Discovery of Leptin and Elucidation of Leptin Gene Expression

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Pubmed search on "leptin" retrieves over 25,200 entries and there is hardly a biological or medical term that has not been linked to leptin. But there was a time when leptin was not around and this chapter describes how this molecule was first imagined and then actually identified. The discovery of the leptin gene is not one of those processes, common in big step forward in the life sciences, that happen almost by chance. It is indeed a vision, a concept that became hard evidence. In this respect it is more similar to the discovery of new subatomic particles predicted by theoretical physics equations than to biological findings as remarkable as penicillin in which a scientist gave a brilliant interpretation of an unpredicted result.

The Milieu Intérieur and Homeostasis

We start by reviewing the conceptual frame that led to leptin imagination. It is first worth mentioning that the contemporary concept of body weight in the so-called industrialized societies is somewhat different from what might have been only 50–60 years ago. Up to the mid-twentieth century less than 10 % of the US population was obese in contrast to the 68 % that is characterized by obesity or overweight today [1]. Maintenance of body weight was thus the most "normal" phenomenon to observe. It is calculated that coefficients of weight variation in the absence of "obesogenic" environments and over relatively short periods are about 0.5–0.6 % and cross sectional observations indicate that they persist over more extended periods [2].

The idea that higher animals possess complex homeostatic mechanisms to maintain a stable internal environment for their living units, the cells, is a fundamental concept of modern physiology. The great physiologist Claude Bernard first conceptualized the existence of a reaction of the organism to external stimuli in the aim to maintain constant conditions within the body [3]. He coined the term *milieu intérieur* which has been translated into English as "internal environment" as opposed to external environment, often referred to as simply "environment." Bernard asserted that "the fixity of the internal environment is the condition of free, independent, life" [2, 4, 5]. Although he focused most of his attention and studies on the regulation of body temperature and to blood, "which should be maintained in a certain composition in order for the organism to live," the idea that energy stores are also part of the stability of the organism is contained in the concept of milieu intérieur. The pancreas being a major focus of his studies,

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Bernard had a keen interest in glucose homeostasis and also conceived a role for the brain in this regulation: according to his work metabolic regulation is achieved through a series of rapid onset–offset signals that maintain a tight control of energy supplies. Afterwards Walter Bradford Cannon gave eating and drinking an explicit role in stabilizing vital functions: according to his theory organisms "…have somehow learned the methods of maintaining constancy and keeping steady" [5–7] and further "the constant conditions which are maintained in the body might be termed *equilibria*… Cannon coined the term homeostasis to synthesize his theory and applied that also to energy balance "The coordinated physiological systems which maintain most of the steady states in the organism are so complex and so peculiar to living beings—involving as they may brain, nerves, the heart …—that I have suggested a special term for these states, *homeostasis*" [6].

