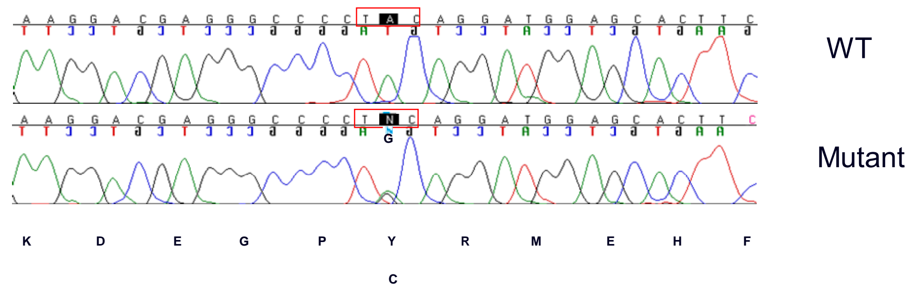
Figure 3-2. A heterozygous missense mutation due to a single base substitution from A to G (highlighted and affected codon boxed), resulting in change of the 5th residue of β-MSH from tyrosine to cysteine (underlined).

WT

H-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-**His-Phe-Arg-Trp**-Gly-Ser-Pro-Pro-Lys-Asp-OH

H-Asp-Glu-Gly-Pro-Cys-Arg-Met-Glu-**His-Phe-Arg-Trp**-Gly-Ser-Pro-Pro-Lys-Asp-OH

Mutant [Cys⁵] ß-MSH



To examine whether this variant cosegregates with obesity/overweight in families, first-degree relatives were contacted, and those who agreed were examined and genotyped (Figure 3-3). Thirteen relatives were heterozygotes for Tyr221Cys, 11 of which were obese, two overweight, and none lean. Five relatives were wild-type, three of which were obese and two lean. Thus, in total, in these families, 0/20 Tyr221Cys carriers and 2/5 wild-type subjects were lean.

Tyr221Cys β-MSH mutation alters three-dimensional structure of β-MSH

Tyr221 is a highly conserved residue, being present in the β-MSH sequence of species from teleost fish through to mammals (accession numbers: Zebrafish NP_852103, Chicken BAA34366, Mouse P01193, Rat AAA41903, Human P01189) (figure 3-4). A tyrosine is also present in the equivalent position in all melanocortins from all mammalian species (figure 3-5), i.e. located 4 residues upstream of the core binding motif His-Phe-Arg-Trp (HFRW), which is essential for binding to the melanocortin receptors. To determine whether Tyr221 is necessary for the normal three-dimensional structure of β-MSH, our collaborator undertook NMR studies of wild-type and a mutant peptide analog replacing Tyr221 with Serine (to avoid intermolecular disulfide formation common with Cys-containing peptides). Chemical Shift Index (CSI) values were used to probe average structural properties (Mielke and Krishnan, 2004; Wishart et al., 1992). Compared to wild-type β-MSH, the mutation resulted in a significantly reduced helical turn propensity for two of the three residues between the mutation site and the His-Phe-Arg-Trp receptor binding motif (Figure 3-6).

Figure 3-3. Cosegregation of Tyr221Cys β-MSH mutation with obesity (black symbols) and overweight (gray symbols) in families. Genotype (N = wild-type allele, M = mutant allele) and BMI sds is denoted. In adults, overweight defined as BMI 25–30 kg/m^2, obesity as BMI > 30 kg/m^2. In children, overweight defined as >91st and obesity as >99th percentile for age-adjusted BMI. Arrows indicate the proband of each family.

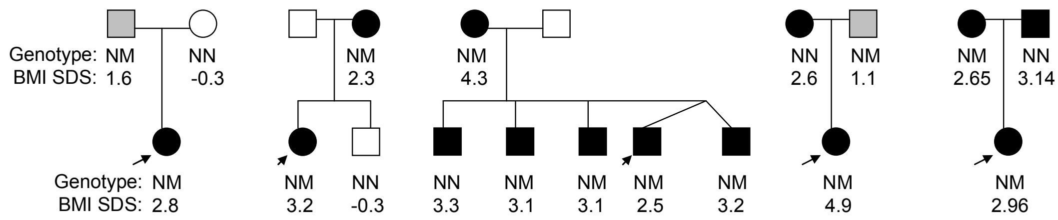


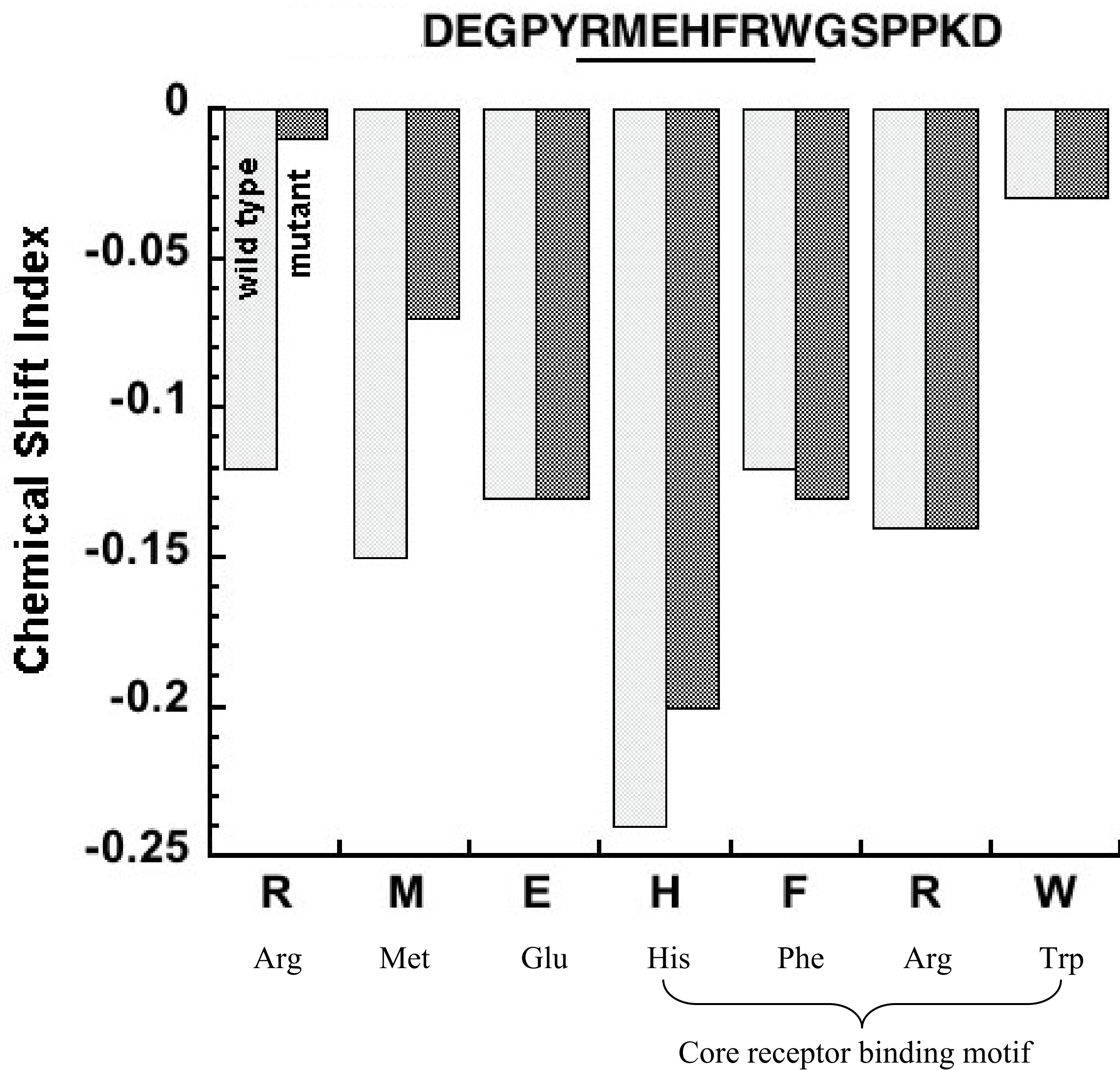
Figure 3-4. Sequence alignment of β-MSH peptides of different species. The tyrosine residue (underlined and in bold) is highly conserved, and invariably located 4 residues upstream of the core binding motif His-Phe-Arg-Trp (HFRW) (in bold).

Mammals	
Human	DEGP**Y**RME**HFRW**GSPPKD
Chimpanzee	DEGP**Y**RME**HFRW**GSPPKD
Dog	DDGP**Y**KME**HFRW**GSPPKD
Sheep	DSGP**Y**KME**HFRW**GSPPKD
Rat	D_GP**Y**RVE**HFRW**GNPPKD
Mouse	DDGP**Y**RVE**HFRW**SNPPKD
Birds	
Chicken	DGGS**Y**RMR**HFRW**HAPLKD
Amphibia	
Bullfrog	_GRK**Y**KMH**HFRW**EGPPKD
African clawed frog	_NGN**Y**RMH**HFRW**GSPPKD
Fish	
Chum salmon	_DGS**Y**RMG**HFRW**GSP
Rainbow trout	_DGS**Y**RMG**HFRW**GSP
Zebrafish	_DPP**Y**KMT**HFRW**SVPPAS
Seabass	_DGT**Y**KMK**HFRW**GGSPAS
White sturgeon	_DGS**Y**KMN**HFRW**SGPPKD

Figure 3-5. Tyrosine residue (underlined and in bold)) is present and located 4 amino acid residues upstream of the core binding motif His-Phe-Arg-Trp (HFRW) (in bold) of various POMC derived peptides, namely the adrenocorticotropic hormone (ACTH), and the three forms of MSH.

ACTH	S**Y**SME**HFRW**GKPV+ 26 amino acids
α-MSH	Ac-S**Y**SME**HFRW**GKPV-NH_2
β-MSH	DEGP**Y**RME**HFRW**GSPPKD
γ_1MSH	**Y**VMG**HFRW**DRF-NH_2

Figure 3-6. Comparison of the Chemical Shift Index (CSI = experimental chemical shift—consensus random coil chemical shift) values between wild-type and mutant β-MSH. Throughout the segment Arg-Met-Glu-His-Phe-Arg-Trp, the CSIs for both peptides were indicating the presence of turns (values ranging from −0.01 to −0.24). For the receptor binding motif, His-Phe-Arg-Trp, both wild-type and mutant peptides gave approximately identical values. However, for the Arg-Met residues following the mutation, but preceding the receptor binding motif, the wild-type sequence showed a significantly greater tendency to form turns relative to the mutant.



Tyr221Cys β-MSH mutation alters [Cys^{5}] β-MSH signaling through MC4R

When compared to wild-type β-MSH, a synthetic peptide incorporating the Tyr221Cys mutation showed reduced binding to MC4R stably expressed in HEK293 cells (Figure 3-7) (IC_{50} of mutant β-MSH = 797.1 ± 261.0 nM compared to 67.2 ± 43.0 nM of wt β-MSH, $p = 0.006$).

Consistent with these findings, the addition of wild-type β-MSH led to a dose-dependent increase in cAMP accumulation in HEK293 cells transiently expressing MC4R, while Tyr221Cys generated significantly less cAMP (Figure 3-8) (EC_{50} mutant β-MSH = 1.95 nM compared to 0.1 nM of wt β-MSH, $p = 0.04$).

Figure 3-7. [Cys^5] β-MSH binds to MC4R with lower affinity than β-MSH. Whole HEK293 cells stably expressing wild-type MC4R were exposed to tracer amounts of [^{125}I]NDP-MSH, and the ability of increasing concentrations of β-MSH or [Cys^5] β-MSH to inhibit radio-ligand binding was measured as described previously. Data are expressed as a percentage of maximum counts of [^{125}I]NDP-MSH binding to MC4R. Each point represents the mean (±SEM) of four independent experiments in triplicate.

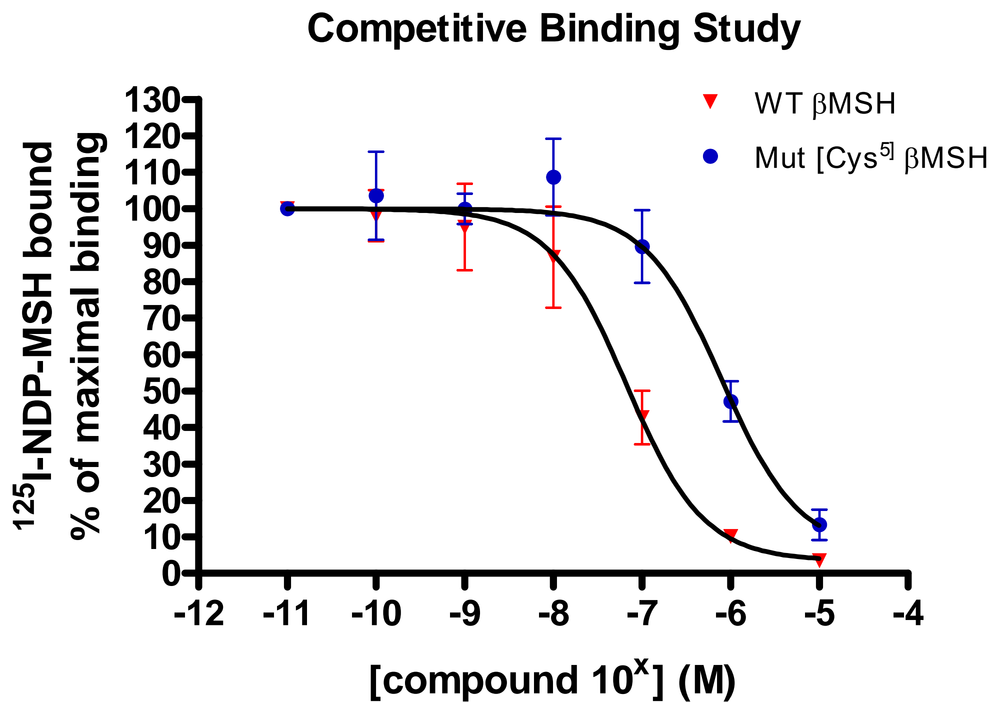
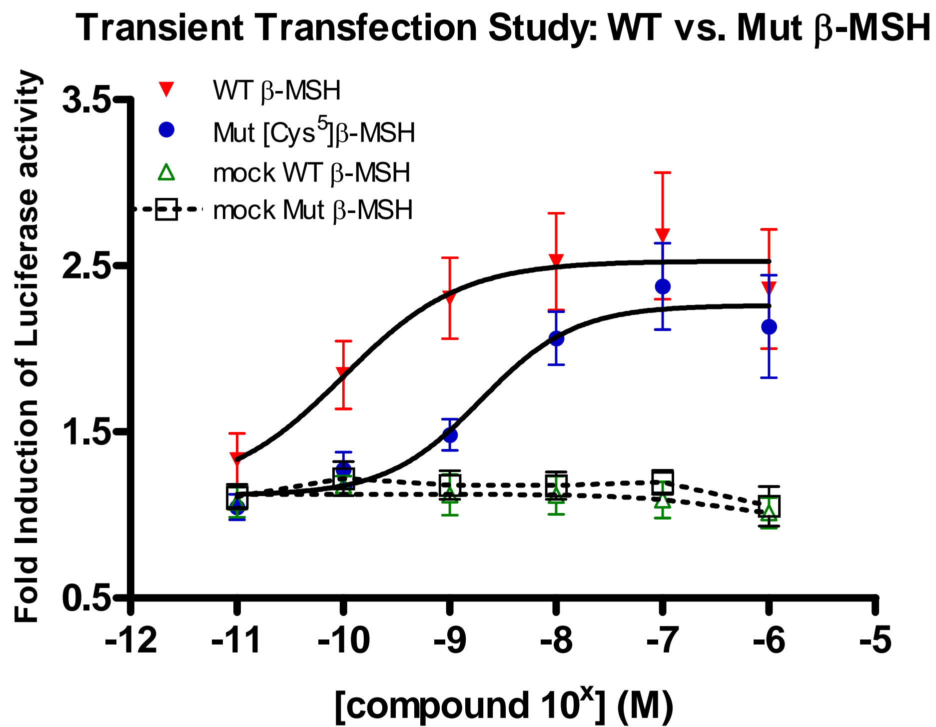


Figure 3-8. [Cys^{5}] β-MSH has a markedly reduced ability to stimulate production of cAMP. Graphs indicate responses of wild-type, mutant, and control constructs to a logarithmic increase in β-MSH concentration. cAMP/Luciferase reporter assays were performed as described previously. Open symbols denote mock transfected controls. Each point represents the mean (±SEM) of eight independent experiments performed in quadruplicate.



Clinical phenotype of subjects with Tyr221Cys mutation in β-MSH

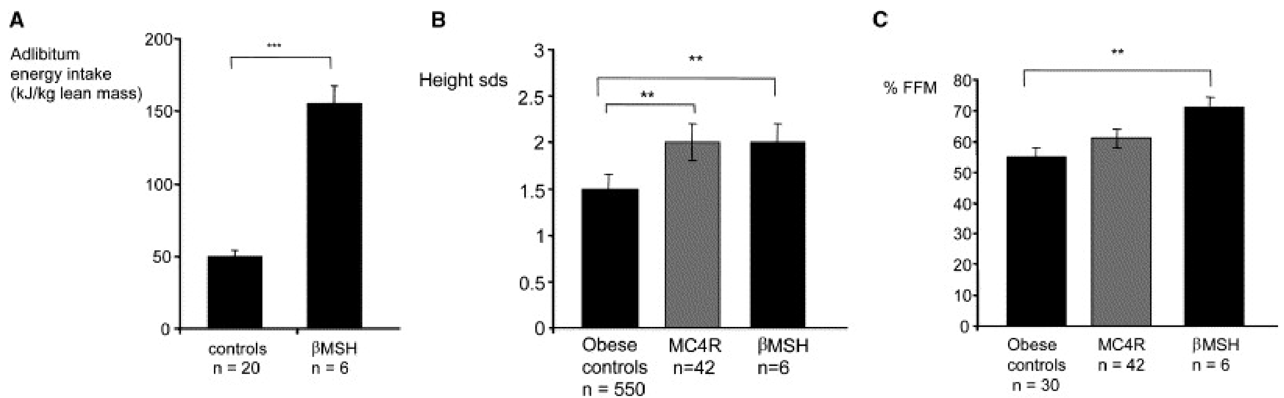
To establish whether obese children carrying the β-MSH mutation were hyperphagic, we admitted six children heterozygous for Tyr221Cys β-MSH (five probands and one relative) to the Addenbrooke's Hospital Clinical Research Facility. The six children underwent an ad libitum test breakfast. Ad libitum caloric intake, expressed per kg lean mass, was markedly increased compared to normal weight control children (Figure 3-9A) and similar to that seen in subjects heterozygous for MC4R mutations (Farooqi et al., 2003). Basal metabolic rate of affected subjects was not significantly different from that predicted using age- and gender-specific equations (data not shown). In order to determine whether obese children carrying the β-MSH mutation had other phenotypic similarities to children with known defects in MC4R signaling, we assessed body composition using standard procedures (Farooqi et al., 2003). The children with the β-MSH mutation more closely resembled *MC4R* heterozygotes than equivalently obese children (with normal MC4R and POMC sequence) in terms of accelerated linear growth and increased fat-free mass (Figures 3-9B and 3-9C).

Figure 3-9. Phenotypes of subjects with Tyr221Cys mutation

A) Food intake at an 18MJ ad libitum test meal compared to normal weight controls. Food intake is expressed per kg lean body mass measured by dual energy X-ray absorptiometry (DXA) to allow comparison between subjects of different ages (Farooqi et al., 2003) $^{***}p < 0.001$.

B) Height SDS in children with the Tyr221Cys mutation in β-MSH compared to heterozygotes for MC4R mutations and controls of comparable age and degree of obesity. $^{**}p < 0.01$

C) Fat-free mass (FFM) measured by DXA in children with the Tyr221Cys mutation in β-MSH compared to heterozygotes for MC4R mutations and controls of comparable age and degree of obesity (unpublished data). $^{**}p < 0.01$



A novel missense mutation His143Gln in α-MSH

As described above, one UK Caucasian proband was found to have a missense *POMC* mutation (His143Gln) located in the region coding for α-MSH (Figures 3-1 and 3-10A). However, this was also present in one of the 300 UK Caucasian controls. The grandfather of the proband carried the mutation and was obese, but the transmitting parent was lean (Figure 3-10B).

The mutation replaces a highly conserved residue within the classical His-Phe-Arg-Trp receptor binding motif of the melanocortins and would be expected to impair function. Consistent with this, a synthetic mutant peptide [Gln^6] α-MSH has marked reduced affinity for the MC4 receptor (Figure 3-11) (IC_{50} of mutant α-MSH = 1624 ± 624 nM compared to 15.2 ± 14.0 nM of wt α-MSH, $p < 0.0001$). The His143Gln mutant α-MSH also had reduced ability to stimulate cAMP generation from MC4R (Figure 3-12) with a reduced maximal response, although the EC50 was not significantly different from wild-type (EC_{50} mutant α-MSH = 3.8 nM compared to 2.2 nM of wt α-MSH, p = ns). Thus, while heterozygous disruption of α-MSH function may play a contributory role in the obesity of the proband, the presence of this mutation in a lean transmitting family member implies that it is insufficient, in itself, to fully explain the obese phenotype.

Figure 3-10A. A heterozygous missense mutation His143Gln due to a single base substitution from C to G (highlighted, and affected codon boxed), resulting in change of the histidine residue of highly conserved core binding motif His-Phe-Arg-Trp (HFRW) to glutamine.

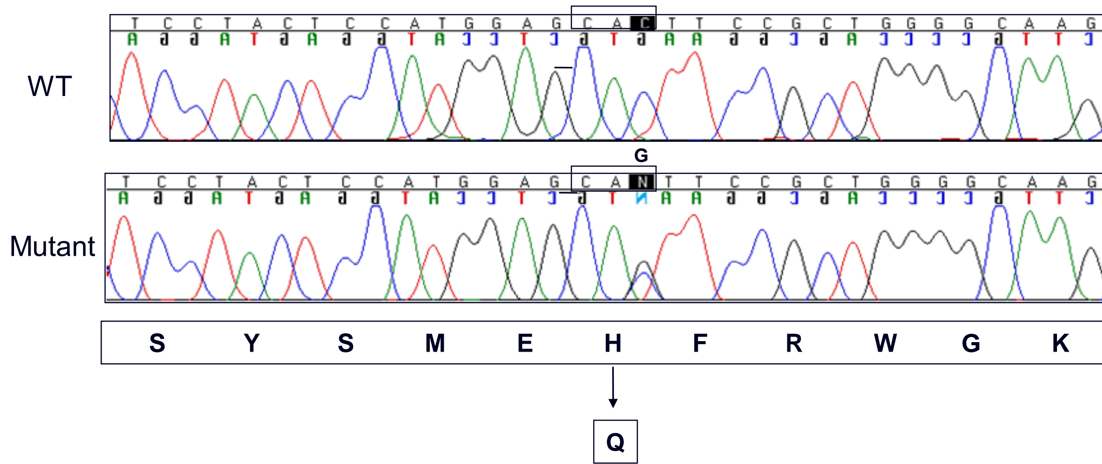


Figure 3-10B. Cosegregation of His143Gln α-MSH mutation with obesity (black symbols). N = wild-type allele, M = Mutant allele. Numbers were BMI SDS of each family member.

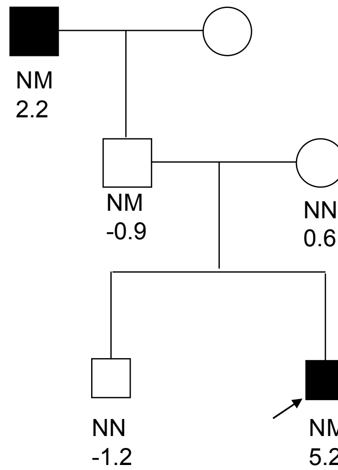


Figure 3-11. [Gln^6] α-MSH binds to MC4R with lower affinity than α-MSH (methods as in Figure 3-7). Data is expressed as a percentage of maximum counts of [^{125}I]NDP-MSH binding to MC4R. Each point represents the mean (±SEM) of four independent experiments in triplicate.

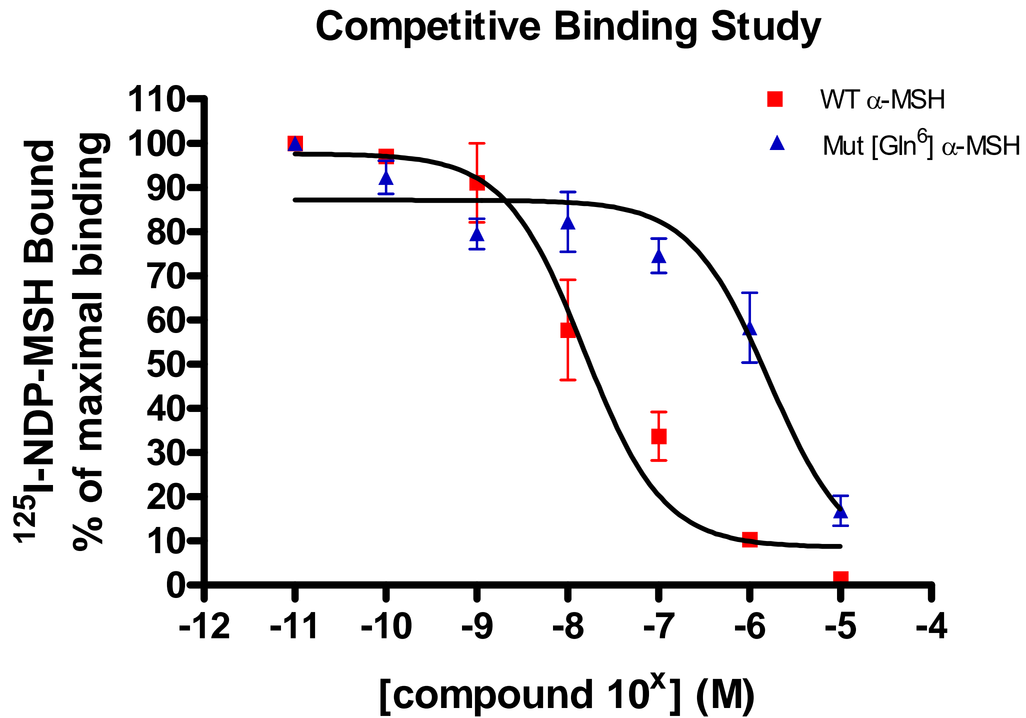
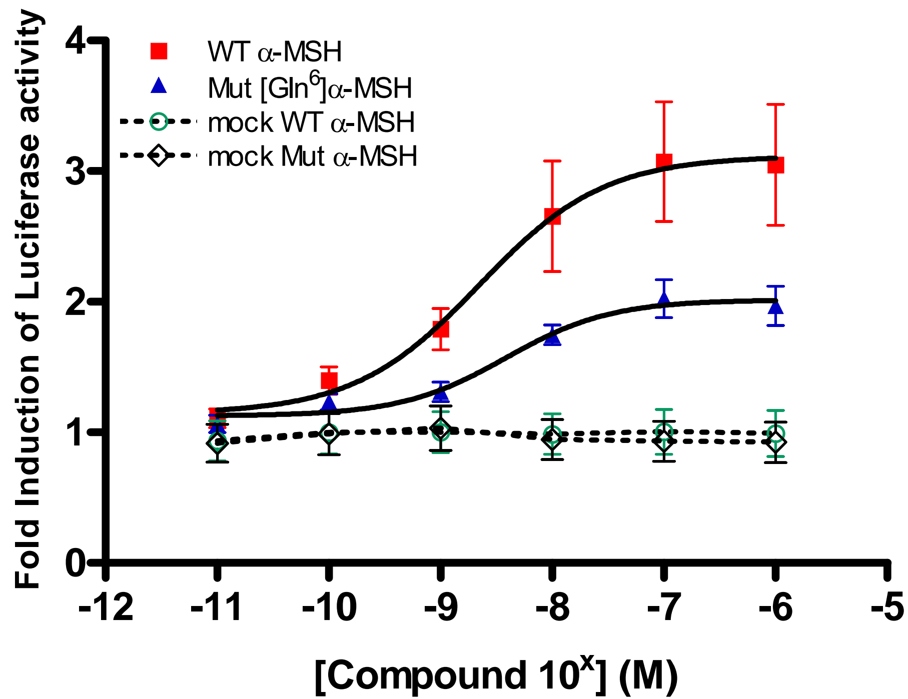


Figure 3-12. [Gln^{6}] α-MSH has a markedly reduced ability to stimulate production of cAMP (methods as in figure 1E). Open symbols denote mock-transfected controls. Each point represents the mean (±SEM) of eight independent experiments performed in quadruplicate.



Discussion

Tyr221Cys mutation in β-MSH is associated with human early-onset obesity

These studies provide compelling evidence that β-MSH is likely to be a physiologically relevant, endogenous ligand for the MC4R in humans. Firstly, the Tyr221Cys variant is enriched in a population of UK Caucasian subjects with early-onset obesity compared to its background prevalence in the UK population. The overrepresentation of this mutation in obese subjects is supported by an independent study in a German population (Biebermann et al., 2006). The fact that we did not find this variant at all in a large French population suggests that this variant may have arisen relatively recently in European ancestry.

Further support for the importance of β-MSH in energy homeostasis is provided by a previous observation of an association of a cleavage site mutation Arg236Gly affecting β-MSH with obesity although, in contrast with Tyr221Cys, that mutation resulted in the production of a β-MSH/β-endorphin fusion peptide with reduced ability to activate MC4R but normal binding affinity, and thus has the potential to interfere with signaling at the MC4R in a dominant-negative manner (Challis et al., 2002). It is unlikely that Tyr221Cys would result in a dominant-negative interference with wild-type β-MSH, so we hypothesize that this mutation simply reduces the amount of β-MSH tone at the MC4R and that this in itself is sufficient to predispose to obesity. The plausibility of such a model is supported by the increased prevalence of obesity and overweight in carriers of POMC null mutations ((Farooqi et al., 2006; Krude et al., 2003).

It is important to note that the Tyr221Cys mutation is unlikely to be a highly penetrant cause of monogenic obesity, but that apparent enrichment in obese populations is likely to occur because it is an allele that increases the risk of obesity. Our conclusion is supported by the fact that human and murine heterozygotes for null mutations in POMC show a propensity to increased adiposity but are not all frankly obese, and some carriers of the Tyr221Cys β-MSH mutation are not obese.

Both α-MSH and β-MSH influence melanocortinergic tone in humans

The vast majority of the literature concerning the POMC system and energy homeostasis focuses on the possible role of α- rather than β-MSH as the endogenous ligand (Coll et al., 2004; Cone, 2005), as the N-terminal cleavage site necessary for the generation of β-MSH is absent in rodents. In contrast, β-MSH has been established to be present in the human hypothalamus as a normal maturation product of POMC (Bertagna et al., 1986). We also found a rare missense mutation in α-MSH in a single proband, which had a major deleterious effect on its function. However, this variant was found in one lean family member and one lean unrelated control. While it is possible that this variant is contributing to the obesity of the proband, it is notable that our human genetic studies provide more compelling evidence for a specific role for β-MSH than α-MSH in the control of human energy balance. Finally, the similarities in clinical phenotype between obese children carrying the Tyr221Cys β-MSH mutation and those with *MC4R* mutations (Farooqi et al., 2003) suggest that both mutations are affecting shared physiological pathways. In conclusion, β-MSH is likely to be an important and physiologically relevant endogenous ligand for the human MC4R. These findings illustrate how studies of human

genetics can provide insights into the important physiological roles of particular species-restricted peptides that are not obtainable through the study of rodent models.

Acknowledgments

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Chapter 4

Novel mutations of the pro-opiomelanocortin (*POMC*) gene which affect POMC sorting to regulated secretory pathway

Summary

The functional loss of both alleles of the human pro-opiomelanocortin (*POMC*) gene leads to hypoadrenalism, red hair, fair skin, and early-onset obesity due to impaired synthesis POMC-derived peptides, and even heterozygous *POMC* mutations which affect the production or functional structure of melanocortin peptides can predispose to human obesity. In order to examine whether more subtle genetic variants in POMC might contribute to early-onset obesity, the coding region of the gene was sequenced in 960 subjects with a history of severe obesity from childhood (GOOS cohort). Two heterozygous missense mutations, Cys28Phe and Leu37Phe, were found in a Turkish subject and a Caucasian subject respectively, and were absent in 100 Turkish and 300 UK Caucasian controls. Both mutations result in substitution of highly conserved residues in a region of the N-terminus (N-terminal peptide) that is considered to be the sorting signal motif of POMC, involved in its sorting to the regulated secretory pathway. This Cys28Phe variant co-segregated with obesity in the family, but not as well for the Leu37Phe. Metabolic labeling with immunoprecipitation, Western blot studies, and immunoassay analysis of lysates were performed using β-TC3 cells transiently transfected with POMC cDNA and co-transfected with prohormone convertase 2 and its chaperone, 7B2. Metabolic labeling studies indicate that while the mutations do not reduce intracellular levels of POMC, both mutations impair the ability of POMC to be

processed to generate bioactive products (Cys28Phe more so than Leu37Phe). Studies of the secretion of POMC products suggest, particularly with Cys28Phe, that the impaired propeptide processing of these mutations results from a mistargeting of mutant POMC to the constitutive rather than the regulated secretory pathway. No dominant negative effect was detected. This is consistent with previous reports that inactivating heterozygous POMC mutations can predispose to human obesity. These mutations in patients with early onset obesity represent a novel molecular mechanism of POMC deficiency whereby naturally occurring mutations in its N-terminal sequence impair the ability of POMC to enter the trafficking pathway in which serial propeptide processing normally occurs.

Introduction

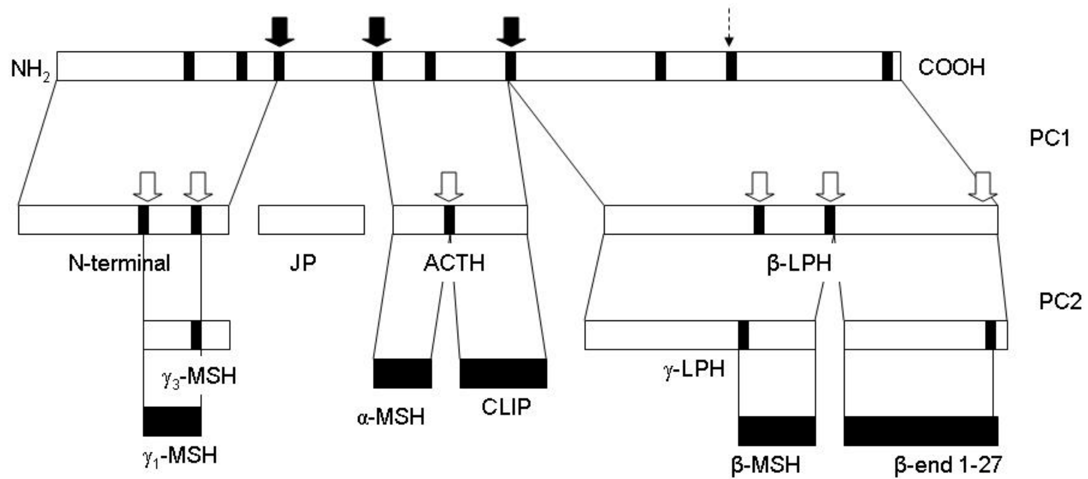
As discussed in the previous chapters, homozygous nonsense or missense mutations in POMC are associated with hyperphagia, early onset obesity, hypopigmentation and isolated ACTH deficiency, and loss of one copy of *POMC* is sufficient to predispose to obesity, likely due to haploinsufficiency (Farooqi et al., 2006; Krude et al., 2003). A variety of heterozygous missense *POMC* mutations are found more commonly in obese children (Challis et al., 2002; Lee et al., 2006).

POMC (accession no. NP_000930) is a pro-peptide synthesized on the rough endoplasmic reticulum, and then transported to the trans-Golgi network (TGN) where it is packaged into immature secretory granules of the regulated secretory pathway (RSP). The POMC-derived peptides are secreted via both constitutive and regulated secretory

pathways. Within these granules, POMC is cleaved by endoproteases to yield the active peptides, which are stored in mature (dense core) granules to be released upon stimulation (Pritchard and White, 2007). Thus POMC undergoes tissue-specific post-translational processing during sorting as it transit through the golgi network and secretory vesicles, the products of which include ACTH and β-lipotropin (β-LPH) after cleavage by prohormone convertase 1 (PC1) localized to the TGN as well as secretory granules (Christie et al., 1991; Hornby et al., 1993; Kirchmair et al., 1992), and then by prohormone convertase 2 (PC2) which acts in a post-Golgi compartment or in secretory granules (Benjannet et al., 1993; Bennett et al., 1992; Johanning et al., 1996; Rouille et al., 1995; Zhou et al., 1993; Zhou et al., 1995) to further yield the melanocortin peptides α, β, and γ-MSH and β-endorphin (β-end) (figure 4-1) (Pritchard et al., 2002; Raffin-Sanson et al., 2003; Seidah and Chretien, 1999). There is evidence to suggest that the N-terminal peptide (NT) of POMC carries a sorting signal motif which directs POMC to the regulated secretory pathway (Cool et al., 1995; Cool et al., 1997). To date, no naturally occurring variants in this region of the human POMC gene have been described.

In a mutation screen of subjects with early onset obesity from the GOOS cohort, two heterozygous missense mutations located in the NT region of *POMC* were found in a Caucasian proband and Turkish proband. Functional studies revealed that both mutations may interfere with sorting of POMC into the regulated pathway and result in reduced secretion of the melanocortin peptides.

Figure 4-1. POMC post-translation processing by PC1 (*black arrow*) and PC2 (*clear arrow*) at dibasic cleavage sites (*solid line*). Tissue specific expression results in a different range of peptides produced in the anterior pituitary (grey) compared with the hypothalamus (black) (figure modified from Coll, A.P., Farooqi, I.S., Challis, B.G., Yeo, G.S., and O'Rahilly, S. [2004]. Proopiomelanocortin and energy balance: insights from human and murine genetics. J Clin Endocrinol Metab 89, 2557-2562)



Methods

Subjects and Human Genetic Studies

More than 960 DNA samples from the GOOS cohort were screened for POMC mutations (in the coding region) using a combination of direct sequencing and dHPLC as described in the previous chapter (Lee et al., 2006). DNA and phenotypic information was obtained from family members of the probands with N-terminal peptide missense mutations and the genotype of relatives was ascertained by direct sequencing. The prevalence of each mutation in an appropriate ethnically matched control group (100 Turkish and 300 UK Caucasian controls) was determined using the same screening method for the GOOS cohort. Body mass index standard deviation scores were calculated using UK reference data (Cole et al., 1995).

Construction of POMC Wildtype, Cys28Phe, and Leu37Phe expression vectors

Construction of a human POMC expression vectors (in pcDNA3; Invitrogen Life Technologies) containing a c-Myc epitope tag near the carboxyterminus has been described previously (Challis et al., 2002). Briefly, the human POMC gene with a c-Myc epitope tag between codons 262 and 263 was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen). The epitope tag precedes the KKGE sequence to avoid removal after cleavage at the KK sequence. This construct was used as template for mutagenesis to introduce the G to T (Cys28Phe) and C to T (Leu37Phe) mutations using a QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the guidelines of the supplier. Mutations were confirmed by nucleotide sequencing.

Biochemical properties of POMC variants

a) Metabolic labelling studies

This experiment was performed by our collaborator Assoc Professor John WM Creemers from the University of Leuven Centre for Human Genetics, Leuven, Belgium. 5-6×10^5 mouse insulinoma β-TC3 cells were transfected with a total of 2 μg expression vector and 6μl Lipofectamine 2000 (Invitrogen Life Technologies) and plated in 10 cm^2 dishes. All experiments described below were performed in triplicate. Metabolic labeling was performed the next day for 1 h, using 200 μCi [^{35}S]-methionine (Perkin Elmer; specific activity 1175 Ci/mmol) in 600 μl methionine-deficient RPMI-1640 medium (Sigma), and chased for 1 h in medium supplemented with excess (0.2 mM) unlabeled methionine. Cells were lysed in 1 ml lysis buffer (50 mM Tris/HCL pH 7.4, 150 mM NaCl, 1% Triton X-100) containing complete protease inhibitors (Roche Molecular Biochemicals). Lysates were pre-cleared with preformed complexes of protein G sepharose (GE Healthcare) and mouse pre-immune IgGs. Specific immunoprecipitation was performed with monoclonal antibody 9E10, directed against the c-Myc epitope. The immunoprecipitated proteins were size separated by SDS-PAGE (Criterion, Bio-Rad) and visualized by autoradiography.

b) Studies of constitutive and regulated secretion

Secretion studies of POMC variants was performed essentially as described previously (Creemers et al., 2006) with minor modifications (performed by Assoc Professor John WM Creemers from the University of Leuven Centre for Human Genetics). Briefly, cells were transfected as described above and then cultured for 16h in serum free medium, to

minimize the tonic release of POMC-derived peptides induced by serum factors. The next day, the cells were first incubated for 3 h with serum free medium (unstimulated fraction) and subsequently for 3 h with medium containing the secretagogues forskolin (10 μM [Sigma]) and IBMX (0.1 mM [Sigma]), which elevate the intracellular cyclic AMP concentration. After collection of this conditioned medium (stimulated fraction), the cells were lysed in laemmli sample buffer.

Western Blot analysis (performed by Assoc Professor John WM Creemers from the University of Leuven Centre for Human Genetics): Culture medium from the experiment above was mixed with 25 μg/ml albumin before precipitation with 4 volumes of methanol at -20°C. Medium precipitates were dissolved in sample buffer and size separated by SDS-PAGE (Criterion, Bio-Rad). Western blotting was performed using monoclonal antibody 9E10 directed against the c-Myc epitope.

POMC and α-MSH immunoassay (performed by Dr Rob L Oliver and Professor Anne White from the University of Manchester, Endocrine Sciences Research Group, Faculties of Life Sciences and Medical and Human Sciences): Culture media and cells from parallel experiments to those for western blotting were collected for POMC and α-MSH analysis. Cells were lysed in 1 ml lysis buffer (50 mM Tris/HCL pH 7.4, 150 mM NaCl, 1% Triton X-100) containing complete protease inhibitors (Roche Molecular Biochemicals). α-MSH was measured by radio-immunoassay (Euro-diagnostica AB, Malmö, Sweden). POMC measurement with a two-antibody ELISA (Rousseau et al.,

2007) based on the immunoradiometric assay previously described (Crosby et al., 1988) was planned, but not performed yet at the time of writing this chapter.

Results

Two novel mutations in the N-terminus of POMC

We identified two novel heterozygous missense mutations in exon 2 of *POMC* (figure 4-2A). Cys28Phe (c.82G>T) was found in a 17 year old boy of Turkish origin with severe obesity and not in 100 control alleles of the same ethnic group. This mutation was also found in two other members of the family (figure 4-2B), both of whom were overweight (BMI 28 and 29 kg/m^2 respectively). Leu37Phe (c.109C>T) was found in a 9 year old girl of UK Caucasian origin with severe obesity and not in 300 control alleles of the same ethnic group. The mutation was inherited from her mother who was overweight (BMI 27 kg/m^2) (figure 4-2B).

Figure 4-2A. Chromatograms for Proband 1 and 2 indicating heterozygosity for missense mutations Cys28Phe (c.82G>T) and Leu37Phe (c.109C>T) respectively (arrow). Affected codons indicated by overbars.

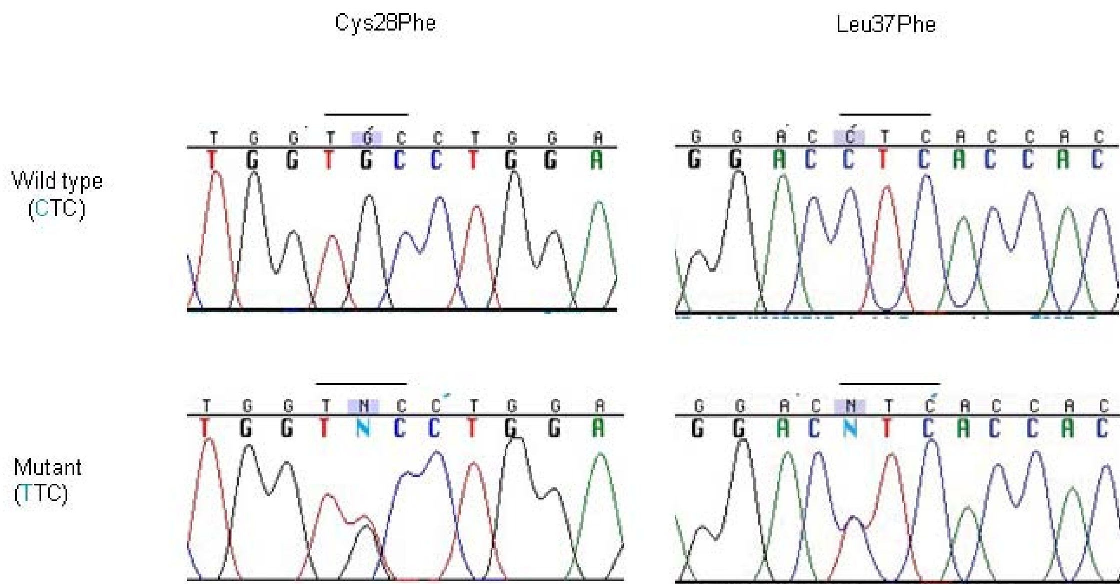
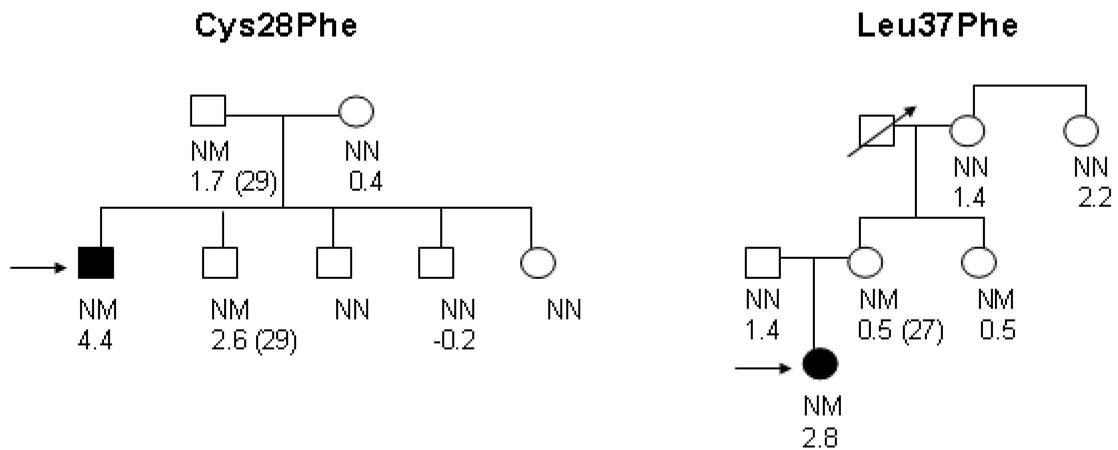


Figure 4-2B. Co-segregation of Cys28Phe and Leu37Phe with overweight/obesity in family members. Filled symbols indicate probands; Genotypes are included where available - N denotes normal allele, M denotes mutant allele. Body mass index (BMI) standard deviation scores are indicated beneath genotype (Cole et al., 1995); BMI (kg/m^2) is indicated in parentheses for subjects > 16yrs of age



Both residues are located in the N-terminal region of POMC (Figure 4-3) which has previously been implicated in the sorting of POMC to the regulated secretory pathway. Both Cys28 and Leu37 are highly conserved across multiple species (accession numbers: Zebrafish NP_852103, Chicken BAA34366, Mouse P01193, Rat AAA41903, Human NP_000930) (Figure 4-4). This region contains two disulfide bridges stabilizing an amphipathic hairpin loop structure between the second and third cysteine residues (figure 4-5) (Cool et al., 1995). The mutation Cys28Phe resulted in the loss of the outer disulphide bridge, while Leu37Phe led to the loss of the highly conserved Leu37 residue, part of the amphipathic motif comprising of four highly conserved hydrophobic and acidic amino acids (Asp^{36}-Leu^{37}-Glu^{40}-Leu^{44}) thought to be vital for the sorting function.