VMH Lesions and the Controller System

Starting from the early 1940s the British Royal Navy financed and planned research on food rations, food needs, and other topics that had been relevant during the Second World War. This research was carried in the department of Experimental Medicine directed by the physiologist McCance at the Addenbrooke's Hospital in Cambridge and led to seminal discoveries on the physiological basis of energy balance regulation [8]. As often happens in science the right combination of factors including expertise, personalities, and random events was key to the advancement of knowledge in this field. We will herein mention some of them starting, from Gordon Kennedy: he conducted pioneer studies on the obesity phenotype resulting from lesions of the ventromedial part of the hypothalamus (VMH) in rodents [9]. Kennedy interpreted the results in terms of a negative feedback loop system controlling food intake in normal animal, and hypothesized that the amount of fat in the body provided the information for the control signal that was targeted to the hypothalamus. This concept is known as lipostatic theory and suggests that the amount of fat in the body is measured and maintained constant. Other theories had been previously formulated to explain energy balance: these included Brobeck's concept [10] postulating body temperature as the main parameter to indicate the status of energy balance and Mayer's glucostatic theory in which the controlled quantity is the difference between arterial and venous plasma concentrations of glucose [11, 12]. Kennedy's concept was further developed by Romaine Hervey, a younger fellow at the Department of Experimental Medicine, who was the first to employ parabiosis in studies concerning energy balance regulation. Parabiosis is the surgical union of two animals to produce a common blood supply and allow the investigation of circulating factors in the regulation of physiological systems [8]. In parabiosis experiments we distinguish a treated animal and a partner: what happens to the partner is the result of some treatment dependent changes in blood borne signals that the animal is able to sense. Hervey applied this technique to VMH lesioned rats. The animals in which the lesions were made showed hyperphagia and obesity. Their parabiotic partners, with normal hypothalami, ate much less, became thin and in some cases starved to death. Hervey concluded "…these results may be evidence for a feedback control of food intake, and may throw some light on the information used in such a system" [13, 14]. These data were in fact taken to imply that the obese rat produced a humoral agent that would normally stimulate the brain to inhibit food intake. Although the lesioned rats could not respond to the signal, the factor had to be carried across the parabiotic union and inhibit the food intake of the normal partner [13, 15]. These results led Hervey to state that maintenance of body weight "requires some precision" and he was the first to express the concept in rigorous thermodynamic terms: "during any period it must be true that energy intake − energy output = energy stores." He thus imagined energy homeostasis as a "controller system" borrowing this concept from a branch of engineering that deals with the behavior of dynamical systems [16]. According to this theory *inputs*, also called reference or afferent signals, are sensed and manipulated by a *controller* in order to obtain

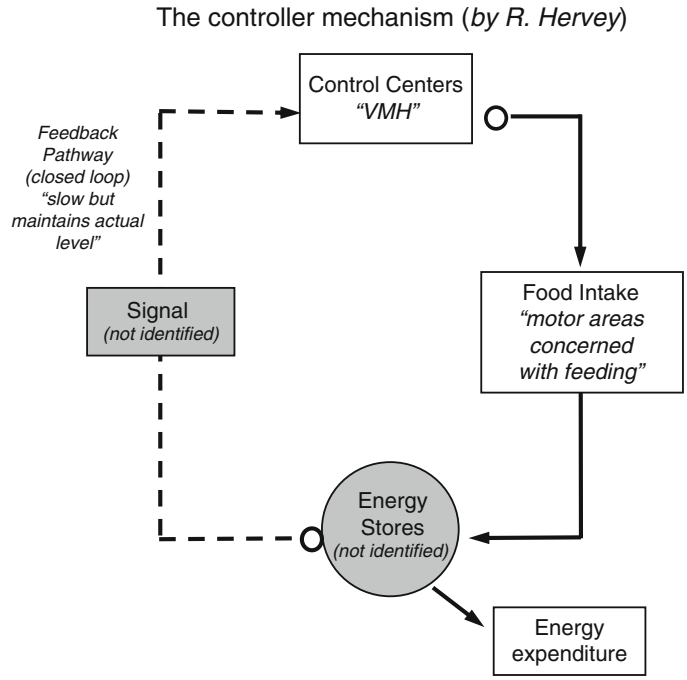

Fig. 1.1 The hypothetical model postulated by Hervey to represent the mechanism regulating energy homeostasis in mammals. Homeostasis can be represented by a controller mechanism, better known as feedback loop: from the energy stores start afferent signals to the center which will in turn produce efferent signals to modulate energy stores. At that stage most of the players were not known. *White box*: known component. *Grey box*: component to be identified

the desired effect on the *output* of the system in a "closed loop system" (Fig. 1.1).

Hervey established the nature of two of the components of this system, namely the VMH as central controller and the output constituted by "motor areas concerned with feeding," while still unknown were "the quantity that the system immediately senses and stabilizes" and the means by which inputs are made to the controller. In the conclusions of the Nature paper published in 1969 Hervey put forward the idea that "regulation of energy balance involves a hormonal feedback signal" and he also discussed the possibility that steroids, already known to affect body weight, could play such a role [16]. The search for the afferent signal had just started.