Figure 4-3. Structure of POMC and location of mutations. Cys28Phe and Leu37Phe are located in the N terminal region of POMC distant from previously reported missense mutations (arrows) associated with obesity. The signal peptide (1st – 26th residues) is necessary for the translocation of the nascent protein through the membrane of the rough endoplasmic reticulcum (RER), and is then rapidly cleaved. The POMC protein is then engaged in the secretory pathway and ready for processing by the prohormone convertases (PC1 & PC2).

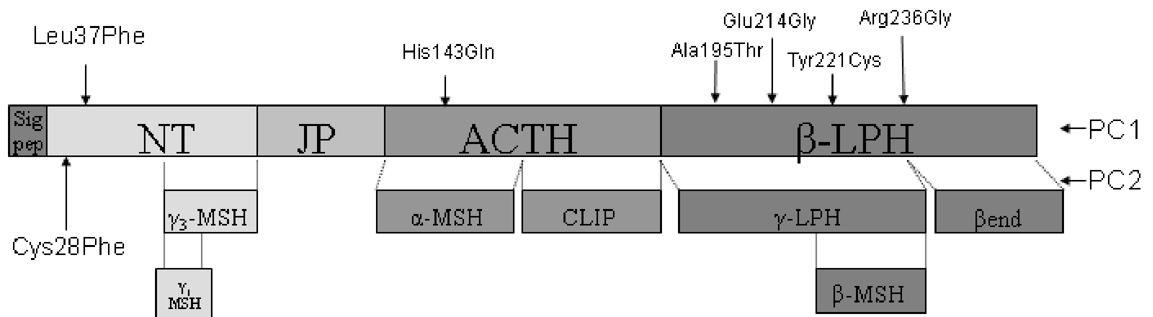


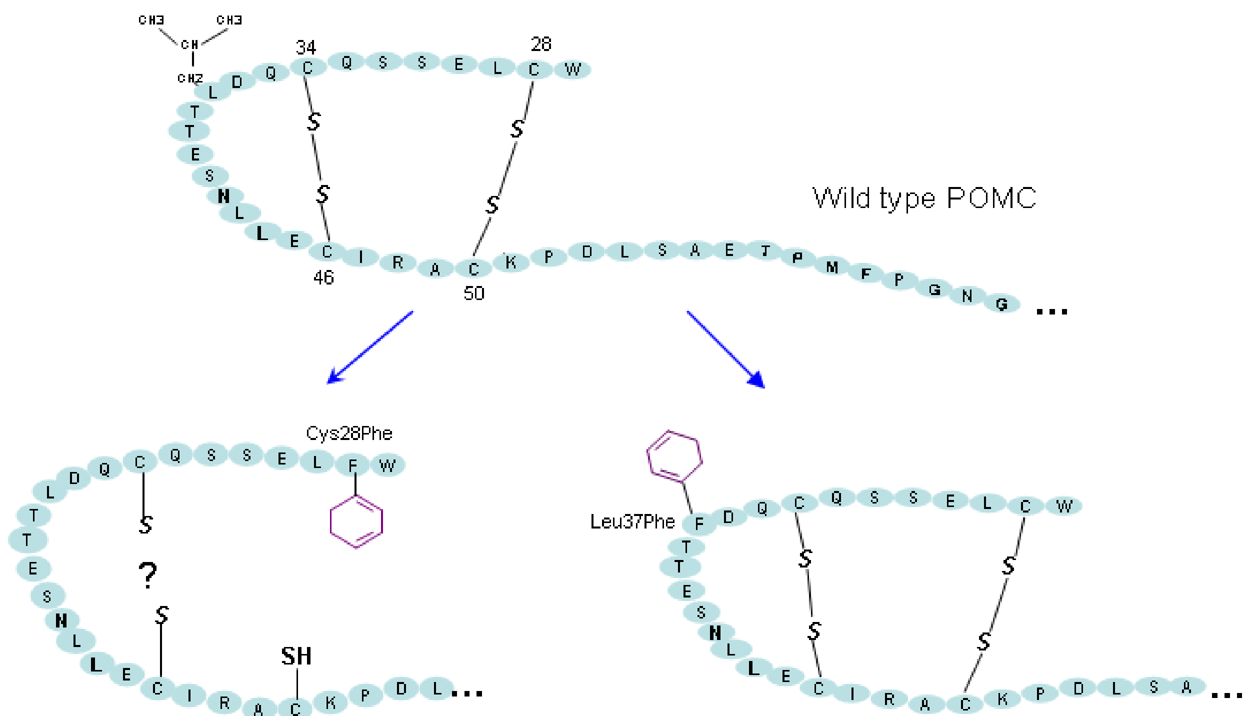
Figure 4-4. Sequence alignment shows that Cys28 and Leu37 residues are highly conserved residues, present in the POMC sequence of species from fish through to mammals (figure modified from Cool, D.R., Fenger, M., Snell, C.R., and Loh, Y.P. [1995]. Identification of the sorting signal motif within pro-opiomelanocortin for the regulated secretory pathway. J Biol Chem 270, 8723-8729). Located of the two mutations are indicated as C28F and L37F.

Alignment of NH_2 terminal regions showing POMC homology

		C28F									L37F																	
Human POMC	W	C	L	E	S	S	Q	C	Q	D	L	T	T	E	S	N	L	L	E	C	I	R	A	C	K	P	D	L
Pig-tailed macaque POMC	W	C	L	E	S	S	Q	C	Q	D	L	T	T	E	S	N	L	L	A	C	I	R	A	C	K	P	D	L
Mouse POMC	W	C	L	E	S	S	Q	C	Q	D	L	T	T	E	S	N	L	L	A	C	I	R	A	C	K	L	D	L
Rat POMC	W	C	L	E	S	S	Q	C	Q	D	L	T	T	E	R	N	L	L	A	C	I	R	A	C	R	L	D	L
Guinea Pig POMC	W	C	L	E	S	S	Q	C	Q	D	L	T	T	E	S	H	L	L	E	C	L	R	A	C	K	P	D	L
Bovine POMC	W	C	L	E	S	S	Q	C	Q	D	L	T	T	E	S	N	L	L	A	C	I	R	A	C	K	P	D	L
Porcine POMC	W	C	L	E	S	S	Q	C	Q	D	L	S	T	E	S	N	L	L	A	C	I	R	A	C	K	P	D	L
African clawed frog POMC	Q	C	W	E	S	S	R	C	A	D	L	S	S	E	D	G	V	L	E	C	I	K	A	C	K	T	D	L
Bullfrog POMC	Q	C	W	E	S	N	R	C	T	D	L	S	S	E	D	G	I	L	E	C	I	K	A	C	K	M	D	L
Frog POMC	Q	C	W	E	S	N	K	C	T	D	L	S	S	E	D	G	I	L	E	C	I	K	A	C	K	M	D	L
Ostrich POMC	P	C	W	E	S	G	K	C	Q	D	L	T	T	E	A	G	V	L	A	C	A	A	A	C	R	S	D	L
Salmon I POMC	E	C	W	E	N	P	R	Q	C	Q	D	L	N	S	E	N	S	I	L	E	C	I	Q	L	C	R	S	D
Salmon II POMC	Q	C	W	D	S	S	H	C	K	D	L	P	S	E	D	J	I	L	E	C	T	H	L	F	R	S	G	L
Trout A POMC	Q	C	W	E	N	P	R	C	H	D	L	S	S	E	N	N	L	L	E	C	I	Q	L	C	R	S	D	L
Trout B POMC	Q	C	W	D	S	S	H	C	K	D	L	P	S	E	D	K	I	L	E	C	I	H	L	F	R	S	G	L

NT peptide residue

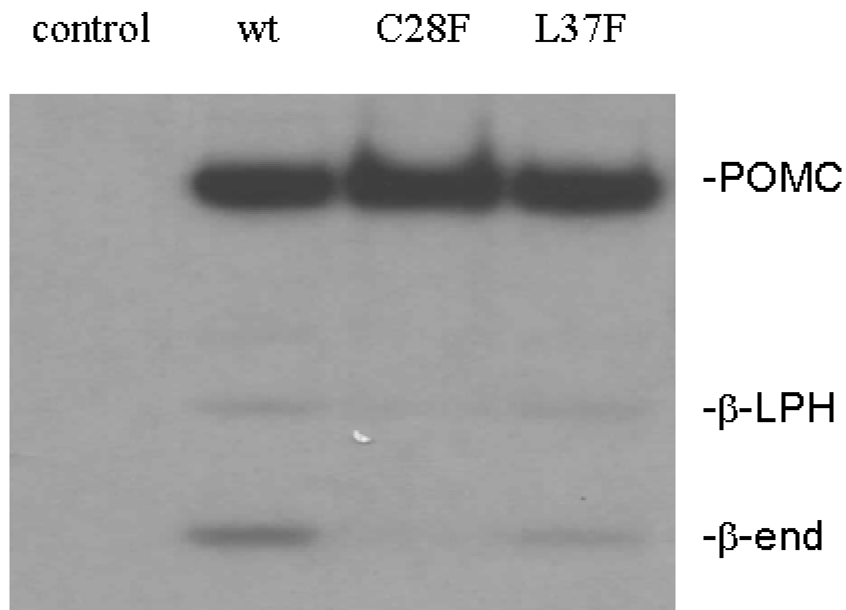
Figure 4-5. The N-terminal peptide (NT) of POMC carries the sorting signal motif which directs POMC to the regulated secretory pathway. The sorting motif is made up of 13 amino acid residues from codons 34 to 46, forming a hairpin loop structure at the N-terminus held by two disulfide bridges (between Cys28 and Cys50, and Cys34 and Cys46), and disruption of this structure leads to mis-sorting. This motif is amphipathic, with four highly conserved hydrophobic and acidic amino acids (Asp^{36}-Leu^{37}-Glu^{40}-Leu^{44}) thought to be vital for the sorting function (Cool et al., 1995; Cool et al., 1997). The mutation Cys28Phe resulted in the loss of the outer disulphide bridge, while Leu37Phe led to the loss of the highly conserved Leu37 residue of the amphipathic motif.



Mutant POMCs were less efficiently processed

To study the effect of the mutations on the maturation and of POMC and processing to its derivative peptides, biosynthetic studies were performed in transfected β-TC3 cells labelled for one hour with ^{35}S-methionine (figure 4-6). Cell lysates were immunoprecipitated with an antibody directed against the carboxyterminal c-Myc tag and therefore recognizing the uncleaved POMC precursor and the cleavage products β-LPH and β-endorphin, the most C-terminal cleavage products. A striking reduction in β-endorphin and to a lesser extent of β-LPH was observed with the Cys28Phe mutant as compared to wild type POMC. A similar, but less severe reduction was also observed with the Leu37Phe mutant. β-LPH is generated by PC1 activity starting in the golgi network and proceeding into the immature secretory granules and dense core secretory granules, and β-endorphin production is typically a late event starting after sorting into immature secretory granules and large dense core vesicles. These results indicate that for both POMC mutants, newly synthesized POMC is less efficiently processed into bioactive peptides than is the case with wild-type POMC.

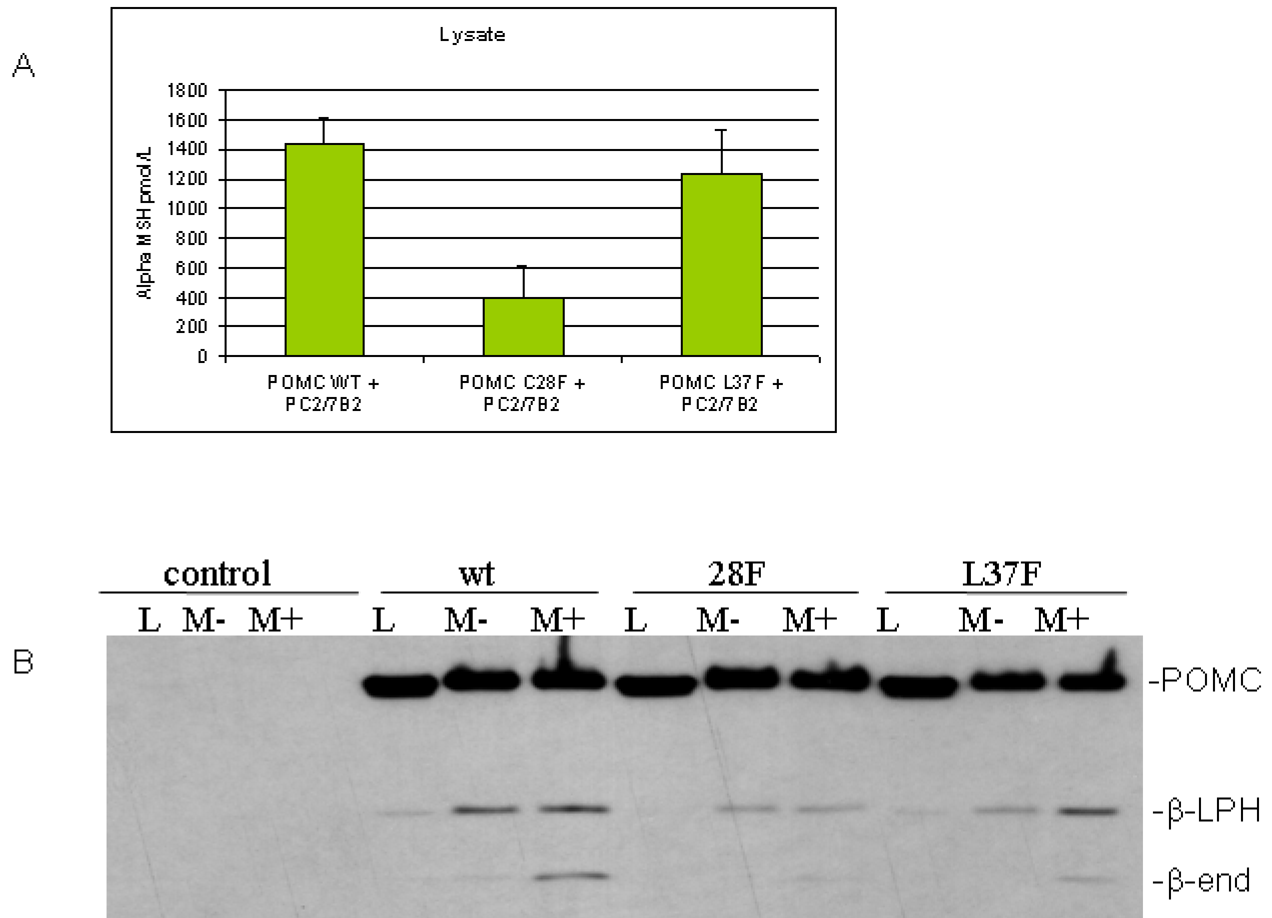
Figure 4-6. Cys28Phe and Leu37Phe mutations impair the intracellular generation of POMC products. Immunoprecipitation of radiolabelled C-terminally Myc-tagged POMC-derived peptides from β-TC3 cells expressing wild-type POMC and mutant POMCs with Cys28Phe and Leu37Phe. Cys28Phe and to a lesser extent Leu37Phe mutant POMCs were processed less efficiently to β-lipotropin (β-LPH) (13 kDa) and β-endorphin (β-end) (4 kDa). Wt=wildtype POMC; control = cells tranfected with empty vectors.



As the N-terminus of POMC has been shown to be important for sorting of POMC into dense-core granules (Tam et al., 1993), our collaborator A/Prof Creemers undertook studies designed to examine the effects of the N-terminal mutations on POMC sorting. POMC-derived peptides are secreted via both constitutive and regulated secretory pathways. Stimulated secretion of POMC products from endocrine cells occur by fusion of dense core granules (DCG), the most mature element of the regulated secretory pathway, with the plasma membrane. Stimulus–independent secretion of POMC related products occurs through the constitutive secretory pathway. β-TC3 cells which endogenously express PC1 and PC2 (and engineered to overexpress PC2 and 7B2 to improve processing efficiency) were transfected with wild-type or mutant POMC and incubated in serum-free medium for 16 hrs to minimise tonic release of POMC by serum factors. Cells were then exposed to fresh serum-free medium for three hours and subsequently with serum-free medium containing forskolin and IBMX for three hours after which the conditioned medium and cell lysates was assayed for alpha-MSH. The absolute amount of αMSH was significantly reduced with the C28F mutant (figure 4-7A) compared to wild type POMC. This was particularly true for the stimulated release but also reduced in the medium from unstimulated cells and in the cell lysates. The results for the L37F variant displayed the same trend.

In parallel transfections, the presence of C-terminally myc-tagged POMC was used to analyse the processing by western blotting. In the case of wild-type POMC, in the absence of a stimulus for regulated secretion, mainly POMC and some β-LPH were secreted (figure 4-7B).

Figure 4-7. Effects of C28F and L37F on constitutive and regulated secretion of POMC products (A) β-TC3 cells transfected with wild type POMC or C28F or L37F mutations were incubated in unstimulated medium (M-), or medium with forskolin and IBMX (M+) to stimulate release of peptides from the regulated secretory pathway. Cell lysates (L) were collected after the stimulation period. The Cys28Phe variant resulted in much less α-MSH, and that of the Leu37Phe variant is intermediate between wild-type and Cys28Phe. (B) Parallel transfections utilized the Myc-Tag on the C-terminal of POMC for Western blotting of samples from medium without secretagogues (M-); medium with secretagogues (M+) and lysates (L). Cys28Phe POMC and to a lesser extent Leu37Phe POMC were not sorted as efficiently as wild-type POMC, with less β-LPH and β-end detected. β-LPH secretion was not increased by secretagogues, indicating that it was mainly processed before sorting, and was constitutively secreted, whereas β-end was mainly secreted after stimulation.



After the addition of secretagogues, a large increase in the secretion of β-endorphin was observed in the cells transfected with wild-type POMC, while levels of secreted POMC and β-endorphin remained the same. These results support the notion that secreted β-endorphin is an appropriate marker for POMC's normal entry into, and processing within, the regulated secretory pathway. With the Cys28Phe mutant the amount of POMC secreted under basal and constitutive states was similar to that seen with wild type, but smaller amounts of β-LPH and β-endorphin were secreted in both conditions (figure 4-7). Importantly, the relative amounts of β-endorphin vs POMC detected in the medium after exposure to secretagogues indicates that considerably less of the mutant POMC is entering the mature elements of the regulated secretory pathway thus suggesting that this mutant POMC is, at least in part, mis-targeted within the cell. Similar, but less marked, results were seen with L37F. In the case of this mutant, however, there was a small but consistent increase in β-LPH in the medium after stimulation by secretagogues, a phenomenon that did not occur with wild-type POMC or C28F. These results as a whole suggest that these mutations in the N terminus of POMC do not influence the synthesis or steady state amounts of POMC propeptide produced. However they both (Cys28Phe > Leu37Phe) impair the processing of the mutant propeptide to mature bioactive peptides. Given the distance of the mutants from the main processing sites the most parsimonious explanation for this is that the mutations interfere with the entry of POMC to the normal regulated secretory pathway, a finding consistent with previous studies of artificial mutants in this region of the molecule (Cool et al., 1995).

Discussion

The melanocortin products of the *POMC* gene are involved in a wide range of biological processes from adrenal steroidogenesis to skin pigmentation (Bertagna, 1994). POMC derived peptides appear to be essential for normal energy homeostasis in a subset of neurons in the arcuate nucleus of the hypothalamus (Schwartz et al., 2000), as mice and humans entirely lacking POMC develop hyperphagia and severe obesity despite lacking circulating glucocorticoids due to ACTH deficiency (Coll et al., 2004). Since the original description of complete human POMC deficiency (Krude et al., 1998) there have been a range of reports describing the association of heterozygous missense mutations in POMC with an increased risk of obesity (Challis et al., 2002; Dubern et al., 2007; Hinney et al., 1998; Lee et al., 2006). The vast majority of these missense mutations affect either the structure or function of α- or β-MSH, or the cleavage from their precursor. The phenotype from loss of function *POMC* mutations appears to involve an early onset of obesity and objective evidence for increased appetite and food intake (Farooqi et al., 2006; Lee et al., 2006). Both probands carrying the two mutations Cys28Phe and Leu37Phe have early onset obesity, and the proband with the Leu37Phe showed increased food intake at an *ad libitum* test meal consistent with loss of melanocortin function. While insufficient family members were available to formally assess co-segregation with these mutations, those who carry the mutations were either overweight or obese.

Cys28Phe and Leu37Phe differ notably from previous reported *POMC* mutations associated with obesity in that they do not lie within, or adjacent to, the melanocortin

peptides known to have an established role in energy homeostasis (namely α- and β-MSH), but lie in the N-terminal region of the POMC molecule. We have shown that these mutations caused loss of POMC-derived signalling peptides by a novel mechanism, which is impaired sorting and processing of the POMC pro-peptide.

The sorting of proteins to the regulated secretory pathway is still a matter of debate (Dikeakos and Reudelhuber, 2007). For POMC it has been shown that the 26 amino acids immediately downstream to the carboxy-terminal of the signal peptide (amino acids 27-52) are sufficient to target a reporter protein to the regulated secretory pathway (Tam et al., 1993). As discussed earlier, two disulfide bridges are formed by Cys28/Cys50 and Cys34/Cys46 within that sequence (figure 4-5). The hairpin loop between Cys28 and Cys50 forms an amphipathic loop and contains highly conserved amino acids, in particular Asp36, Leu37, Glu40 and Leu44 (Cool et al., 1995), which may be part of a vital sorting signal binding site for the sorting process (Cool and Loh, 1998). Sorting of POMC is required for efficient processing to its bioactive peptides, which starts in the TGN and proceeds in the regulated secretory pathway (Schnabel et al., 1989). The two main enzymes involved in its processing are the PC1 and PC2. Cleavage by PC1 starts in the late Golgi subcompartments and immature secretory granules, and results in the generation of β-LPH and some ACTH (Schnabel et al., 1989). PC2 requires a more acidic environment and therefore becomes active only upon maturation of the immature secretory granules, resulting in the production of melanocortins and β-endorphin (Pritchard et al., 2002).

Sorting of POMC from the trans-Golgi network (TGN) to immature secretory granules may be a key regulatory step which determines the extent of POMC processing (Pritchard and White, 2007). There are different theories regarding the mechanisms by which POMC is sorted and targeted to the regulated secretory pathway. Currently, the majority of studies support a sorting-for-entry model where targeting to granules is determined in the TGN (Dumermuth and Moore, 1998). The amino-terminal amphipathic loop on POMC is important as a sorting signal motif (Cool et al., 1995), which has been proposed to bind to carboxypeptidase E as a putative sorting receptor (Cool et al., 1997). Therefore disruption of this region by changes in amino acids (Leu37Phe) is likely to impede sorting to secretory granules and the effective processing of POMC to bioactive peptides.

Cys28 has previously been mutated by others without observing a deleterious effect on sorting (Cool et al., 1995; Roy et al., 1991). This discrepancy with our observations can be explained because Cys28 was replaced by the physico-chemically similar amino acid serine in these studies. The Cys28Phe mutation results in substitution by the much larger phenylalanine with a bulky aromatic side chain (Cys28Phe), which is likely to cause steric hindrance and destabilize the overall structure of the hairpin loop (figure 4-5).

Leu37 is highly conserved in POMC and the replacement of aliphatic leucine by phenylalanine with an aromatic side chain might have been expected to have a severe effect. The modest effect observed here might be the consequence of the experimental

set-up. POMC sorting is a multi-step process including oligomerization/aggregation in the TGN, which is independent of the N-terminal loop (Cawley et al., 2000). Furthermore, the influence of each domain might be cell-type specific (Gorr et al., 2001). For instance, cell-type specific effects on processing are found in the mouse model carrying a mutation in the *PC1* gene resulting in a N222D substitution (Lloyd et al., 2006). In this model, carboxy-terminal processing of PC1 was much more affected in the hypothalamus than in brain and pancreas. The experiments in the current study were performed in pancreatic β cells to circumvent the problem of endogenous POMC, but it is possible that the observed differences in sorting and α-MSH production are more pronounced in hypothalamic neuronal cells.

Csy28Phe and Leu37Phe represent a novel mechanism of disruption of human POMC function, and support the notion that the N-terminal component of human POMC is important for targeting of the propeptide to the regulated secretory pathway. These naturally occurring mutations act as illuminating "experiments of nature", which validate the sequence requirements for normal POMC targeting and processing as demonstrated *in vitro* is just as critical in humans *in vivo*.

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Chapter 5

The role of Melanocortin 3 Receptor Gene in Childhood Obesity

Summary

Melanocortin 3 receptor (MC3R) plays a critical role in weight regulation of rodents, but its role in humans remains unclear. To identify genetic variants of the *MC3R* gene and determine its association with childhood obesity, we screened 201 obese children for *MC3R* gene mutations, with anthropometric measurements, blood tests, feeding behaviour and body composition assessment. We identified three novel heterozygous missense mutations (Ile183Asn, Ala70Thr, and Met134Ile) in three unrelated subjects, which were not found in 188 controls, and two common polymorphisms Thr6Lys and Val81Ile (single nucleotide polymorphisms). *In-vitro* functional studies of the resultant mutant receptors revealed impaired signaling activity but normal ligand binding and cell surface expression. The heterozygotes demonstrated higher leptin levels and adiposity, and less hunger, compared to obese controls, reminiscent of the *MC3R* knockout mice. Family studies showed that these mutations may be associated with childhood or early onset obesity. The common variants Thr6Lys and Val81Ile were in complete linkage disequilibrium, and *in-vitro* studies revealed reduced signaling activity compared to wildtype MC3R. Obese subjects with the 6Lys/81Ile haplotype had significantly higher leptin levels, percentage body fat, and insulin sensitivity, and the causative role of the 6Lys/81Ile variants is supported by the presence of an additive effect, where heterozygotes had an intermediate phenotype compared to homozygotes. *MC3R* mutations may not result in autosomal dominant forms of obesity, but may contribute as a predisposing factor to childhood

obesity, and exert an effect on the human phenotype. Our study supports the role of *MC3R* in human weight regulation.

Introduction

The melanocortin pathway mediates leptin action and regulates energy balance by inhibiting feeding, increasing energy expenditure and reducing energy storage. The Melanocyte Stimulating Hormone (MSH) is the principal agonist of the neuronal melanocortin receptors, melanocortin 3 receptor (MC3R) and melanocortin 4 receptor (MC4R), both of which are critical for weight regulation in rodents (Butler et al., 2000; Chen et al., 2000; Huszar et al., 1997).

MC3R is a seven-transmembrane G-protein coupled receptor (Gantz et al., 1993a) expressed in hypothalamic nuclei known to regulate energy homeostasis. It exhibits a more restricted distribution than MC4R in the central nervous system (Roselli-Rehfuss et al., 1993), and has a dominant role in inhibition of energy storage (Butler et al., 2000; Chen et al., 2000). *Mc3r*$^{-/-}$ mice homozygous for knockout mutations of *MC3R* gene had increased body fat (Butler et al., 2000; Chen et al., 2000) not caused by increased food intake but by increased feed efficiency. The *Mc3r*$^{-/-}$ mice were hypophagic with hyperleptinemia compared to wildtype littermates (Chen et al., 2000). These mice were unusually susceptible to high fat diet-induced obesity, and were relatively inactive, which partly explained the obesity. The mice showed no perturbations in metabolic rate, thyroid hormone levels, respiratory exchange ratio or body temperature. Male mice developed mild hyperinsulinaemia. Mice lacking both MC3R and MC4R have

exacerbated obesity, which supports the notion that both are important and non-redundant (Chen et al., 2000).

MC4R mutations causing human obesity are well described (Farooqi et al., 2003; Vaisse et al., 2000), but the search for human *MC3R* mutation has been largely unsuccessful (Feng et al., 2005; Hani et al., 2001; Li et al., 2000; Schalin-Jantti et al., 2003; Yiannakouris et al., 2004). In this chapter, we report three novel missense mutations in three unrelated probands, as well as the association of the common *MC3R* variants with obesity related phenotypes in our cohort of obese children (Lee et al., 2007b; Lee et al., 2002).

Methods

Study subjects and assessment

The MC3R gene was analysed in 201 unrelated children and adolescents with early onset severe obesity, recruited from the general clinics of the nationwide School Health Service. Inclusion criteria were percentage ideal weight for height (WFH) of more than 140%, and onset of obesity before 6 years of age. There were 128 boys (63.7%); ethnic distribution was as follows: Chinese 105 (52.2%), Malay 69 (34.3%), Indian 21 (10.4%) and Others 6 (3%). The mean (SD) age was 11.1 (3.0) years, WFH 170.5 (22.7) %, BMI 31.9 (5.5) kg/m^2, and % body fat 40.7 (5.2) % by dual energy x-ray absorptiometry (DEXA) or 45.7 (9.1) % by bipedal bioimpedance analysis for body fat composition (BIA). This research was approved by our hospital's Research and Ethics Committee, and informed written consent was obtained from all subjects and parents.

Blood samples were obtained in the fasted state. Leptin was measured using DSL-10-23100 Human Leptin Enzyme-Linked Immunosorbent Kit (Diagnostic Systems Laboratories, Texas). Insulin resistance was calculated using the homeostasis model assessment (HOMA) (Matthews et al., 1985), and insulin sensitivity by Quantitative Insulin Sensitivity Check Index (QUICKI) (Katz et al., 2000). Bone density and body fat composition were assessed using DEXA (Norland DEXA model XR-36, Fort Atkinson, Wisconsin). BIA was performed using the portable Tanita Body Composition Analyzer (TBF-300GS, Tanita Corporation, Tokyo).

Qualitative food-intake evaluation was performed using the Three-Factor Eating Questionnaire, a psychometric instrument to study eating behaviour, measuring three dimensions: restraint, disinhibition and hunger (Stunkard and Messick, 1985). Restrained eating is defined as the tendency to restrict food intake in order to control body weight. Disinhibition is the inability to resist emotional and social eating cues. Hunger is the subjective feeling of hunger. Numeric scores for each of the three factors were derived for each subject. Data concerning estimation of their physical activities were also obtained by using a physical activity questionnaire modified from the Modified Activity Questionnaire for Adolescents and validated in our local population (Aaron et al., 1995; Schmidt et al., 1998), and each subject was categorized depending on physical activity for the past month: categories 1 (inactive), 2 (relatively inactive), 3 (light physical activity), 4 (moderately active), and 5 (vigorous physical activity).

DNA analysis

Genomic DNA was extracted from peripheral leukocytes using standard techniques. The single exon was amplified by polymerase chain reaction (PCR) using the following primers: forward primer 5'-GGGAGACAGAAGGAAGACAGC-3', and reverse primer 5'-GAGCAGATGAGCAGACAGGC-3'. The PCR was performed with a thermal cycler using the following conditions: denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C x 45 seconds, annealing at 57°C x 45 seconds, elongation at 72°C x 1 minute, followed by final elongation at 72°C for 5 minutes. The amplicons were directly sequenced in 2 fragments using the following sequencing primers: 5'-TCTCTCTACCCTCCCCATCC-3' and 5'-TGCAACCTCCTGGCCATCGC-3'. Sequencing was performed using dRhodamine Terminator Cycle Sequencing Kit (PE Applied Biosystems) and analysed on the ABI 377 automated sequencer. All mutations found were verified by reverse sequencing.

Three novel mutations, Ile183Asn, Ala70Thr, and Met134Ile were found during the screening process. 188 genomic DNA samples from 99 healthy children with normal height and weight [mean age (SD) = 7.1 (4.6) years; mean BMI (SD) = 16.8 (3.4) kg/m^2; 63 males], and 89 healthy adults (mean BMI < 25 kg/m^2, 30 males) were analysed as normal controls. The three novel mutations were not found in these 188 normal controls. Allele-specific PCR for Ile183Asn was used to amplify mutant *MC3R* gene with Ile183Asn (T548A) in these control samples. Allele-specific PCR was also used to study the pedigree with the mutation. Multiplex PCR by concomitant PCR amplification of a marker DNA is performed to exclude PCR failure (a short fragment of MC4R gene). Primers for mutant-

specific PCR for Ile183Asn: forward primer 5'-TCTCTCTACCCTCCCCATCC-3', reverse (selective) primer 5'-GTA GCGGAGCGCGTAAAAGT-3'. Primers for marker DNA: forward primer 5'-CTGCTTTCAATTGCAGTGGA-3', reverse primer 5'-ATGGTCAAGGTAATCGCTCC-3'. PCR conditions: denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C x 45 seconds, annealing at 54°C x 45 seconds, elongation at 72°C x 45 seconds, followed by final elongation at 72°C for 5 minutes. The other methods used are restriction enzyme digest using *Hha* I for Ala70Thr (G208A) and allele-specific PCR for Met134Ile using similar conditions as above.

Two common variants Thr6Lys and Val81Ile were always found together, and we confirmed they resided on the same allele (haplotype) in the heterozygotes. Allele specific PCR of Thr6 and Lys6 alleles were performed using selective forward primers, and amplified DNA was then incubated with *Bse*DI. Only amplicons with Val81 (triplet GTT) would be cut.

In-vitro receptor function studies

Construction of *MC3R* plasmids and expression

Wild type, variant (Thr6Lys and Val81Ile) and mutant *MC3R* (Ala70Thr, Met134Ile and Ile183Asn) were directly amplified from individual genomic DNA using Pfx DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA). Forward and reverse primers were designed with *Hin*d III and *Xba* I enzyme restriction sequences at the 5' end. The *Renilla* luciferase reporter gene was amplified from the phRG-TK reporter vector (Promega Corp, Madison, WI) with a pair of primers containing the *Kpn* I and *Xho* I

restriction sites at the 5' end. All generated amplicons were TA cloned into the pDrive vector using the QIAGEN PCR Cloning Plus Kit (Qiagen GmbH, Hilden, Germany).

All *MC3R* inserts were excised from the pDrive/*MC3R* constructs using *Hin*d III and *Xba* I, and ligated into the pBudCE4 mammalian dual expression vector (Invitrogen Life Technologies, Carlsbad, CA) under the control of the CMV promoter while the *Renilla* luciferase reporter gene was ligated into the *Kpn* I and *Xho* I site of the vector under the control of the EF-1α promoter. For creating stable cell lines expressing the receptors, amplified *MC3R* gene was TA cloned into the pcDNA5/FRT/V5-His vector (Invitrogen Life Technologies, Carlsbad, CA).

One day prior to transfection, subconfluent Griptite human embryonic kidney cells (Invitrogen Life Technologies, Carlsbad, CA) were trypsinized and seeded in 96-well plates at a density of $1x10^4$ cells/well. Transient transfection was performed using the Effectene transfection reagent (Qiagen GmbH, Hilden, Germany) with 90 ng pCRE-Luc (Stratagene, La Jolla, CA) and 10 ng pBudCE4/*MC3R*/*Renilla* construct per well.

Stably expressed MC3R cells were created with the Flp-In System (Invitrogen Life Technologies, Carlsbad, CA) as recommended by the manufacturer. Briefly, pcDNA5/FRT/V5-His/*MC3R* and pOG44 vectors were transfected into Flp-In 293 cells at a ratio of 9 to 1. After 48 hours, the cells were trypsinized and seeded into T75 flasks containing complete DMEM media and 100 ug/ml hygromycin B for selection.

MC3R stimulation, luciferase assay, and dimerisation

24 hours after transient transfection, cells were washed with PBS and stimulated with α-MSH (Sigma-Aldrich, St. Louis, Missouri, USA) with concentrations ranging from 1 pM to 1 uM in DMEM media containing 0.2% BSA for 6 hours. Cells were then washed and lysed with 1x passive lysis buffer, then assayed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega Corp, Madison, WI) in the TD-20/20 luminometer (Turner Biosystems Inc, Sunnyvale, USA). cAMP induced firefly luciferase readings from pCRE-Luc expression were normalized with *Renilla* luciferase readings expressed from the pBudCE4/*MC3R*/*Renilla* plasmid construct. Normalized luciferase readings were then expressed as fold activity over basal (non-stimulated transfected cells). Data points were fitted by non-linear regression analysis using GraphPad Prism software (version 4.00 for Windows, GraphPad, San Diego, California, USA).

To investigate effects of receptor dimerization between wild type and mutant receptors, stable cells expressing wild type receptors were transfected with pBudCE4/mutant constructs, pCRE-Luc and phRG-TK reporter vector. Receptor stimulation and luciferase assay were then performed similarly as above.

Membrane preparation

Stable cell lines expressing receptors were grown in selective media until subconfluent. Cells were washed with ice cold PBS and scraped off, pelleted, then resuspended in 2 mls of ice cold homogenization buffer (20 mM HEPES, pH 7.0, 1 mM MgCl2, 1 mM EDTA, 1 mM PMSF). The cell suspension was homogenized in Dounce glass homogenizer on ice.

The homogenate was centrifuged at 1700xg for 10 minutes at 4^0C, the supernatant was then collected, and centrifuged at 40,000xg for 40 minutes at 4^0C. The pellet containing crude cell membranes was then resuspended in homogenization buffer, aliquoted and stored at –80^0C immediately. Protein concentration of crude membranes was determined using Bio-Rad's Protein Assay reagent (Bio-Rad, Hercules, CA) with BSA as the standard.

Receptor binding studies

Binding studies were performed using time-resolved fluorometry (TRF) technology with europium labeled ligand. 24 hours after transient transfection in 96-well plates, cells were washed once in PBS and incubated for two hours with increasing concentrations of europium labeled NDP-αMSH (PerkinElmer, Boston, MA) in binding buffer (25 mM HEPES, pH 7.0, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 100 mM NaCl, 25 uM EDTA, 0.2% BSA). Non-specific binding was determined in the presence of 3 μM unlabeled NDP-αMSH. After incubation, cells were washed four times with wash buffer (50 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$) and incubated with 100 uL/well of DELFIA Enhancement Solution (PerkinElmer, Boston, MA) at room temperature for 15 minutes. Fluorescence were measured in the Wallac Victor^2-V 1420 multilabel HTS counter (PerkinElmer, Boston, MA) fitted with filters for europium TRF measurements.

Saturation studies using crude cell membranes were performed similarly in 100 ul of HEPES binding buffer using 2 ug of membrane protein, in Acrowell filter plates (Pall Corp, East Hills, NY). Reactions were terminated by rapid filtration using a vacuum

manifold with four washes of wash buffer before the addition of the DELFIA enhancement solution.

Competitive ligand binding studies were performed in 100 ul of HEPES binding buffer containing 2 ug of crude membranes, europium labeled ligand at a concentration close to the Kd values and competitor ligands (NDP-αMSH and α-MSH). Incubation conditions were similar as for saturation studies and plates were processed as described above.

Immunofluorescence staining of *MC3R*

Griptite 293 cells grown on glass coverslips were transiently transfected with 200 ng of pBudCE4/*MC3R*/*Renilla* using the Effectene reagent. After 48 hours incubation, the cells were washed once in PBS and fixed with 3.7% paraformaldehyde for 15 mins at room temperature. The cells were washed three times with PBS and then incubated with blocking buffer (5% BSA, 2% FBS) for 30 minutes at room temperature. After rinsing in PBS, cells were incubated with polyclonal rabbit anti-MC3R IgG (Santa Cruz Biotechnology, Santa Cruz, CA) in 1% BSA for 2 hours at room temperature. Cells were washed 4 times with PBS and then incubated with goat anti-rabbit IgG conjugated with Alexa fluor 488 dye (Molecular Probes Inc, Eugene, OR) in 1% BSA for 1 hour at room temperature. Cells were then subjected to 5 washes with PBS and coverslips were mounted with ProLong Gold Antifade reagent (Molecular Probes Inc, Eugene, OR). Fluorescence was visualized with the Carl Zeiss Axioskop microscope (100x).

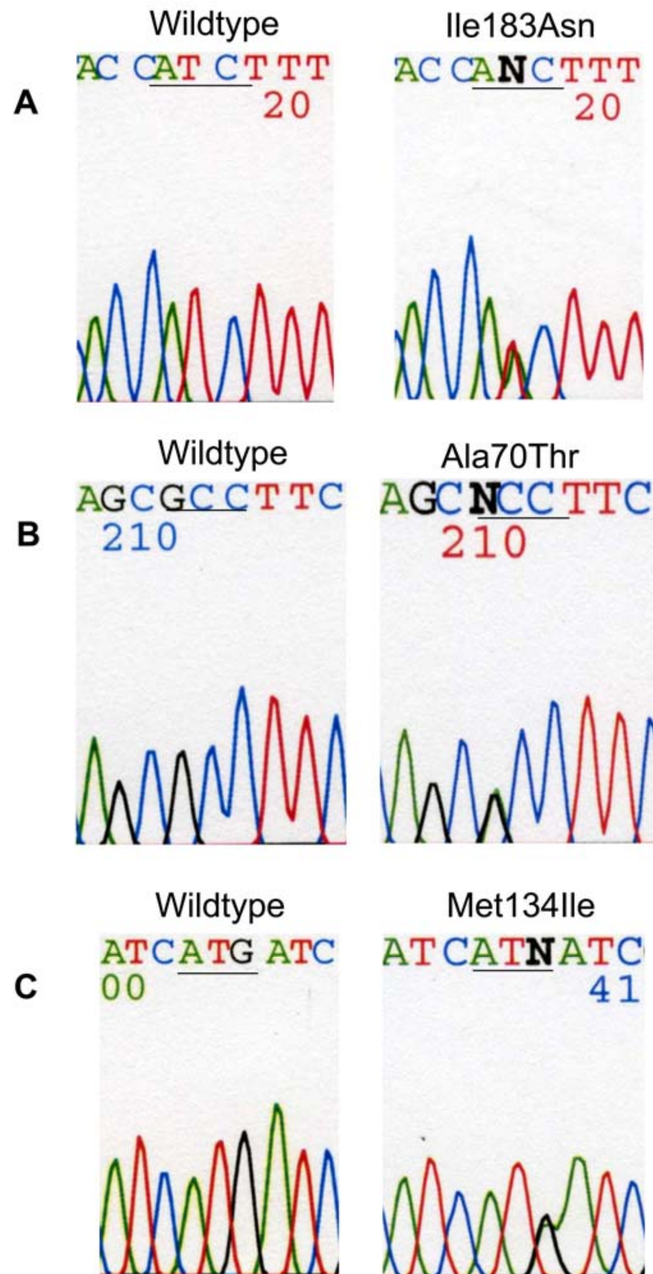
Statistical analysis

Comparison of parameters was performed with Student's *t* test, General Linear Model with covariates analyses to adjust for age, gender and ethnicity, One way ANOVA trend analysis, Mann Whitney U test, Fisher's exact test and chi-square test where appropriate (SPSS Inc., Chicago, Ill., USA). Graph fit curves and EC_{50}/IC_{50} were obtained and performed using GraphPad Prism 4 for Windows (ver 4.02 GraphPad software, San Diego, California, USA), and curves were fitted using the logistical equation. Ki values were determined from IC_{50} values using the equation of Cheng and Prusoff (Cheng and Prusoff, 1973).

Results

We found three rare variants or potentially novel missense mutations, Ile183Asn (T548A), Ala70Thr (G208A), and Met134Ile (G402A), in the heterozygous state in three unrelated subjects (figures 5-1A, B and C), as well as two common variants Thr6Lys and Val81Ile. The three potentially novel mutations Ile183Asn, Ala70Thr, and Met134Ile were not found in 188 non-obese normal controls.

Figure 5-1 A. Sequencing of the *MC3R* gene in the proband revealed T to A transition in heterozygous state at nucleotide position 548 (N), leading to the substitution of isoleucine by asparagine at codon position 183. **B.** G to A change in heterozygous state at nucleotide position 208 causing change of alanine to threonine at codon position 70. **C.** G substitution by A in heterozygous state at nucleotide position 402, leading to change of methionine to isoleucine at codon 134.