Douglas Coleman and Parabiosis

VMH lesioned rats are experimental models of obesity obtained by a very drastic and unphysiological treatment: finding the humoral agent hypothesized on the basis of parabiosis results in these models was like looking for a needle in a haystack. But murine models of obesity existed also as a result of spontaneous mutations, and the search was then steered by genetics. In 1949 a few animals in the non-inbred V strain at the Jackson laboratory (Bar Harbor, Maine) were observed to be plump early in life and to become markedly obese and diabetic thereafter. Breeding experiments revealed that the obesity syndrome was caused by a single autosomal recessive gene, *obese* (gene symbol *ob*). The mutation was later transferred on the congenic C57BL/6J background on which it has been propagated since [17, 18]. Dickie and Lane [19] positioned the *ob*6J mutation on proximal chromosome 6 between *Microphthalmia* and *waved-1* loci. The mice were infertile, exhibited hyperphagia and early onset obesity, but on this genetic background only a transient form of hyperglycemia appeared, in association with elevated plasma insulin [20]. A second co-isogenic allele, known as *ob*2J, resulting in a similar obese phenotype, was later identified in the SM/Ckc-Dac mouse strain (Table 1.1).

A few years later in 1965 a new spontaneous obese mutant was identified at the Jackson laboratory [21]. The mutation, arisen on the

Table 1.1 *Ob* and *Db* alleles, genetic background of propagation and effect on glucose metabolism

Allele	Strain of propagation	Obesity	Glucose metabolism
Ob (*obese*) first reported by Ingalls et al. [17]			
Ob	Non-inbred stock	Severe	Diabetes
Ob	C57BL6/J	Severe	Hyperinsulinemia
			Transient
			Hyperglycemia
Ob	C57BL/KsJ	Severe	Severe diabetes
Ob^{2J}	SM/CKc-+^{Dac}	Severe	Hyperinsulinemia
			Transient
			Hyperglycemia
Db (*diabetes*) first reported by Hummel et al. [21]			
Db	C57BL/KsJ	Severe	Severe diabetes
Db	C57BL6/J	Severe	Hyperinsulinemia
			Transient
			Hyperglycemia
Db^{2J}	C57BL/KsJ	Severe	Severe diabetes
Db^{3J}	129/J	Severe	Hyperinsulinemia
			Hypoglycemia
Db^{ad}	C57BL/KsJ	Severe	Severe diabetes

C57BL/ks strain and inherited as an autosomal recessive gene located on chromosome 4, was called *diabetes* (symbol *db)*. Mutant mice were characterized by infertility, hyperphagia, and early onset obesity just like the *ob/ob* mutant, but unlike them they developed a severe form of diabetes that reduced their life span. Several alleles of the *db* gene were eventually found on other strains, namely the 2J allele that appeared on the BL6 background and the 3J allele on the 129/J strain. The severity of diabetes differed depending on the background with a stronger phenotype being expressed in the Ks strain [18].

Coleman was intrigued by the differences that the different genetic backgrounds conferred to the expression of the phenotype, especially in terms of the onset and severity of diabetes. He noticed that on the C57BL6 background in which the *ob* original mutation was propagated and in which one of the *db* alleles had spontaneously arisen, the *db/db* and *ob/ob* phenotypes were identical with mild diabetes and markedly elevated plasma insulin sustained throughout a nearly normal life span [22]. Similarly, when he later transferred the *ob* mutant allele to the C57BL/ks background he obtained a mutant with a severe early form of diabetes that compromised its life span, exactly as in the case of the original C57BL/ks *db/db* mutant [23]. Coleman's conclusion was that the wide spectrum of phenotypes observed in *db/db* and *ob/ob* mice depended exclusively on genetic background and that the two genetic defects led to an identical phenotype [24].