Common variants

We confirmed the Thr6Lys (17C>A) and Val81Ile (241G>A) variants were in complete linkage disequilibrium and located on the same allele (haplotype) in our cohort and normal controls. Of 198 subjects (excluding the three with novel variants), 121 were homozygous for the wildtype allele, 70 subjects were heterozygous, and 7 subjects were homozygous for the allele (haplotype) with common variants. 88 DNA samples from the 99 non-obese children (controls) were randomly selected for genotyping for the common variants, and 5 DNA samples failed to yield satisfactory PCR products or sequence readings. Among these 83 non-obese children, 31 were found to be heterozygous and 2 homozygous for the 6Lys/81Ile haplotype (henceforth identified as 6K/81I). No significant difference in genotypic frequencies was detected when compared to the obese group (p=0.843).

Obese subjects with 6K/81I had significantly higher leptin levels, percentage body fat, and insulin sensitivity index QUICKI, with lower insulin resistance index HOMA. The causative role of the 6K/81I variants is further supported by the presence of an additive effect, where the heterozygotes had an intermediate phenotype compared to homozygotes (table 5-1).

Table 5-1. Additive effect of the 6K/81I variants on the adiposity, leptin levels and insulin resistance indices, in (A) all subjects, and (B) Chinese subjects.

(A) all subjects

	Wildtype	Heterozygous for 6K/81I	Homozygous for 6K/81I	*p*-value[#]	*p*-value[+]
N	121	70	7	-	-
Age (years)	11.0 (3.0)	11.2 (3.0)	12.2 (3.5)	-	0.371 (ns)
WFH (%)	168.5 (18.6)	172.0 (24.8)	174.1 (21.0)	0.495 (ns)	0.222 (ns)
Leptin (mcg/L)	45.4 (31.3)	54.9 (37.9)	82.6 (64.8)	0.041	<0.001
body fat (%)	44.4 (9.0)	48.7 (8.7)	54.1 (14.5)	0.002	<0.001
HOMA	5.180 (1.914)	5.076 (2.397)	2.707 (2.060)	0.031	0.042
QUICKI	0.303 (0.025)	0.306 (0.035)	0.331 (0.035)	0.016	0.047

All values are expressed as mean (standard deviation).

[#]General linear model with age, gender and race as covariates

[+]One-way ANOVA trend analysis.

(B) Chinese subjects

	Wildtype	Heterozygous for 6K/81I	Homozygous for 6K/81I	*p*-value#	*p*-value+
N	64	38	3	-	-
Age (years)	11.5 (3.0)	11.6 (2.7)	12..0 (2.4)	-	0.71
WFH (%)	167.3 (18.0)	165.0 (19.0)	166.0 (15.4)	0.841	0.818
Leptin (mcg/L)	41.5 (31.5)	45.1 (32.7)	48.8 (4.0)	0.07	0.091
body fat (%)	44.4 (8.9)	48.8 (8.7)	55.3 (13.3)	0.002	<0.001
HOMA	5.975 (1.851)	5.701 (1.944)	2.494 (1.576)	0.018	0.069
QUICKI	0.297 (0.023)	0.299 (0.025)	0.334 (0.023)	0.009	0.038

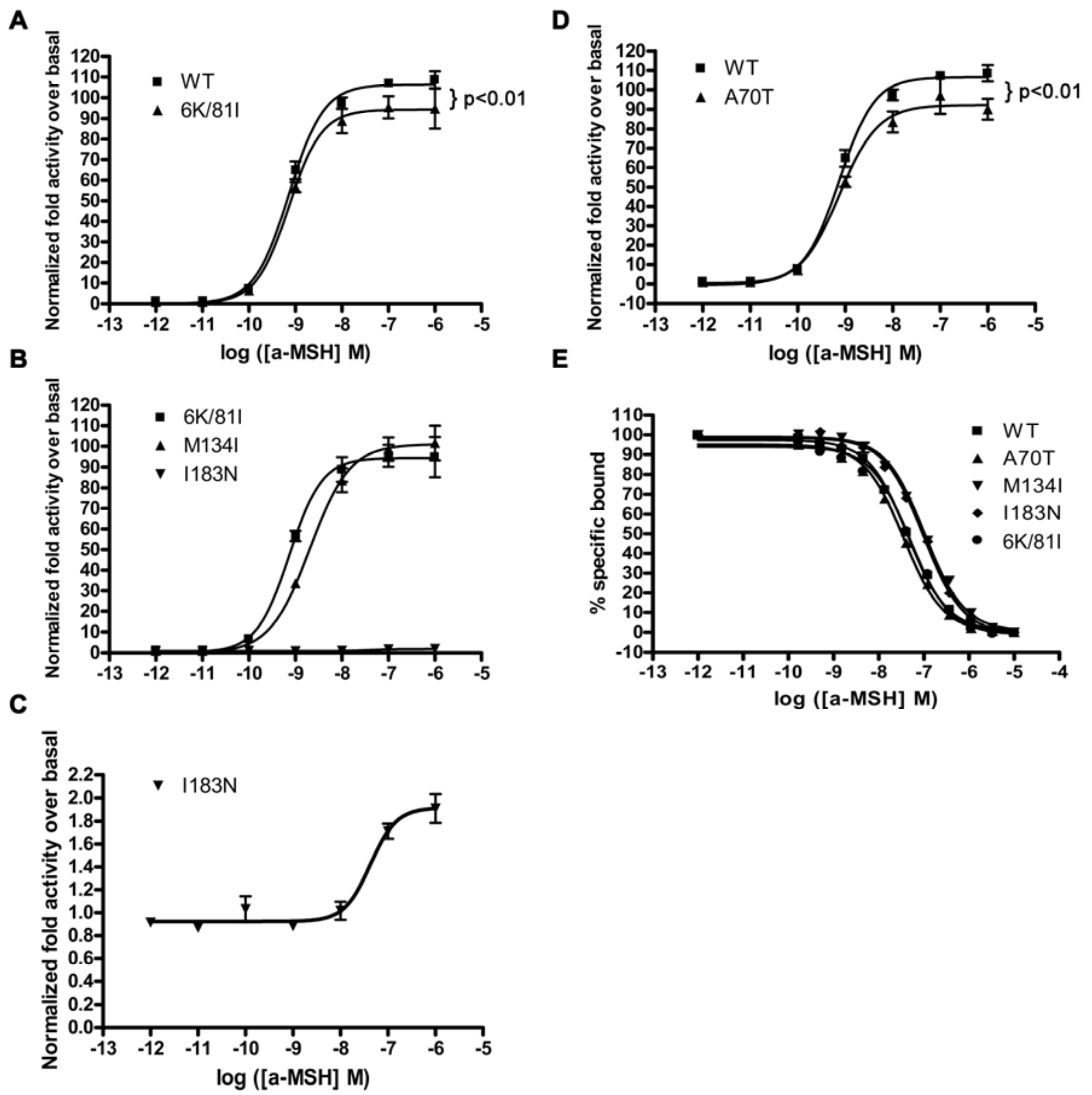
All values are expressed as mean (standard deviation).

#General linear model with age and gender as covariates.

+One-way ANOVA trend analysis.

In addition, the obese subjects heterozygous and homozygous for 6K/81I had significantly lower triglyceride levels (1.19 + 0.50 mmol/L vs. 1.41 $\pm$ 0.73 mmol/L, p=0.012) and lower fasting glucose levels (4.4 $\pm$ 0.5 mmol/L vs. 4.8 $\pm$ 0.8 mmol/L, p=0.024) compared to obese subjects with wildtype *MC3R* alleles. We did not detect any significant differences in other parameters, including insulin and c-peptide levels, insulin-glucose ratios, frequency of diabetes mellitus/impaired glucose tolerance, physical activity pattern, or feeding behaviour scores. Our clinical findings are supported by *in-vitro* studies which revealed a modest but significant decrease (p<0.01) in maximal activity of the MC3 receptor with variants 6K/81I compared to wildtype MC3 receptor (figure 5-2A), without any significant difference in binding affinity to α-MSH or NDP-MSH (figure 5-2E). Based on the *MC3R* knockout mouse model, reduced MC3 receptor activity is expected to lead to increased body fat (feed efficiency), and leptin levels (Butler et al., 2000; Chen et al., 2000).

Figure 5-2. Constructs with wildtype and variant *MC3R* were co-transfected with pCRE-Luc into HEK293 cells and stimulated with varying amounts of α-MSH. Y axis represents the fold increase over basal Relative Light Units (RLU), which is the ratio of luminescence generated by Firefly luciferase (from pCRE-Luc) to Renilla luciferase (from pBudCE4/Renilla/*MC3R* construct); the data is expressed as such to normalize for transfection efficiency and amount of MC3R produced. This represents the amount of cyclic AMP generated with increasing concentration of the ligand α-MSH (represented by x-axis). Each data point represents the mean and standard error range of at least 3 independent experiments performed in triplicates. **A.** There is a significant reduction in 6K/81I MC3 receptor compared to wildtype (normalized fold activity over basal 94.42 [95% CI 88.26 – 100.6] vs. 106.2 [95% CI 102.4-110.1]; $p<0.01$) **B.** There was severely impaired generation by the Ile183Asn mutant MC3 receptor (with 6K/81I) compared to the MC3R with 6K/81I. The Met134Ile mutant MC3 receptor (with 6K/81I) dose response curve is shifted to the right, with a significantly higher EC_{50} value compared to receptor with 6K/81I (2.051×10^{-9} [95% CI 1.358×10^{-9} – 3.097×10^{-9}] vs. 7.408×10^{-10} [95% CI 5.008×10^{-10} – 1.096×10^{-9}]; $p=0.0005$). **C.** Ile183Asn mutant receptor actually exhibits a small response to MSH stimulation. Note magnified scale of y-axis. **D.** Ala70Thr mutant receptor has significantly reduced response to MSH stimulation compared to wildtype MC3R (normalized fold activity 92.7 [95% CI 86.0-99.3] vs. 106.2 [95% CI 102.4-110.1]; $p<0.01$). **E.** Competitive ligand binding studies using crude membranes and europium labeled NDP-MSH at a concentration close to the Kd values and competitor αMSH. Data is expressed as a percentage of the maximum counts of europium labeled NDP-αMSH binding to MC3R. Each point represents the mean and standard error range of at least 6 independent experiments in triplicates.

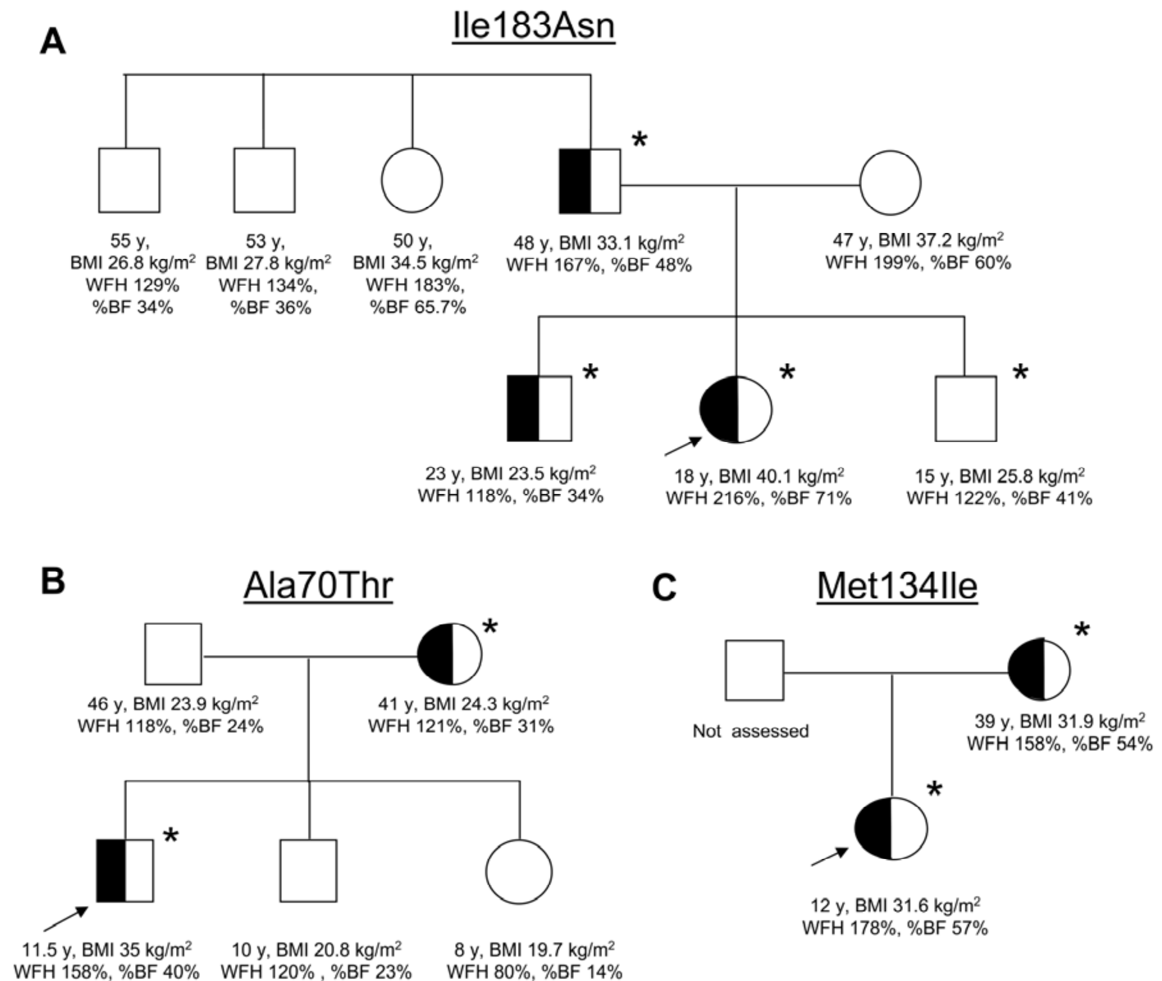

A
Normalized fold activity over basal
WT
6K/81I
} p<0.01
log ([a-MSH] M)
B
6K/81I
M134I
I183N
C
I183N
D
WT
A70T
} p<0.01
E
% specific bound
WT
A70T
M134I
I183N
6K/81I

Ile183Asn

This novel mutation was found in an Indian family, and is the first report of human *MC3R* mutation (Lee et al., 2002). A 13-year-old Indian girl (proband) with early onset severe obesity was heterozygous for this mutation (figure 5-3A). She was first screened at 13 years of age, when she already had severe obesity (WFH 182%) and irregular menstrual cycles due to clinically diagnosed polycystic ovarian syndrome. The proband and her father who was also heterozygous for the same mutation did not have any distinguishing phenotype. Although the proband's percentage body fat was excessive at 49%, it was not distinguishing, when compared to other obese patients in general. Her dietary history was not suggestive of excessive caloric intake, and despite careful dieting she continued to gain weight. Although she had tried to engage in regular physical exercises, she was not able to sustain her efforts. These aspects are reminiscent of the $Mc3r^{-/-}$ mice. The father was previously very obese, but he was more motivated and had engaged in weight losing measures successfully. His WFH was 157% but percentage body fat was only 30% by DEXA; but there was significant hypertriglyceridemia. Her mother and younger brother without the Ile183Asn mutation were available for evaluation at that time, and both were also obese (WFH 170% and 132% respectively), and thus it was impossible to study genotype-phenotype co-segregation. Obesity is such a common condition that, and even within a given family, a varying combination of environmental, behavioural and multiple genetic factors other than *MC3R* mutation can be contributory. Four years later, at 17 years of age, the proband was re-evaluated and found to have developed morbid obesity with high percentage ideal body weight for height (WFH) of 216%, and percentage body fat (%BF) of 71%. Her heterozygous father's WFH has increased to 167%, and %BF was

48%. The proband was the most overweight of the family with the highest %BF. The eldest sibling (not available for the first evaluation four years ago) was found to be heterozygous for this mutation. At 23 years old, he was not obese, with BMI of 23.5 kg/m^2. However, he was overweight as a child. At 7 years old, his weight was documented as 27 kg with WFH of 125% (>120% defined as mild obesity). He only started to lose weight in his teenage years as he was physically active, and underwent army training for two years. He has since maintained a very active lifestyle with regular exercise and sports, and is careful with his diet. The paternal uncles and aunt had late onset obesity or overweight phenotypes, and genotyping revealed wildtype *MC3R* alleles. An interesting observation was the co-segregation of early onset childhood obesity with the mutant *MC3R* allele (figure 5-3A). Among the four siblings (paternal uncles and aunt), the proband's father was the only one who was obese since early childhood. His documented weight and BMI at 16 years were 86 kg and 33 kg/m^2. The other three sibs were overweight only after 30 years of age: the eldest uncle was 64 kg at 18 years of age, and second uncle was 60 kg at 20 years old.

Figure 5-3. Family pedigrees of affected subjects with *MC3R* mutations. The age (in years), body mass index (BMI), percentage of ideal weight for height (WFH), and percentage body fat (%BF) are listed. Heterozygotes are half-shaded. Subjects with history of childhood or early onset obesity are highlighted with asterisk (*). **A.** Family with Ile183Asn mutation. **B.** Family with Ala70Thr mutation. **C.** Family with Met134Ile mutation.



Ala70Thr

The Ala70Thr mutation was found in an 11-year-old boy and his mother (figure 5-3B). The proband was overtly obese, whereas his heterozygous mother had mild obesity (% wt for ht 121%). His mother was overweight as a child, but documented weight and height measurements were unavailable. She has been careful with her diet and exercises regularly, as she is very conscious of her weight and body image.

Met134Ile

The heterozygous Met134Ile mutation was found in a 12-year-old Indian girl and her obese mother, and both mother and child have type 2 diabetes mellitus (figure 5-3C). The proband was asymptomatic but was diagnosed by abnormal oral glucose tolerance test.

The 3 probands were compared to groups of obese controls of similar age and same gender as shown in table 5-2. The controls selected were 2-3 years younger or older than the subjects with rare variants. This age range was chosen arbitrarily to include a sizeable number of controls for this comparison. All subjects who fulfilled the criteria are included in the analysis. The probands had significantly higher leptin levels (p=0.018), and also appeared to have higher BMI, WFH and %BF, although these did not reach statistical significance. We also observed that heterozygotes had significantly lower Hunger scores (less subjective feeling of hunger) compared to that of the obese controls (p=0.026) (table 5-3). The scores for Restrained Eating and Disinhibition were not significantly different between the heterozygotes and the obese controls without *MC3R* mutations (p >0.05 for each factor analysed). This is reminiscent of the *MC3R* knockout mice which were

hypophagic with high leptin levels and body fat mass (Butler et al., 2000; Chen et al., 2000). We did not detect any significant difference in HOMA or insulin/glucose ratio, or physical activity levels.

Impaired signaling activities of the mutant receptors

As wildtype MC3R and MC3R with common variants 6K/81I (haplotype) showed significant differences in response to α-MSH stimulation (Feng et al., 2005), we compared the signaling properties of the mutant MC3Rs to the corresponding MC3R with or without 6K/81I. This is also in consideration of possible conformational changes induced by interaction of these variants. The Ile183Asn and Met134Ile mutant alleles of our two subjects carried the 6K/81I (haplotype) concomitantly, and were thus compared to MC3R with 6K/81I (haplotype) in our transfection studies. This was determined by cloning each of the two *MC3R* alleles from the subject's DNA sample into pDrive vector followed by direct sequencing, and also by allele-specific PCR for Lys6 followed by direct sequencing as described above.

Table 5-2. Comparison of the age, body mass index (BMI), percentage ideal weight for height (WFH), waist-hip ratio (WHR), and percentage body fat (%BF) of each proband with a group of controls of similar age, same gender, and 6K/81I carrier status.

	Met134Ile	controls† N=25	Ala70Thr	controls‡ N=37	Ile183Asn	controls§ N=4	All 3 probands	Controls N=110	*P*
Age (years)	11.9	11.1 (3.2)	11.7	11.1 (1.3)	13.1	13.6 (1.9)	12.3 (0.7)	12.3 (1.7)	0.929
BMI (kg/m^2)	31.6	30.7 (3.6)	35.0	32.0 (3.6)	36.6	33.1 (5.7)	34.4 (2.6)	33.5 (5.1)	0.769
WFH (%)	178.0	166.1 (19.8)	173	166.5 (15.8)	182	180.5 (22.9)	177.7 (4.5)	171.0 (23.2)	0.633
WHR	0.990	0.890 (0.072)	0.972	0.950 (0.062)	0.954	0.870 (0.035)	0.981 (0.013)	0.926 (0.072)	0.281
%BF	57.3	47.0 (6.7)	40.0	41.1 (3.9)	61.3	58.4 (12.5)	52.2 (12.5)	47.8 (9.4)	0.428
Leptin (mcg/L)	116.4	53.7 (36.4)	71.9	45.8 (29.6)	142.8	100.9 (75.1)	110.4 (35.8)	54.7 (39.7)	0.018*

Values expressed as mean (standard deviaton).

† Females selected based on age 3 years above or below 11.9 years, and heterozygous for 6K/81I, as subject with Met134Ile is heterozygous for 6K/81I.

‡Males selected based on age 2 years above or below 11.7 years, and with wildtype *MC3R* alleles 6T/81V, as subject with Ala70Thr is homozygous for wildtype allele.

§Females selected based on age 2 years above or below 13.1 years, and homozygous for 6K/81I, as subject with Ile183Asn is homozygous for 6K/81I.

All controls that fulfilled age criteria are included in the analysis.

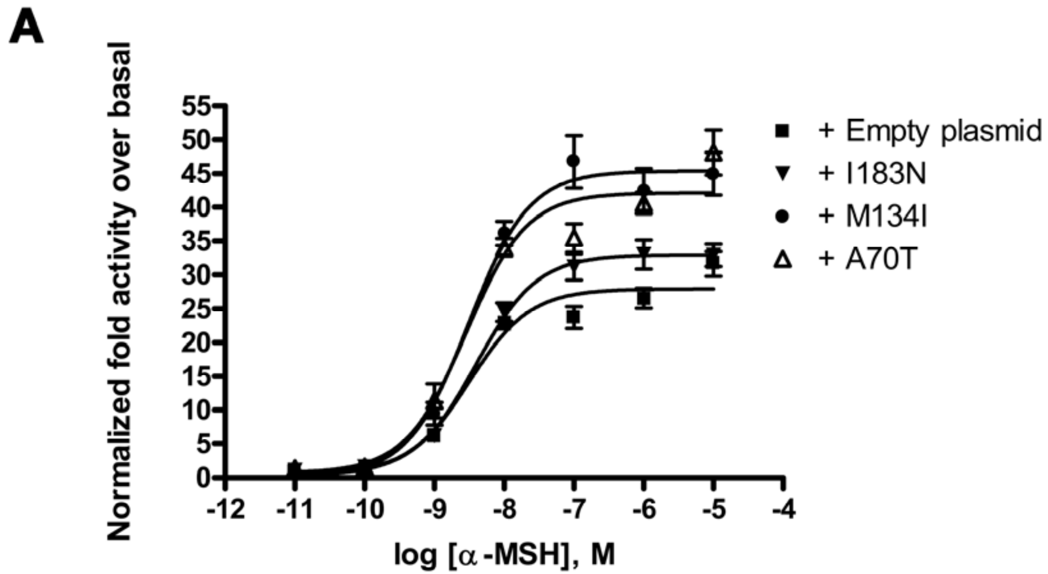
Table 5-3. Feeding behaviour assessment of the three probands and obese heterozygote parents, compared to obese controls without *MC3R* mutations.

	Obese subjects without *MC3R* mutations (n=161)	Proband Ile183Asn	Father Ile183Asn	Proband Ala70Thr	Proband Met134Ile	Mother Met134Ile	All heterozygotes
Restraint	9 (0-20)	11	16	7	9	11	11 (7-16)
Disinhibition	7 (0-14)	2	8	6	4	6	6 (2-8)
Hunger	7 (0-14)	3	4	7	3	2	3 (2-7)*

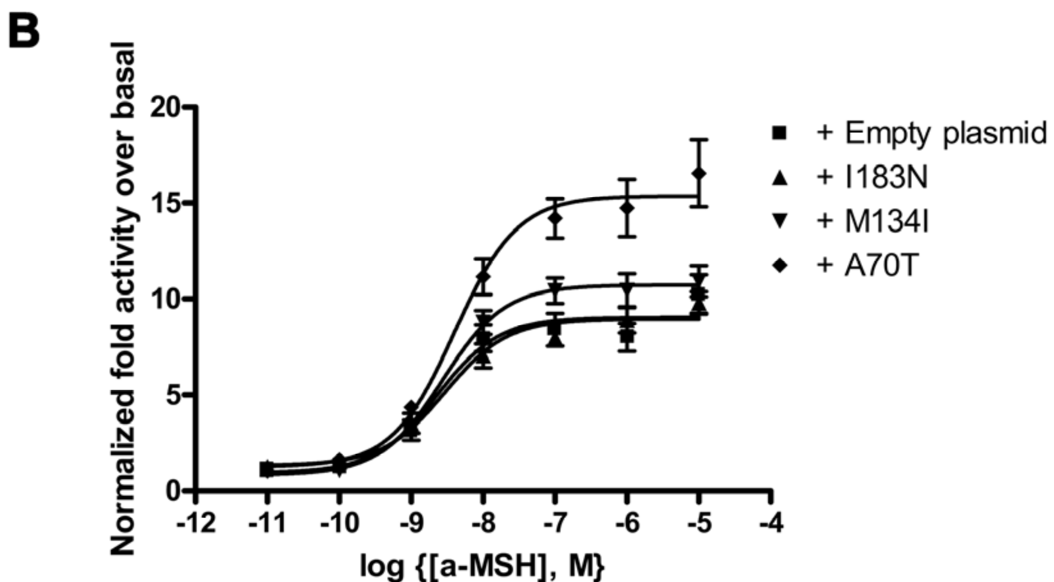
Scores expressed as median (range).

Both transient and stable (not shown) transfection studies revealed severely impaired signaling response of the Ile183Asn mutant receptor to α-MSH. The cells transiently expressing the mutant MC3 receptor Ile183Asn showed negligible response to increasing α-MSH concentration (figures 5-2B and C). Mutant receptor Met134Ile demonstrated a modest but significantly higher EC_{50} compared to MC3R with 6K/81I (figure 5-2B). The mutant Ala70Thr receptor also demonstrated significantly reduced cAMP response to MSH stimulation (figure 5-2D). Our competitive ligand binding studies did not find any significant differences in IC_{50} between the wildtype, 6K/81I, Ala70Thr, Met134Ile and Ile183Asn MC3 receptors (figure 5-2E). The three mutant MC3 receptors did not exert any dimerisation effect on wildtype or 6K/81I MC3 receptors (figure 5-4). The findings were similar using NDP-MSH instead of α-MSH (data not shown). Immunofluorescence staining of HEK cells transiently transfected with these variant MC3 receptors revealed good level of cell surface expression comparable to wildtype MC3R qualitatively (figure 5-5).

Figure 5-4 Mutant MC3 receptors were transfected into stable cell lines expressing **a.** wildtype MC3R. **b** MC3R with 6K/81I. There was no significant reduction in cAMP generation by these cells to suggest dimerisation as a result of concomitant expression of the mutant receptors.

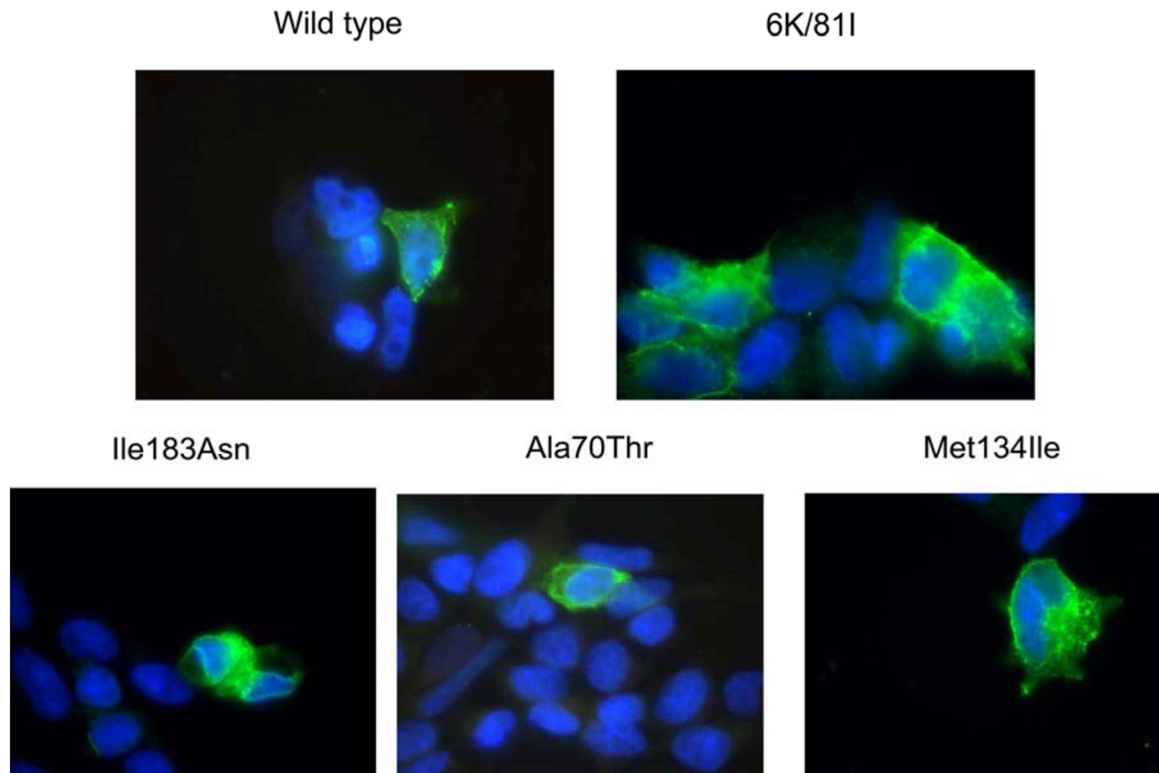


Mutant receptors transfected into stable cell lines expressing wild type MC3R



Mutant receptors transfected into stable cells expressing MC3R with 6K/81I

Figure 5-5. Cell surface expression of MC3R detected by immunofluorescence staining. Griptite 293 cells were grown in coverslips and transiently transfected with indicated wild type and variants. Cells were not permeabilized and stained with anti-MC3R antibody followed by secondary antibody conjugated to Alexa 468 dye.



Discussion

Common obesity is a polygenic trait resulting from interaction of multiple genetic loci with the environment. Sequence variants in a large set of genes implicated in energy regulation could predispose an individual to excessive weight gain in a given environment. While *MC3R* mutations are unlikely to result in an autosomal dominant form of monogenic obesity, this study provides evidence that *MC3R* can be one of the predisposing genes which contributes to increased adiposity, and the wide variation in the adiposity of the individuals with common and rare variants may be due to other modifying genetic and environmental factors.

MSH, a derivative of pro-opiomelanocortin (POMC), acts on MC4R and MC3R to reduce feeding and feed efficiency. The *Mc3r*$^{-/-}$ mice demonstrated mild obesity but increased body fat and leptin, while the phenotype of the heterozygous *Mc3r*$^{+/-}$ mice did not appear to differ significantly from wildtype mice (Butler et al., 2000; Chen et al., 2000). In comparison, the *Pomc*$^{-/-}$ mice also exhibit an obese phenotype while the heterozygous *Pomc*$^{+/-}$ mice appeared to have a similar phenotype to wildtype mice on standard chow (Challis et al., 2004; Yaswen et al., 1999). However, *Pomc*$^{+/-}$ mice developed obesity when put on a high-fat diet (Challis et al., 2004), exhibiting an intermediate obese phenotype between wild-type and *Pomc*$^{-/-}$ mice, demonstrating that a single functional copy of the *POMC* gene is not sufficient for maintaining normal energy homeostasis under certain environmental conditions, and haploinsufficiency can interact with dietary factors to increase body weight. There is evidence to suggest that loss of one *POMC* allele, and even genetic variants with subtle effects on POMC function, could influence susceptibility to

obesity in humans in our modern ‘obesogenic’ environment. Heterozygous parents of obese children homozygous for *POMC* null mutations had high normal to high BMI (Krude et al., 2003), and other family members heterozygous for *POMC* null allele were more overweight than those with wildtype alleles (Farooqi et al., 2006). Heterozygous partially inactivating *POMC* mutations Arg236Gly and Tyr221Cys were also found more frequently in obese children, and the phenotype is reminiscent of MC4R deficiency (Challis et al., 2002; Lee et al., 2006). Therefore, partially inactivating genetic variants of *MC3R* may likewise exert a significant effect on the phenotype even in the heterozygous state, in the ‘obesogenic’ environment. This notion was supported by the linkage of a locus encoding *MC3R* on human chromosome 20q13.2 to the regulation of BMI, subcutaneous fat mass and fasting insulin (Lembertas et al., 1997). We report common and rare novel variants at the *MC3R* locus which result in partially reduced activity of the MC3R in response to MSH, and demonstrate that the common variants, and possibly the rare variants, are associated with increased body fat and leptin levels (with additive effect), and perhaps decreased hunger, in human subjects, congruous with the phenotype of the *Mc3r*$^{-/-}$ mice. Of note is the presence of childhood or early onset obesity in all the carriers of the rare mutations (figure 5-3), although the significance of this is uncertain.

There were varying reports of increased insulin-glucose ratio, HOMA, leptin, BMI, and body fat in humans with 6K/81I (Feng et al., 2005; Schalin-Jantti et al., 2003; Yiannakouris et al., 2004). Our finding of decreased insulin resistance despite increased body fat in subjects with 6K/81I appeared counterintuitive. However, our finding is supported by a recently reported murine study comparing *mc4r*$^{-/-}$ and *mc3r*$^{-/-}$ mice which

developed severe obesity when fed with high fat chow, and showed that the *MC4R* knockout mice developed low adiponectin and increased insulin resistance as expected, but the *MC3R* knock mice maintained relatively normal adiponectin levels with mild insulin resistance only (Trevaskis et al., 2007). This interesting murine phenotype lend support to our finding and suggests that this improved insulin resistance may be mediated by adiponectin, and this is an obvious area of further research for our cohort. Our report reinforced the evidence that these two variants contribute significantly to the human phenotype by demonstrating a significant additive effect of 6K/81I on adiposity and leptin. Feng et al reported that the MC3R with 6K/81I resulted in reduced receptor activity, binding affinity, and protein expression compared to wildtype MC3R (Feng et al., 2005). Tao et al however reported there was no difference in receptor activity and ligand binding affinity for these two variants (Tao and Segaloff, 2004). Our report has furthered this debate; we found that MC3R with 6K/81I had reduced receptor activity in response to MSH as reported by Feng, but we did not detect any difference in ligand binding, and there was qualitative evidence that the variant MC3R was well expressed on the cell surface.

The three rare variants were not found in other similar studies (Feng et al., 2005; Hani et al., 2001; Li et al., 2000; Schalin-Jantti et al., 2003; Yiannakouris et al., 2004). Isoleucine residue 183 of MC3R is located in the second intracytoplasmic loop of this G-protein coupled receptor (Gantz et al., 1993a). Isoleucine at codon 183 is a highly conserved residue, present in the *MC3R* sequence of many other species, from teleost fish through to mammals (accession numbers: Zebrafish NP_851303, Chicken BAA32555, Mouse AAI03670, Rat NP_001020441, Human NP063941). This hydrophobic

isoleucine was substituted by hydrophilic asparagine in Ile183Asn MC3R. The transmembrane domain directly interacts with G proteins and controls cAMP production; this mutation may cause partial reduction in receptor function resulting from abnormal G protein interaction. After our first communication of this mutation (Lee et al., 2002), Tao et al (Tao and Segaloff, 2004) and Rached et al (Rached et al., 2004) subsequently reported that the Ile183Asn MC3R had total abolished cAMP response to MSH, and Rached also reported a dominant negative effect on wildtype MC3R, and complete intracellular retention with no cell surface expression. Our studies demonstrated near total but not complete loss of cAMP generation in response to MSH stimulation (figure 5-2C), with normal cell surface expression, normal ligand binding affinity, and absence of dominant negative activity on wildtype MC3R.

The Ala70Thr mutation affects the extracellular domain (Gantz et al., 1993a) within the critical region for binding activity (Schioth et al., 1997), where the hydrophobic alanine residue was changed to hydrophilic threonine. The hydrophobic alanine residue at codon 70 is replaced by another hydrophobic residue Glycine in the MC3R of chicken, mouse, rat and spiny dogfish (accession number: AAS66720); thus it may be possible that the substitution by a hydrophilic residue (threonine) may result in a significant change in conformation and function of the receptor. The met134Ile mutation is located at the second transmembrane region of the seventh transmembrane domain. The hydrophobic methionine is conserved in chicken, mouse, and rat.

The three novel rare mutations were not found in the population sample, nor reported elsewhere, and the clinical characteristics of our subjects coupled with the *in-vitro* studies support their pathogenic role. We believe that our report has shed light on the human *MC3R* mutation phenotype, and further studies with bigger numbers will continue to unravel the phenotype and support the role of MC3R in human weight regulation and pathogenesis of obesity.

The data in this chapter is published in Lee, Y.S., Poh, L.K., Kek, B.L., and Loke, K.Y. (2007). The role of melanocortin 3 receptor gene in childhood obesity. Diabetes 56, 2622-2630.

Chapter 6

Unraveling the biology of human weight regulation through human obesity genetic studies: the quest to complete the unfinished jigsaw puzzle

Human and animal genetics studies performed in the quest to elucidate the genes governing obesity have improved our understanding of weight regulation. Whether this knowledge can be effectively applied to clinical practice remains to be seen. What is clear from these genetic studies is that they have challenged our traditional views of the pathogenesis of obesity, and changed our view of obesity as just a psychological disorder arising from sheer gluttony and poor discipline. The multitude of evidence now supports a physiological basis for weight dysregulation, where individuals may be predisposed to varying degree of obesity due to their genetic makeup, especially when exposed to the "right" environmental conditions. Some individuals may be simply predisposed to obesity at multiple genetic levels, and this may also make it very difficult for them to lose weight. Current research reinforces the view that obesity is caused by both genetic and environment determinants.

There are various classifications of human obesity, the commonest of which is the classification into early onset or childhood obesity, and late or adult onset obesity. While the environment, behaviour and genetic make up contributes the obese phenotype, their role and contribution may vary between these two groups. Our hypothesis is that genetic factors have dominant pathogenic role in early onset obesity, while late onset obesity may be predominantly due to environmental factors. Our studies and others have thus far supported the view that genetic variants and mutations are the predominant contributory

factor in childhood obesity. Early onset obesity can be further subclassified into either due to single gene defects (monogenic form of obesity) such as MC4R mutations, or combination of several predisposing genetic variants, such as the *POMC* mutation Tyr221Cys and *MC3R* variant 6Lys/81Ile.

Although much attention has been focused on leptin-melanocortin system, there are a large number of other unidentified hormones, receptors, enzymes that are involved in the complex regulating mechanism governing energy homeostasis, waiting to be discovered. While the weight regulation mechanism is similar between rodents and man, differences may exist, and thus mere experimentation with knockout obese mouse models may not be adequate. The search for human genetic mutations in candidate genes predicted to affect weight regulation serves a very important purpose. By showing that loss-of-function mutations in these candidate genes, which occur naturally in humans, result in or predispose to human obesity, these "experiments of nature" validate the products of these genes as critical mediators of the weight regulation mechanism in humans. The discovery of these novel gene products will also collectively help to complete this unfinished jigsaw puzzle. These critical mediators in turn will become the focuses of intense, million dollar research efforts by pharmaceutical companies to produce new drugs to combat the worldwide epidemic of obesity. Indeed, elucidation of the complete human weight regulation mechanism and the pathogenesis of obesity will facilitate the design of novel therapeutic agents as adjuncts in the treatment of obesity. Obesity gene research contributes to our understanding of the molecular circuitry controlling appetite and energy balance. By identifying these genes and their products, and understanding how they confer

susceptibility and interact with other factors to lead to obesity, we can then devise more effective prevention and treatment strategies to combat this epidemic.

Related Publications by candidate

Journal articles

1. Y S Lee, L K S Poh, BLK Kek and K Y Loke, "The role of Melanocortin 3 Receptor Gene in Childhood Obesity". *DIABETES*, 56, no.10, (2007): 2622-2630.

2. Y S Lee, B Challis, D Thompson, G Yeo, J Keogh, M Madonna, V Wraight, M Sims, V Vatin, D Meyre, J Shield, C Burren, Z Ibrahim, T Cheetham, P Swift, A Blackwood, C C Hung, N J Wareham, P Froguel, G L Millhauser, S O'Rahilly and I S Farooqi, "A POMC variant implicates beta-melanocyte-stimulating hormone in the control of human energy balance". *CELL METABOLISM*, 3, no.2, (2006): 135-140.

3. Y S Lee, L K S Poh and K Y Loke, "A novel melanocortin 3 receptor gene (MC3R) mutation associated with severe obesity". *JOURNAL OF CLINICAL ENDOCRINOLOGY AND METABOLISM*, 87, no.3, (2002): 1423-1426.

4. Y S Lee, L K S Poh, BLK Kek and K Y Loke, "Novel melanocortin 4 receptor gene mutations in severely obese children". *CLINICAL ENDOCRINOLOGY,* (2002): 529-535.

5. JW Creemers, YS Lee, RL Oliver, M Bahceci, A Tuzcu, D Gokalp, J Keogh, S Herber, A White, S O'Rahilly, IS Farooqi. Mutations in the amino-terminal

region of proopiomelanocortin (POMC) in patients with early-onset obesity impair POMC sorting to the regulated secretory pathway. *J CLIN ENDOCRINOL METAB*, 93, no.11, (2008):4494-9 (the first 3 authors contributed equally to the work).

6. Y S Lee and K Y Loke, "The molecular pathogenesis of obesity: An unfinished jigsaw puzzle". *ANNALS OF ACADEMY OF MEDICINE SINGAPORE*, 29, no.3, (2000): 388-395.

<u>Conference papers</u>

7. Lee, Y S, "Recent advances in the genetics of obesity". *Journal of the Asean Federation of Endocrine Societies*, 23, no. 1 (Suppl. issue on The 2005 Congress of the Asean Federation of Endocrine Societies [Philippines] Book of Abstracts) (2005): S23. Manila: The Philippine Society of Endocrinology and Metabolism. (2005 AFES Congress "Linking Molecular and Clinical Endocrinology", 7 - 10 Dec 2005, Philippine International Convention Center, Manila, Philippines)

8. Lee, Y S, "Recent advances in the genetics of severe obesity". *Final Program and Abstracts of the 3rd Biennial Scientific Meeting Asia Pacific Paediatric Endocrine Society* (2004): 46. Japn: APPES. (Invited paper) (Final Program and Abstracts of the 3rd Biennial Scientific Meeting Asia Pacific Paediatric Endocrine Society, 24 - 26 Sep 2004, Kobe, Japan)

9. Lee, Y S, "Recent advances in the genetics of severe obesity". *Second Asia-Oceania Conference on Obesity (MASO 2003): Combating the obesity epidemic - a shared responsibility* (2003): 8. KL: Asia-Oceania Association for the Study of Obesity. (Invited paper) (Second Asia-Oceania Conference on Obesity [MASO 2003]: Combating the obesity epidemic - a shared responsibility, 7 - 9 Sep 2003, Kuala Lumpur, KL, Malaysia)

10. Lee Y S, L K S Poh, S M E Ng, L K B Kek, A M M Ling, R Vaithinathan and K Y Loke, "Prevalence of melanocortin-3 receptor (MC3R) and melanocortin-4 receptor (MC4R) gene mutations in obese Singapore children". *Journal of the ASEAN Federation of Endocrine Societies*, 21, no. 1/2 (Suppl. on Abstract Book of the 12th Congress of the ASEAN Federation of Endocrine Societies) (2003): 37. 1 Jan 2003

11. Lee Y S, L K S Poh, L K B Kek, S M E Ng and K Y Loke, "Novel mutations of the melanocortin 3 receptor (MC3R) and melanocortin 4 receptor (MC4R) genes associated with severe obesity: deciphering the biology of weight regulation". *1st NHG Scientific Congress* (2002): 130. Singapore: NHG. (1st NHG Scientific Congress, 17 - 18 Aug 2002)

12. Lee Y S, L K S Poh, L K B Kek, S M E Ng, R Vaithinathan and K Y Loke, "Autosomal dominant obesity associated with novel melanocortin 3 receptor

(MC3R) and melanocortin 4 receptor (MC4R) gene mutations causing reduced receptor activity : unraveling the biology of weight regulation". *6th NUS-NUH Annual Scientific Meeting: "From laboratory to clinic"* (2002): 42. Singapore. (6th NUS-NUH Annual Scientific Meeting: "From laboratory to clinic", 16 - 17 Aug 2002, Singapore)

13. Farooqi I S, J Keogh, E Lank, Y S Lee, A Blackwood and S O'Rahilly, "Role of leptin signalling in human obesity". *Hormone Research*, 64 (Suppl. issue on Abstracts of the ESPE/LWPES 7th Joint Meeting Paediatric Endocrinology in collaboration with APEG, APPES, JSPE and SLEP) (2005): 275. Basel: S Karger AG. (ESPE/LWPES 7th Joint Meeting Paediatric Endocrinology in collaboration with APEG, APPES, JSPE and SLEP, 21 - 24 Sep 2005, Palais des Congres, Lyon, France)

14. Lee Y S, I S Farooqi, V L Wraight, J Keogh, G S H Yeo, B G Challis and S O'Rahilly, "Mutations of the proopiomelanocortin (POMC) gene associated with severe early onset obesity". *Hormone Research*, 61 (Suppl. 1 on Abstracts of the ESPE/LWPES 7th Joint Meeting Paediatric Endocrinology in collaboration with APEG, APPES, JSPE and SLEP) 2005): 377. Basel: Karger. (ESPE/LWPES 7th Joint Meeting Paediatric Endocrinology in collaboration with APEG, APPES, JSPE and SLEP), 21 - 24 Sep 2005, Lyon, France)

15. Lee Y S, L K S Poh, S M E Ng, L K B Kek and K Y Loke, "Mutations of the

melanocortin-3 receptor and melanocortin-4 receptor genes associated with obesity in humans". *International Journal of Obesity and related metabolic disorders*, 26 (Suppl. 1 on Abstracts : "Ninth International Congress on Obesity") (2002): S76 (Ninth International Congress on Obesity, 24 - 29 Aug 2002)

16. Lee Y S, L K S Poh and K Y Loke, "A novel melanocortin 4 receptor gene (MC4R) mutation associated with severe obesity". *Program and Abstracts of the Endocrine Society's 84th Annual Meeting* (2002): 589. United States: The Endocrine Society. (Program and Abstracts of the Endocrine Society's 84th Annual Meeting, 19 - 22 Jun 2002, San Francisco, United States)

17. Lee Y S, "Advances in the genetics of severe obesity". *Journal of the ASEAN Federation of Endocrine Societies*, 21, no. 1/2 (Suppl. on Abstract Book of the 12th Congress of the ASEAN Federation of Endocrine Societies) (2003): 31 (Journal of the ASEAN Federation of Endocrine Societies, 1 Jan 2003)

Bibliography

Aaron, D.J., Kriska, A.M., Dearwater, S.R., Cauley, J.A., Metz, K.F., and LaPorte, R.E. (1995). Reproducibility and validity of an epidemiologic questionnaire to assess past year physical activity in adolescents. Am J Epidemiol 142, 191-201.