The juxtaposition of the two strains of obese mice provided Coleman with a brilliant intuition. He devised a clever set of experiments based on parabiosis experiments (see scheme in Fig. 1.2) that once again were inspired by deductive reasoning, interdisciplinary insights, and of course serendipity. In the first set of parabiosis experiments *db/db* animals were joined to wild type mice. In the very precise Coleman's recollection of his experimental design he explains that this first set was dictated by the need of having parabiosed animals on the same genetic background to minimize vigorous immune rejection [24]. As occasionally observed for partners of VMH lesioned rats, the +/+ members of the pairs expired, their blood glucose decreased to starvation levels and subsequent carcass analysis revealed absence of food remnants in their guts and a liver completely depleted of glycogen. Conversely, the obese *db/db* retained normal blood glucose and presented food in the stomach and in the intestine. These observations led Coleman to a "eureka moment," as he defines it [24]. He postulated that the *db/db* mouse produced a "blood borne factor so powerful that it could induce the normal partner to starve to death," also in consideration of the relatively small portion of blood that is exchanged between two parabiosed animals. Then he parabiosed *ob/ob* with +/+ mice and observed marked improvements in glycaemic control and decreased energy intake in the *ob/ob* mice. When he finally managed to place the two mutations (*ob* and *db*) on the same genetic background he could perform the key experiments by joining the blood circle of *db/db* and *ob/ob* mice. It is interesting, and almost incredible. To read in the Coleman's narrative written for the Lasker Award [24] that in his mind this experiment involving two mice of comparable weight was also aimed at excluding the possibility

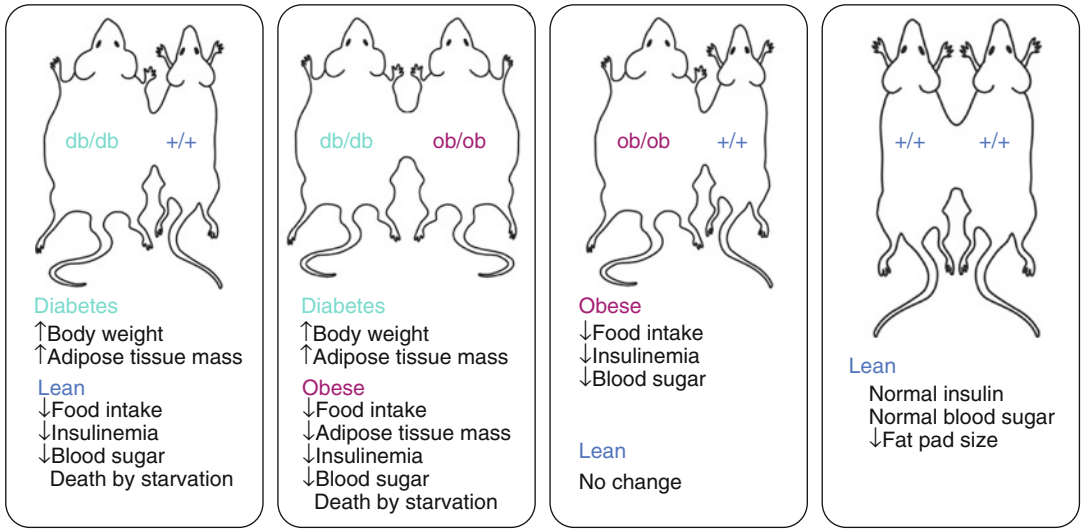

Fig. 1.2 Schematic representation of Coleman's experiments. Reproduced with permission [22]

that in case of union between an obese and a lean animal, the latter was losing weight because of being dragged around by the heavy partner. What Coleman obtained was severe weight loss, hypoglycemia, and in some cases death in the *ob/ob* members of the pairs. Carcass analysis revealed that weight decrease was entirely due to loss of adipose mass. Also in this case *db/db* animals were not affected by parabiosis and kept gaining weight according to their normal growth curve. Coleman's overall conclusion as reported in its own words "…the *db/db* mutant mouse overproduces a satiety factor but cannot respond to it—perhaps owing to a defective receptor—whereas the *ob/ob* mutant recognizes and responds to the factor but cannot produce it. The normal wild type mouse also produces this factor but in insufficient amounts to be lethal."

These findings along with previous knowledge obtained using parabiosis on hypothalamus lesioned rodents laid the basis to build an identikit of the factor missing in the *ob/ob* mouse. The requirements that this factor had to satisfy included the following:

1. A blood borne signal since it is exchanged between parabionts.
2. A bioactive molecule since it causes reduction of food intake and weight.
3. It has to reflect the amount of energy stores since the obese animal overproduces this factor as compared to the lean one.
4. Its receptor is in the hypothalamus since parabiosis performed with rodents carrying lesions in the VMH and in the arcuate nucleus cause starvation and weight loss in the untreated partner, similar to those observed for partners of the *db/db* mouse.