Abbott, C.R., Rossi, M., Kim, M., AlAhmed, S.H., Taylor, G.M., Ghatei, M.A., Smith, D.M., and Bloom, S.R. (2000). Investigation of the melanocyte stimulating hormones on food intake. Lack Of evidence to support a role for the melanocortin-3-receptor. Brain Res 869, 203-210.

Adams, T.D., Heath, E.M., LaMonte, M.J., Gress, R.E., Pendleton, R., Strong, M., Smith, S.C., and Hunt, S.C. (2007). The relationship between body mass index and per cent body fat in the severely obese. Diabetes Obes Metab 9, 498-505.

Allison, D.B., Faith, M.S., and Nathan, J.S. (1996a). Risch's lambda values for human obesity. Int J Obes Relat Metab Disord 20, 990-999.

Allison, D.B., Kaprio, J., Korkeila, M., Koskenvuo, M., Neale, M.C., and Hayakawa, K. (1996b). The heritability of body mass index among an international sample of monozygotic twins reared apart. Int J Obes Relat Metab Disord 20, 501-506.

Bell, C.G., Walley, A.J., and Froguel, P. (2005). The genetics of human obesity. Nat Rev Genet 6, 221-234.

Benjannet, S., Rondeau, N., Paquet, L., Boudreault, A., Lazure, C., Chretien, M., and Seidah, N.G. (1993). Comparative biosynthesis, covalent post-translational modifications and efficiency of prosegment cleavage of the prohormone convertases PC1 and PC2: glycosylation, sulphation and identification of the intracellular site of prosegment cleavage of PC1 and PC2. Biochem J 294 (Pt 3), 735-743.

Bennett, D.L., Bailyes, E.M., Nielsen, E., Guest, P.C., Rutherford, N.G., Arden, S.D., and Hutton, J.C. (1992). Identification of the type 2 proinsulin processing endopeptidase as PC2, a member of the eukaryote subtilisin family. J Biol Chem 267, 15229-15236.

Bertagna, X. (1994). Proopiomelanocortin-derived peptides. Endocrinol Metab Clin North Am 23, 467-485.

Bertagna, X., Lenne, F., Comar, D., Massias, J.F., Wajcman, H., Baudin, V., Luton, J.P., and Girard, F. (1986). Human beta-melanocyte-stimulating hormone revisited. Proc Natl Acad Sci U S A 83, 9719-9723.

Biebermann, H., Castaneda, T.R., van Landeghem, F., von Deimling, A., Escher, F., Brabant, G., Hebebrand, J., Hinney, A., Tschop, M.H., Gruters, A., and Krude, H. (2006). A role for beta-melanocyte-stimulating hormone in human body-weight regulation. Cell Metab 3, 141-146.

Bouchard, C. (1991). Current understanding of the etiology of obesity: genetic and nongenetic factors. Am J Clin Nutr 53, 1561S-1565S.

Bouchard, C., Perusse, L., Leblanc, C., Tremblay, A., and Theriault, G. (1988). Inheritance of the amount and distribution of human body fat. Int J Obes 12, 205-215.

Boutin, P., and Froguel, P. (2001). Genetics of human obesity. Best Pract Res Clin Endocrinol Metab 15, 391-404.

Bray, G.A., and York, D.A. (1971). Genetically transmitted obesity in rodents. Physiol Rev 51, 598-646.

Butler, A.A., Kesterson, R.A., Khong, K., Cullen, M.J., Pelleymounter, M.A., Dekoning, J., Baetscher, M., and Cone, R.D. (2000). A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. Endocrinology 141, 3518-3521.

Campfield, L.A., Smith, F.J., Guisez, Y., Devos, R., and Burn, P. (1995). Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 269, 546-549.

Caro, J.F., Kolaczynski, J.W., Nyce, M.R., Ohannesian, J.P., Opentanova, I., Goldman, W.H., Lynn, R.B., Zhang, P.L., Sinha, M.K., and Considine, R.V. (1996). Decreased cerebrospinal-fluid/serum leptin ratio in obesity: a possible mechanism for leptin resistance. Lancet 348, 159-161.

Cawley, N.X., Normant, E., Chen, A., and Loh, Y.P. (2000). Oligomerization of pro-opiomelanocortin is independent of pH, calcium and the sorting signal for the regulated secretory pathway. FEBS Lett 481, 37-41.

Challis, B.G., Coll, A.P., Yeo, G.S., Pinnock, S.B., Dickson, S.L., Thresher, R.R., Dixon, J., Zahn, D., Rochford, J.J., White, A., Oliver, R.L., Millington, G., Aparicio, S.A., Colledge, W.H., Russ, A.P., Carlton, M.B., and O'Rahilly, S. (2004). Mice lacking pro-opiomelanocortin are sensitive to high-fat feeding but respond normally to the acute anorectic effects of peptide-YY(3-36). Proc Natl Acad Sci U S A 101, 4695-4700.

Challis, B.G., Pritchard, L.E., Creemers, J.W., Delplanque, J., Keogh, J.M., Luan, J., Wareham, N.J., Yeo, G.S., Bhattacharyya, S., Froguel, P., White, A., Farooqi, I.S., and O'Rahilly, S. (2002). A missense mutation disrupting a dibasic prohormone processing site in pro-opiomelanocortin (POMC) increases susceptibility to early-onset obesity through a novel molecular mechanism. Hum Mol Genet 11, 1997-2004.

Chen, A.S., Marsh, D.J., Trumbauer, M.E., Frazier, E.G., Guan, X.M., Yu, H., Rosenblum, C.I., Vongs, A., Feng, Y., Cao, L., Metzger, J.M., Strack, A.M., Camacho, R.E., Mellin, T.N., Nunes, C.N., Min, W., Fisher, J., Gopal-Truter, S., MacIntyre, D.E.,

Chen, H.Y., and Van der Ploeg, L.H. (2000). Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. Nat Genet 26, 97-102.

Cheng, Y., and Prusoff, W.H. (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22, 3099-3108.

Choi, S., and Dallman, M.F. (1999). Hypothalamic obesity: multiple routes mediated by loss of function in medial cell groups. Endocrinology 140, 4081-4088.

Christie, D.L., Batchelor, D.C., and Palmer, D.J. (1991). Identification of kex2-related proteases in chromaffin granules by partial amino acid sequence analysis. J Biol Chem 266, 15679-15683.

Chua, S.C., Jr., Chung, W.K., Wu-Peng, X.S., Zhang, Y., Liu, S.M., Tartaglia, L., and Leibel, R.L. (1996). Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor. Science 271, 994-996.

Clement, K., Vaisse, C., Lahlou, N., Cabrol, S., Pelloux, V., Cassuto, D., Gourmelen, M., Dina, C., Chambaz, J., Lacorte, J.M., Basdevant, A., Bougneres, P., Lebouc, Y., Froguel, P., and Guy-Grand, B. (1998). A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. Nature 392, 398-401.

Cole, T.J., Freeman, J.V., and Preece, M.A. (1995). Body mass index reference curves for the UK, 1990. Arch Dis Child 73, 25-29.

Coleman, D.L. (1973). Effects of parabiosis of obese with diabetes and normal mice. Diabetologia 9, 294-298.

Coll, A.P., Farooqi, I.S., Challis, B.G., Yeo, G.S., and O'Rahilly, S. (2004). Proopiomelanocortin and energy balance: insights from human and murine genetics. J Clin Endocrinol Metab 89, 2557-2562.

Collins, S., Kuhn, C.M., Petro, A.E., Swick, A.G., Chrunyk, B.A., and Surwit, R.S. (1996). Role of leptin in fat regulation. Nature 380, 677.

Comuzzie, A.G., Hixson, J.E., Almasy, L., Mitchell, B.D., Mahaney, M.C., Dyer, T.D., Stern, M.P., MacCluer, J.W., and Blangero, J. (1997). A major quantitative trait locus determining serum leptin levels and fat mass is located on human chromosome 2. Nat Genet 15, 273-276.

Cone, R.D. (2005). Anatomy and regulation of the central melanocortin system. Nat Neurosci 8, 571-578.

Considine, R.V., Considine, E.L., Williams, C.J., Hyde, T.M., and Caro, J.F. (1996a). The hypothalamic leptin receptor in humans: identification of incidental sequence polymorphisms and absence of the db/db mouse and fa/fa rat mutations. Diabetes 45, 992-994.

Considine, R.V., Considine, E.L., Williams, C.J., Nyce, M.R., Magosin, S.A., Bauer, T.L., Rosato, E.L., Colberg, J., and Caro, J.F. (1995). Evidence against either a premature stop codon or the absence of obese gene mRNA in human obesity. J Clin Invest 95, 2986-2988.

Considine, R.V., Sinha, M.K., Heiman, M.L., Kriauciunas, A., Stephens, T.W., Nyce, M.R., Ohannesian, J.P., Marco, C.C., McKee, L.J., Bauer, T.L., and et al. (1996b). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 334, 292-295.

Cool, D.R., Fenger, M., Snell, C.R., and Loh, Y.P. (1995). Identification of the sorting signal motif within pro-opiomelanocortin for the regulated secretory pathway. J Biol Chem 270, 8723-8729.

Cool, D.R., and Loh, Y.P. (1998). Carboxypeptidase E is a sorting receptor for prohormones: binding and kinetic studies. Mol Cell Endocrinol 139, 7-13.

Cool, D.R., Normant, E., Shen, F., Chen, H.C., Pannell, L., Zhang, Y., and Loh, Y.P. (1997). Carboxypeptidase E is a regulated secretory pathway sorting receptor: genetic obliteration leads to endocrine disorders in Cpe(fat) mice. Cell 88, 73-83.

Cossrow, N., and Falkner, B. (2004). Race/ethnic issues in obesity and obesity-related comorbidities. J Clin Endocrinol Metab 89, 2590-2594.

Cowley, M.A., Smart, J.L., Rubinstein, M., Cerdan, M.G., Diano, S., Horvath, T.L., Cone, R.D., and Low, M.J. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature 411, 480-484.

Creemers, J.W., Pritchard, L.E., Gyte, A., Le Rouzic, P., Meulemans, S., Wardlaw, S.L., Zhu, X., Steiner, D.F., Davies, N., Armstrong, D., Lawrence, C.B., Luckman, S.M., Schmitz, C.A., Davies, R.A., Brennand, J.C., and White, A. (2006). Agouti-related protein is posttranslationally cleaved by proprotein convertase 1 to generate agouti-related protein (AGRP)83-132: interaction between AGRP83-132 and melanocortin receptors cannot be influenced by syndecan-3. Endocrinology 147, 1621-1631.

Crosby, S.R., Stewart, M.F., Ratcliffe, J.G., and White, A. (1988). Direct measurement of the precursors of adrenocorticotropin in human plasma by two-site immunoradiometric assay. J Clin Endocrinol Metab 67, 1272-1277.

Dikeakos, J.D., and Reudelhuber, T.L. (2007). Sending proteins to dense core secretory granules: still a lot to sort out. J Cell Biol 177, 191-196.

Dubern, B., Lubrano-Berthelier, C., Mencarelli, M., Ersoy, B., Frelut, M.L., Bougle, D., Costes, B., Simon, C., Tounian, P., Vaisse, C., and Clement, K. (2007). Mutational Analysis of the Pro-opiomelanocortin Gene in French Obese Children Led to the Identification of a Novel Deleterious Heterozygous Mutation Located in the alpha-Melanocyte Stimulating Hormone Domain. Pediatr Res.

Dumermuth, E., and Moore, H.P. (1998). Analysis of constitutive and constitutive-like secretion in semi-intact pituitary cells. Methods 16, 188-197.

Echwald, S.M., Sorensen, T.I., Andersen, T., Tybjaerg-Hansen, A., Clausen, J.O., and Pedersen, O. (1999). Mutational analysis of the proopiomelanocortin gene in Caucasians with early onset obesity. Int J Obes Relat Metab Disord 23, 293-298.

Farooqi, I.S., Drop, S., Clements, A., Keogh, J.M., Biernacka, J., Lowenbein, S., Challis, B.G., and O'Rahilly, S. (2006). Heterozygosity for a POMC-null mutation and increased obesity risk in humans. Diabetes 55, 2549-2553.

Farooqi, I.S., Keogh, J.M., Kamath, S., Jones, S., Gibson, W.T., Trussell, R., Jebb, S.A., Lip, G.Y., and O'Rahilly, S. (2001). Partial leptin deficiency and human adiposity. Nature 414, 34-35.

Farooqi, I.S., Keogh, J.M., Yeo, G.S., Lank, E.J., Cheetham, T., and O'Rahilly, S. (2003). Clinical spectrum of obesity and mutations in the melanocortin 4 receptor gene. N Engl J Med 348, 1085-1095.

Farooqi, I.S., Matarese, G., Lord, G.M., Keogh, J.M., Lawrence, E., Agwu, C., Sanna, V., Jebb, S.A., Perna, F., Fontana, S., Lechler, R.I., DePaoli, A.M., and O'Rahilly, S. (2002).

Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. J Clin Invest 110, 1093-1103.

Farooqi, I.S., and O'Rahilly, S. (2004). Monogenic human obesity syndromes. Recent Prog Horm Res 59, 409-424.

Farooqi, I.S., Wangensteen, T., Collins, S., Kimber, W., Matarese, G., Keogh, J.M., Lank, E., Bottomley, B., Lopez-Fernandez, J., Ferraz-Amaro, I., Dattani, M.T., Ercan, O., Myhre, A.G., Retterstol, L., Stanhope, R., Edge, J.A., McKenzie, S., Lessan, N., Ghodsi, M., De Rosa, V., Perna, F., Fontana, S., Barroso, I., Undlien, D.E., and O'Rahilly, S. (2007). Clinical and molecular genetic spectrum of congenital deficiency of the leptin receptor. N Engl J Med 356, 237-247.

Farooqi, I.S., Yeo, G.S., Keogh, J.M., Aminian, S., Jebb, S.A., Butler, G., Cheetham, T., and O'Rahilly, S. (2000). Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. J Clin Invest 106, 271-279.

Feng, N., Young, S.F., Aguilera, G., Puricelli, E., Adler-Wailes, D.C., Sebring, N.G., and Yanovski, J.A. (2005). Co-occurrence of two partially inactivating polymorphisms of MC3R is associated with pediatric-onset obesity. Diabetes 54, 2663-2667.

Flegal, K.M., Carroll, M.D., Ogden, C.L., and Johnson, C.L. (2002). Prevalence and trends in obesity among US adults, 1999-2000. JAMA 288, 1723-1727.

French, S.A., Story, M., and Jeffery, R.W. (2001). Environmental influences on eating and physical activity. Annu Rev Public Health 22, 309-335.

Friedman, J. (2002). Fat in all the wrong places. Nature 415, 268-269.

Friedman, J.M. (2003). A war on obesity, not the obese. Science 299, 856-858.

Friedman, J.M., Leibel, R.L., Siegel, D.S., Walsh, J., and Bahary, N. (1991). Molecular mapping of the mouse ob mutation. Genomics 11, 1054-1062.

Gantz, I., and Fong, T.M. (2003). The melanocortin system. Am J Physiol Endocrinol Metab 284, E468-474.

Gantz, I., Konda, Y., Tashiro, T., Shimoto, Y., Miwa, H., Munzert, G., Watson, S.J., DelValle, J., and Yamada, T. (1993a). Molecular cloning of a novel melanocortin receptor. J Biol Chem 268, 8246-8250.

Gantz, I., Miwa, H., Konda, Y., Shimoto, Y., Tashiro, T., Watson, S.J., DelValle, J., and Yamada, T. (1993b). Molecular cloning, expression, and gene localization of a fourth melanocortin receptor. J Biol Chem 268, 15174-15179.

Geffroy, S., De Vos, P., Staels, B., Duban, B., Auwerx, J., and de Martinville, B. (1995). Localization of the human OB gene (OBS) to chromosome 7q32 by fluorescence in situ hybridization. Genomics 28, 603-604.

Gibson, W.T., Farooqi, I.S., Moreau, M., DePaoli, A.M., Lawrence, E., O'Rahilly, S., and Trussell, R.A. (2004). Congenital leptin deficiency due to homozygosity for the Delta133G mutation: report of another case and evaluation of response to four years of leptin therapy. J Clin Endocrinol Metab 89, 4821-4826.

Gorr, S.U., Jain, R.K., Kuehn, U., Joyce, P.B., and Cowley, D.J. (2001). Comparative sorting of neuroendocrine secretory proteins: a search for common ground in a mosaic of sorting models and mechanisms. Mol Cell Endocrinol 172, 1-6.

Gotoda, T., Manning, B.S., Goldstone, A.P., Imrie, H., Evans, A.L., Strosberg, A.D., McKeigue, P.M., Scott, J., and Aitman, T.J. (1997). Leptin receptor gene variation and

obesity: lack of association in a white British male population. Hum Mol Genet 6, 869-876.

Gropp, E., Shanabrough, M., Borok, E., Xu, A.W., Janoschek, R., Buch, T., Plum, L., Balthasar, N., Hampel, B., Waisman, A., Barsh, G.S., Horvath, T.L., and Bruning, J.C. (2005). Agouti-related peptide-expressing neurons are mandatory for feeding. Nat Neurosci 8, 1289-1291.

Hadley, M.E., and Haskell-Luevano, C. (1999). The proopiomelanocortin system. Ann N Y Acad Sci 885, 1-21.

Hager, J., Dina, C., Francke, S., Dubois, S., Houari, M., Vatin, V., Vaillant, E., Lorentz, N., Basdevant, A., Clement, K., Guy-Grand, B., and Froguel, P. (1998). A genome-wide scan for human obesity genes reveals a major susceptibility locus on chromosome 10. Nat Genet 20, 304-308.

Halaas, J.L., Gajiwala, K.S., Maffei, M., Cohen, S.L., Chait, B.T., Rabinowitz, D., Lallone, R.L., Burley, S.K., and Friedman, J.M. (1995). Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269, 543-546.

Hani, E.H., Dupont, S., Durand, E., Dina, C., Gallina, S., Gantz, I., and Froguel, P. (2001). Naturally occurring mutations in the melanocortin receptor 3 gene are not associated with type 2 diabetes mellitus in French Caucasians. J Clin Endocrinol Metab 86, 2895-2898.

Harris, R.B., and Martin, R.J. (1984). Specific depletion of body fat in parabiotic partners of tube-fed obese rats. Am J Physiol 247, R380-386.

Harrold, J.A., Widdowson, P.S., and Williams, G. (2003). beta-MSH: a functional ligand that regulated energy homeostasis via hypothalamic MC4-R? Peptides 24, 397-405.

Heisler, L.K., Cowley, M.A., Tecott, L.H., Fan, W., Low, M.J., Smart, J.L., Rubinstein, M., Tatro, J.B., Marcus, J.N., Holstege, H., Lee, C.E., Cone, R.D., and Elmquist, J.K. (2002). Activation of central melanocortin pathways by fenfluramine. Science 297, 609-611.

Hervey, G.R. (1969). Regulation of energy balance. Nature 222, 629-631.

Heymsfield, S.B., Greenberg, A.S., Fujioka, K., Dixon, R.M., Kushner, R., Hunt, T., Lubina, J.A., Patane, J., Self, B., Hunt, P., and McCamish, M. (1999). Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. JAMA 282, 1568-1575.

Hinney, A., Becker, I., Heibult, O., Nottebom, K., Schmidt, A., Ziegler, A., Mayer, H., Siegfried, W., Blum, W.F., Remschmidt, H., and Hebebrand, J. (1998). Systematic mutation screening of the pro-opiomelanocortin gene: identification of several genetic variants including three different insertions, one nonsense and two missense point mutations in probands of different weight extremes. J Clin Endocrinol Metab 83, 3737-3741.

Hinney, A., Bettecken, T., Tarnow, P., Brumm, H., Reichwald, K., Lichtner, P., Scherag, A., Nguyen, T.T., Schlumberger, P., Rief, W., Vollmert, C., Illig, T., Wichmann, H.E., Schafer, H., Platzer, M., Biebermann, H., Meitinger, T., and Hebebrand, J. (2006). Prevalence, spectrum, and functional characterization of melanocortin-4 receptor gene mutations in a representative population-based sample and obese adults from Germany. J Clin Endocrinol Metab 91, 1761-1769.

Hinney, A., Hohmann, S., Geller, F., Vogel, C., Hess, C., Wermter, A.K., Brokamp, B., Goldschmidt, H., Siegfried, W., Remschmidt, H., Schafer, H., Gudermann, T., and

Hebebrand, J. (2003). Melanocortin-4 receptor gene: case-control study and transmission disequilibrium test confirm that functionally relevant mutations are compatible with a major gene effect for extreme obesity. J Clin Endocrinol Metab 88, 4258-4267.

Hinney, A., Schmidt, A., Nottebom, K., Heibult, O., Becker, I., Ziegler, A., Gerber, G., Sina, M., Gorg, T., Mayer, H., Siegfried, W., Fichter, M., Remschmidt, H., and Hebebrand, J. (1999). Several mutations in the melanocortin-4 receptor gene including a nonsense and a frameshift mutation associated with dominantly inherited obesity in humans. J Clin Endocrinol Metab 84, 1483-1486.

Hixson, J.E., Almasy, L., Cole, S., Birnbaum, S., Mitchell, B.D., Mahaney, M.C., Stern, M.P., MacCluer, J.W., Blangero, J., and Comuzzie, A.G. (1999). Normal variation in leptin levels in associated with polymorphisms in the proopiomelanocortin gene, POMC. J Clin Endocrinol Metab 84, 3187-3191.

Hornby, P.J., Rosenthal, S.D., Mathis, J.P., Vindrola, O., and Lindberg, I. (1993). Immunocytochemical localization of the neuropeptide-synthesizing enzyme PC1 in AtT-20 cells. Neuroendocrinology 58, 555-563.

Huszar, D., Lynch, C.A., Fairchild-Huntress, V., Dunmore, J.H., Fang, Q., Berkemeier, L.R., Gu, W., Kesterson, R.A., Boston, B.A., Cone, R.D., Smith, F.J., Campfield, L.A., Burn, P., and Lee, F. (1997). Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88, 131-141.

Jacobson, P., Ukkola, O., Rankinen, T., Snyder, E.E., Leon, A.S., Rao, D.C., Skinner, J.S., Wilmore, J.H., Lonn, L., Cowan, G.S., Jr., Sjostrom, L., and Bouchard, C. (2002). Melanocortin 4 receptor sequence variations are seldom a cause of human obesity: the

Swedish Obese Subjects, the HERITAGE Family Study, and a Memphis cohort. J Clin Endocrinol Metab 87, 4442-4446.

Jegou, S., Boutelet, I., and Vaudry, H. (2000). Melanocortin-3 receptor mRNA expression in pro-opiomelanocortin neurones of the rat arcuate nucleus. J Neuroendocrinol 12, 501-505.

Johanning, K., Mathis, J.P., and Lindberg, I. (1996). Role of PC2 in proenkephalin processing: antisense and overexpression studies. J Neurochem 66, 898-907.

Katz, A., Nambi, S.S., Mather, K., Baron, A.D., Follmann, D.A., Sullivan, G., and Quon, M.J. (2000). Quantitative insulin sensitivity check index: a simple, accurate method for assessing insulin sensitivity in humans. J Clin Endocrinol Metab 85, 2402-2410.

Khaw, K.T., Wareham, N., Luben, R., Bingham, S., Oakes, S., Welch, A., and Day, N. (2001). Glycated haemoglobin, diabetes, and mortality in men in Norfolk cohort of european prospective investigation of cancer and nutrition (EPIC-Norfolk). BMJ 322, 15-18.

Kirchmair, R., Gee, P., Hogue-Angeletti, R., Laslop, A., Fischer-Colbrie, R., and Winkler, H. (1992). Immunological characterization of the endoproteases PC1 and PC2 in adrenal chromaffin granules and in the pituitary gland. FEBS Lett 297, 302-305.

Krude, H., Biebermann, H., Luck, W., Horn, R., Brabant, G., and Gruters, A. (1998). Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. Nat Genet 19, 155-157.

Krude, H., Biebermann, H., Schnabel, D., Tansek, M.Z., Theunissen, P., Mullis, P.E., and Gruters, A. (2003). Obesity due to proopiomelanocortin deficiency: three new cases and

treatment trials with thyroid hormone and ACTH4-10. J Clin Endocrinol Metab 88, 4633-4640.

Larsen, L.H., Echwald, S.M., Sorensen, T.I., Andersen, T., Wulff, B.S., and Pedersen, O. (2005). Prevalence of mutations and functional analyses of melanocortin 4 receptor variants identified among 750 men with juvenile-onset obesity. J Clin Endocrinol Metab 90, 219-224.

Lee, G.H., Proenca, R., Montez, J.M., Carroll, K.M., Darvishzadeh, J.G., Lee, J.I., and Friedman, J.M. (1996). Abnormal splicing of the leptin receptor in diabetic mice. Nature 379, 632-635.

Lee, Y.S., Challis, B.G., Thompson, D.A., Yeo, G.S., Keogh, J.M., Madonna, M.E., Wraight, V., Sims, M., Vatin, V., Meyre, D., Shield, J., Burren, C., Ibrahim, Z., Cheetham, T., Swift, P., Blackwood, A., Hung, C.C., Wareham, N.J., Froguel, P., Millhauser, G.L., O'Rahilly, S., and Farooqi, I.S. (2006). A POMC variant implicates beta-melanocyte-stimulating hormone in the control of human energy balance. Cell Metab 3, 135-140.

Lee, Y.S., Poh, L.K., Kek, B.L., and Loke, K.Y. (2007a). Novel melanocortin 4 receptor gene mutations in severely obese children. Clin Endocrinol (Oxf).

Lee, Y.S., Poh, L.K., Kek, B.L., and Loke, K.Y. (2007b). The role of melanocortin 3 receptor gene in childhood obesity. Diabetes 56, 2622-2630.

Lee, Y.S., Poh, L.K., and Loke, K.Y. (2002). A novel melanocortin 3 receptor gene (MC3R) mutation associated with severe obesity. J Clin Endocrinol Metab 87, 1423-1426.

Leibel, R.L. (2006). The molecular genetics of the melanocortin pathway and energy homeostasis. Cell Metab 3, 79-81.

Leibel, R.L., Chung, W.K., and Chua, S.C., Jr. (1997). The molecular genetics of rodent single gene obesities. J Biol Chem 272, 31937-31940.

Lembertas, A.V., Perusse, L., Chagnon, Y.C., Fisler, J.S., Warden, C.H., Purcell-Huynh, D.A., Dionne, F.T., Gagnon, J., Nadeau, A., Lusis, A.J., and Bouchard, C. (1997). Identification of an obesity quantitative trait locus on mouse chromosome 2 and evidence of linkage to body fat and insulin on the human homologous region 20q. J Clin Invest 100, 1240-1247.

Levin, N., Nelson, C., Gurney, A., Vandlen, R., and de Sauvage, F. (1996). Decreased food intake does not completely account for adiposity reduction after ob protein infusion. Proc Natl Acad Sci U S A 93, 1726-1730.

Li, W.D., Joo, E.J., Furlong, E.B., Galvin, M., Abel, K., Bell, C.J., and Price, R.A. (2000). Melanocortin 3 receptor (MC3R) gene variants in extremely obese women. Int J Obes Relat Metab Disord 24, 206-210.

Lloyd, D.J., Bohan, S., and Gekakis, N. (2006). Obesity, hyperphagia and increased metabolic efficiency in Pc1 mutant mice. Hum Mol Genet 15, 1884-1893.

Maes, H.H., Neale, M.C., and Eaves, L.J. (1997). Genetic and environmental factors in relative body weight and human adiposity. Behav Genet 27, 325-351.

Maffei, M., Halaas, J., Ravussin, E., Pratley, R.E., Lee, G.H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S., and et al. (1995). Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1, 1155-1161.

Maffei, M., Stoffel, M., Barone, M., Moon, B., Dammerman, M., Ravussin, E., Bogardus, C., Ludwig, D.S., Flier, J.S., Talley, M., and et al. (1996). Absence of mutations in the human OB gene in obese/diabetic subjects. Diabetes 45, 679-682.

Matthews, D.R., Hosker, J.P., Rudenski, A.S., Naylor, B.A., Treacher, D.F., and Turner, R.C. (1985). Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 28, 412-419.

Mei, Z., Grummer-Strawn, L.M., Pietrobelli, A., Goulding, A., Goran, M.I., and Dietz, W.H. (2002). Validity of body mass index compared with other body-composition screening indexes for the assessment of body fatness in children and adolescents. Am J Clin Nutr 75, 978-985.

Meyre, D., Bouatia-Naji, N., Tounian, A., Samson, C., Lecoeur, C., Vatin, V., Ghoussaini, M., Wachter, C., Hercberg, S., Charpentier, G., Patsch, W., Pattou, F., Charles, M.A., Tounian, P., Clement, K., Jouret, B., Weill, J., Maddux, B.A., Goldfine, I.D., Walley, A., Boutin, P., Dina, C., and Froguel, P. (2005). Variants of ENPP1 are associated with childhood and adult obesity and increase the risk of glucose intolerance and type 2 diabetes. Nat Genet 37, 863-867.

Mielke, S.P., and Krishnan, V.V. (2004). An evaluation of chemical shift index-based secondary structure determination in proteins: influence of random coil chemical shifts. J Biomol NMR 30, 143-153.

Millington, G.W., Tung, Y.C., Hewson, A.K., O'Rahilly, S., and Dickson, S.L. (2001). Differential effects of alpha-, beta- and gamma(2)-melanocyte-stimulating hormones on hypothalamic neuronal activation and feeding in the fasted rat. Neuroscience 108, 437-445.

Minokoshi, Y., Kim, Y.B., Peroni, O.D., Fryer, L.G., Muller, C., Carling, D., and Kahn, B.B. (2002). Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. Nature 415, 339-343.

Miraglia Del Giudice, E., Cirillo, G., Nigro, V., Santoro, N., D'Urso, L., Raimondo, P., Cozzolino, D., Scafato, D., and Perrone, L. (2002). Low frequency of melanocortin-4 receptor (MC4R) mutations in a Mediterranean population with early-onset obesity. Int J Obes Relat Metab Disord 26, 647-651.

Miraglia del Giudice, E., Cirillo, G., Santoro, N., D'Urso, L., Carbone, M.T., Di Toro, R., and Perrone, L. (2001). Molecular screening of the proopiomelanocortin (POMC) gene in Italian obese children: report of three new mutations. Int J Obes Relat Metab Disord 25, 61-67.

Montague, C.T., Farooqi, I.S., Whitehead, J.P., Soos, M.A., Rau, H., Wareham, N.J., Sewter, C.P., Digby, J.E., Mohammed, S.N., Hurst, J.A., Cheetham, C.H., Earley, A.R., Barnett, A.H., Prins, J.B., and O'Rahilly, S. (1997). Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature 387, 903-908.

Mountjoy, K.G., Mortrud, M.T., Low, M.J., Simerly, R.B., and Cone, R.D. (1994). Localization of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol Endocrinol 8, 1298-1308.

Mountjoy, K.G., and Wild, J.M. (1998). Melanocortin-4 receptor mRNA expression in the developing autonomic and central nervous systems. Brain Res Dev Brain Res 107, 309-314.

Mountjoy, K.G., and Wong, J. (1997). Obesity, diabetes and functions for proopiomelanocortin-derived peptides. Mol Cell Endocrinol 128, 171-177.

Neel, J.V. (1962). Diabetes mellitus: a 'thrifty' genotype rendered detrimental by 'progress'? . Am J Hum Genet 14, 353-362.

O'Rahilly, S., Farooqi, I.S., Yeo, G.S., and Challis, B.G. (2003). Minireview: human obesity-lessons from monogenic disorders. Endocrinology 144, 3757-3764.

Oh, I.S., Shimizu, H., Satoh, T., Okada, S., Adachi, S., Inoue, K., Eguchi, H., Yamamoto, M., Imaki, T., Hashimoto, K., Tsuchiya, T., Monden, T., Horiguchi, K., Yamada, M., and Mori, M. (2006). Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature 443, 709-712.

Ohshiro, Y., Sanke, T., Ueda, K., Shimajiri, Y., Nakagawa, T., Tsunoda, K., Nishi, M., Sasaki, H., Takasu, N., and Nanjo, K. (1999). Molecular scanning for mutations in the melanocortin-4 receptor gene in obese/diabetic Japanese. Ann Hum Genet 63, 483-487.

Ohtake, M., Bray, G.A., and Azukizawa, M. (1977). Studies on hypothermia and thyroid function in the obese (ob/ob) mouse. Am J Physiol 233, R110-115.

Pelleymounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., and Collins, F. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269, 540-543.

Persani, L., Tonacchera, M., Beck-Peccoz, P., Vitti, P., Mammoli, C., Chiovato, L., Elisei, R., Faglia, G., Ludgate, M., Vassart, G., and et al. (1993). Measurement of cAMP accumulation in Chinese hamster ovary cells transfected with the recombinant human TSH receptor (CHO-R): a new bioassay for human thyrotropin. J Endocrinol Invest 16, 511-519.

Poggioli, R., Vergoni, A.V., and Bertolini, A. (1986). ACTH-(1-24) and alpha-MSH antagonize feeding behavior stimulated by kappa opiate agonists. Peptides 7, 843-848.

Pritchard, L.E., Turnbull, A.V., and White, A. (2002). Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signalling and obesity. J Endocrinol 172, 411-421.

Pritchard, L.E., and White, A. (2007). Neuropeptide processing and its impact on melanocortin pathways. Endocrinology 148, 4201-4207.

Rached, M., Buronfosse, A., Begeot, M., and Penhoat, A. (2004). Inactivation and intracellular retention of the human I183N mutated melanocortin 3 receptor associated with obesity. Biochim Biophys Acta 1689, 229-234.

Raffin-Sanson, M.L., de Keyzer, Y., and Bertagna, X. (2003). Proopiomelanocortin, a polypeptide precursor with multiple functions: from physiology to pathological conditions. Eur J Endocrinol 149, 79-90.

Rau, H., Reaves, B.J., O'Rahilly, S., and Whitehead, J.P. (1999). Truncated human leptin (delta133) associated with extreme obesity undergoes proteasomal degradation after defective intracellular transport. Endocrinology 140, 1718-1723.

Rong, R., Tao, Y.X., Cheung, B.M., Xu, A., Cheung, G.C., and Lam, K.S. (2006). Identification and functional characterization of three novel human melanocortin-4 receptor gene variants in an obese Chinese population. Clin Endocrinol (Oxf) 65, 198-205.

Roselli-Rehfuss, L., Mountjoy, K.G., Robbins, L.S., Mortrud, M.T., Low, M.J., Tatro, J.B., Entwistle, M.L., Simerly, R.B., and Cone, R.D. (1993). Identification of a receptor for gamma melanotropin and other proopiomelanocortin peptides in the hypothalamus and limbic system. Proc Natl Acad Sci U S A 90, 8856-8860.

Rosenbaum, M., and Leibel, R.L. (1999). The role of leptin in human physiology. N Engl J Med 341, 913-915.

Rosenbaum, M., Nicolson, M., Hirsch, J., Heymsfield, S.B., Gallagher, D., Chu, F., and Leibel, R.L. (1996). Effects of gender, body composition, and menopause on plasma concentrations of leptin. J Clin Endocrinol Metab 81, 3424-3427.

Rouille, Y., Martin, S., and Steiner, D.F. (1995). Differential processing of proglucagon by the subtilisin-like prohormone convertases PC2 and PC3 to generate either glucagon or glucagon-like peptide. J Biol Chem 270, 26488-26496.

Rousseau, K., Kauser, S., Pritchard, L.E., Warhurst, A., Oliver, R.L., Slominski, A., Wei, E.T., Thody, A.J., Tobin, D.J., and White, A. (2007). Proopiomelanocortin (POMC), the ACTH/melanocortin precursor, is secreted by human epidermal keratinocytes and melanocytes and stimulates melanogenesis. FASEB J 21, 1844-1856.

Roy, P., Chevrier, D., Fournier, H., Racine, C., Zollinger, M., Crine, P., and Boileau, G. (1991). Investigation of a possible role of the amino-terminal pro-region of proopiomelanocortin in its processing and targeting to secretory granules. Mol Cell Endocrinol 82, 237-250.

Saper, C.B., Chou, T.C., and Elmquist, J.K. (2002). The need to feed: homeostatic and hedonic control of eating. Neuron 36, 199-211.

Schalin-Jantti, C., Valli-Jaakola, K., Oksanen, L., Martelin, E., Laitinen, K., Krusius, T., Mustajoki, P., Heikinheimo, M., and Kontula, K. (2003). Melanocortin-3-receptor gene variants in morbid obesity. Int J Obes Relat Metab Disord 27, 70-74.

Schioth, H.B., Muceniece, R., and Wikberg, J.E. (1996). Characterisation of the melanocortin 4 receptor by radioligand binding. Pharmacol Toxicol 79, 161-165.

Schioth, H.B., Petersson, S., Muceniece, R., Szardenings, M., and Wikberg, J.E. (1997). Deletions of the N-terminal regions of the human melanocortin receptors. FEBS Lett 410, 223-228.

Schmidt, G.J., Walkuski, J.J., and Stensel, D.J. (1998). The Singapore Youth Coronary Risk and Physical Activity Study. Med Sci Sports Exerc 30, 105-113.

Schnabel, E., Mains, R.E., and Farquhar, M.G. (1989). Proteolytic processing of pro-ACTH/endorphin begins in the Golgi complex of pituitary corticotropes and AtT-20 cells. Mol Endocrinol 3, 1223-1235.

Schwartz, M.W., Baskin, D.G., Bukowski, T.R., Kuijper, J.L., Foster, D., Lasser, G., Prunkard, D.E., Porte, D., Jr., Woods, S.C., Seeley, R.J., and Weigle, D.S. (1996a). Specificity of leptin action on elevated blood glucose levels and hypothalamic neuropeptide Y gene expression in ob/ob mice. Diabetes 45, 531-535.

Schwartz, M.W., Peskind, E., Raskind, M., Boyko, E.J., and Porte, D., Jr. (1996b). Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. Nat Med 2, 589-593.

Schwartz, M.W., Woods, S.C., Porte, D., Jr., Seeley, R.J., and Baskin, D.G. (2000). Central nervous system control of food intake. Nature 404, 661-671.

Seidah, N.G., and Chretien, M. (1999). Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. Brain Res 848, 45-62.

Strobel, A., Issad, T., Camoin, L., Ozata, M., and Strosberg, A.D. (1998). A leptin missense mutation associated with hypogonadism and morbid obesity. Nat Genet 18, 213-215.

Stunkard, A.J., Foch, T.T., and Hrubec, Z. (1986a). A twin study of human obesity. JAMA 256, 51-54.

Stunkard, A.J., and Messick, S. (1985). The three-factor eating questionnaire to measure dietary restraint, disinhibition and hunger. J Psychosom Res 29, 71-83.

Stunkard, A.J., Sorensen, T.I., Hanis, C., Teasdale, T.W., Chakraborty, R., Schull, W.J., and Schulsinger, F. (1986b). An adoption study of human obesity. N Engl J Med 314, 193-198.

Sundaramurthy, D., Campbell, D.A., Leek, J.P., Markham, A.F., and Pieri, L.F. (1998). Assignment of the melanocortin 4 receptor (MC4R) gene to human chromosome band 18q22 by in situ hybridisation and radiation hybrid mapping. Cytogenet Cell Genet 82, 97-98.

Swerdloff, R.S., Batt, R.A., and Bray, G.A. (1976). Reproductive hormonal function in the genetically obese (ob/ob) mouse. Endocrinology 98, 1359-1364.

Tam, W.W., Andreasson, K.I., and Loh, Y.P. (1993). The amino-terminal sequence of pro-opiomelanocortin directs intracellular targeting to the regulated secretory pathway. Eur J Cell Biol 62, 294-306.

Tambs, K., Moum, T., Eaves, L.J., Neale, M.C., Midthjell, K., Lund-Larsen, P.G., and Naess, S. (1992). Genetic and environmental contributions to the variance of body height in a sample of first and second degree relatives. Am J Phys Anthropol 88, 285-294.

Tao, Y.X., and Segaloff, D.L. (2004). Functional characterization of melanocortin-3 receptor variants identify a loss-of-function mutation involving an amino acid critical for G protein-coupled receptor activation. J Clin Endocrinol Metab 89, 3936-3942.

Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K.J., Smutko, J.S., Mays, G.G., Wool, E.A., Monroe, C.A., and Tepper, R.I. (1995). Identification and expression cloning of a leptin receptor, OB-R. Cell 83, 1263-1271.

Trayhurn, P., Thurlby, P.L., and James, W.P. (1977). Thermogenic defect in pre-obese ob/ob mice. Nature 266, 60-62.

Trevaskis, J.L., Gawronska-Kozak, B., Sutton, G.M., McNeil, M., Stephens, J.M., Smith, S.R., and Butler, A.A. (2007). Role of adiponectin and inflammation in insulin resistance of mc3r and mc4r knockout mice. Obesity (Silver Spring) 15, 2664-2672.

Vaisse, C., Clement, K., Durand, E., Hercberg, S., Guy-Grand, B., and Froguel, P. (2000). Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. J Clin Invest 106, 253-262.

Vogler, G.P., Sorensen, T.I., Stunkard, A.J., Srinivasan, M.R., and Rao, D.C. (1995). Influences of genes and shared family environment on adult body mass index assessed in an adoption study by a comprehensive path model. Int J Obes Relat Metab Disord 19, 40-45.

Wang, C.L., Liang, L., Wang, H.J., Fu, J.F., Hebebrand, J., and Hinney, A. (2006). Several mutations in the melanocortin 4 receptor gene are associated with obesity in Chinese children and adolescents. J Endocrinol Invest 29, 894-898.

Wareham, N.J., Wong, M.Y., and Day, N.E. (2000). Glucose intolerance and physical inactivity: the relative importance of low habitual energy expenditure and cardiorespiratory fitness. Am J Epidemiol 152, 132-139.

Weigle, D.S., Bukowski, T.R., Foster, D.C., Holderman, S., Kramer, J.M., Lasser, G., Lofton-Day, C.E., Prunkard, D.E., Raymond, C., and Kuijper, J.L. (1995). Recombinant ob protein reduces feeding and body weight in the ob/ob mouse. J Clin Invest 96, 2065-2070.

Wishart, D.S., Sykes, B.D., and Richards, F.M. (1992). The chemical shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. Biochemistry 31, 1647-1651.

Xu, B., Goulding, E.H., Zang, K., Cepoi, D., Cone, R.D., Jones, K.R., Tecott, L.H., and Reichardt, L.F. (2003). Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. Nat Neurosci 6, 736-742.

Yaswen, L., Diehl, N., Brennan, M.B., and Hochgeschwender, U. (1999). Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. Nat Med 5, 1066-1070.

Yeo, G.S., Connie Hung, C.C., Rochford, J., Keogh, J., Gray, J., Sivaramakrishnan, S., O'Rahilly, S., and Farooqi, I.S. (2004). A de novo mutation affecting human TrkB associated with severe obesity and developmental delay. Nat Neurosci 7, 1187-1189.

Yeo, G.S., Farooqi, I.S., Aminian, S., Halsall, D.J., Stanhope, R.G., and O'Rahilly, S. (1998). A frameshift mutation in MC4R associated with dominantly inherited human obesity. Nat Genet 20, 111-112.

Yeo, G.S., Lank, E.J., Farooqi, I.S., Keogh, J., Challis, B.G., and O'Rahilly, S. (2003). Mutations in the human melanocortin-4 receptor gene associated with severe familial obesity disrupts receptor function through multiple molecular mechanisms. Hum Mol Genet 12, 561-574.

Yiannakouris, N., Melistas, L., Kontogianni, M., Heist, K., and Mantzoros, C.S. (2004). The Val81 missense mutation of the melanocortin 3 receptor gene, but not the 1908c/T nucleotide polymorphism in lamin A/C gene, is associated with hyperleptinemia and hyperinsulinemia in obese Greek caucasians. J Endocrinol Invest 27, 714-720.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994). Positional cloning of the mouse obese gene and its human homologue. Nature 372, 425-432.

Zhou, A., Bloomquist, B.T., and Mains, R.E. (1993). The prohormone convertases PC1 and PC2 mediate distinct endoproteolytic cleavages in a strict temporal order during proopiomelanocortin biosynthetic processing. J Biol Chem 268, 1763-1769.

Zhou, A., Paquet, L., and Mains, R.E. (1995). Structural elements that direct specific processing of different mammalian subtilisin-like prohormone convertases. J Biol Chem 270, 21509-21516.