Molecular Genetics: A Novel Approach to Identify the ob Gene

Reverse Genetics and Positional Cloning

Much had been achieved since the theoretical homeostasis and controller models postulated by Cannon and Hervey: there was a factor, a protein, a gene, indeed two, that when altered caused obesity and these molecules had defined features of a satiety signal and its receptor. This was a breakthrough also in the clinical and popular view that had accompanied obesity, considered up to that point a behavioral rather than a physiological problem; instead, science was demonstrating

that it was not a matter determined by lack of will or by laziness, but a condition attributable to biological causes yet to be defined. Thus the hunt started. In the years that followed various factors were considered good candidates including cholecystokinin, somatostatin, and pancreatic polypeptide.

Molecular genetics was at its dawn in the 1970s and identifying a gene starting from a mammalian phenotype was almost an impossible task. The subsequent years witnessed a rapid and revolutionary advancement in this discipline, something similar to what was taking place in computer science, with a fruitful mixture of progress in knowledge and technology that led to a real explosion of the field. There are people who start what look like impossible enterprises, because they somewhat view that what appears undoable today will not be such in the near future. These people are called visionaries and sometimes they are considered insane in their pursuit of far too ambitious plans. Jeffrey M Friedman a young MD–PhD at the Rockefeller University was such a person. Dr. Friedman was 30 years old when in 1984 he started his laboratory as a Howard Hughes Medical Institute investigator at the Rockefeller University, focusing his research on the molecular identification of the *ob* and the *db* genes.

Among his first contributions to the genetics of obesity is a paper published in Genomics in 1989, that describes the chromosome mapping of the cholecystokinin (*CCK*) gene [25]. CCK is a peptide hormone, originally found in the small intestine and released in response to nutrient ingestion [26]. The high concentration of this hormone present in the brain and its capability to suppress appetite when peripherally administered to rodents, had led to the hypothesis that CCK could act as the satiety signal missing in the *ob/ob* mice. Friedman and colleagues' work ruled out this possibility by mapping *CCK* locus on murine chromosome 9, whereas the *ob* gene had been previously mapped on chromosome 6.

Friedman and collaborators were aware that the multiple metabolic and physiologic abnormalities present in the *ob/ob* and *db/db* mice made it difficult to separate primary gene effects from secondary metabolic alterations: it was therefore difficult to proceed with classical techniques to isolate the satiety signal on the basis of its being overproduced by the *db/db* mouse or based on its biochemical properties. In addition the site of synthesis of the signal had remained unknown: they then decided to take a completely novel approach that required no specific assumption on the biological nature of the defect, called "reverse genetics" [27]. Nowadays this term defines the studies that investigate which phenotypes arise from a specific sequence but at the time this was how Orkin [28] defined reverse genetics: "the isolation of a gene without reference to a specific protein or without any reagents or functional assays useful in its detection…this requires: first establishing the map position of the gene and then identifying a specific gene within this region in which mutations are strictly correlated with the disease. Restriction fragment length polymorphisms (RFLPs) in combination with cytogenetic methods provide the key to map assignment with a resolution of roughly several million base pairs. Identifying the gene of interest is the practical issue." A few years later a development of reverse research called positional cloning that deals effectively with the "practical issue" was successfully applied by Riordan to identify the gene responsible for cystic fibrosis [29]. Positional cloning was used to identify the *ob* and the *db* genes by Friedman and colleagues: it implies three major steps that will be here first briefly outlined in general and then specifically explained in the case of *ob* (and *db*) identification:

1. Genetic mapping. Based on the assumption that for loci sitting on the same chromosome, and thus not segregating independently, the number of recombination events is inversely related to their physical distance on the DNA, it is possible to map a locus within a DNA region using polymorphic markers. Centimorgan (cM) is the unit that defines this distance: one cM equals a one percent chance that a marker on a chromosome will become separated from a second marker on the same chromosome due to crossing over in a single generation; it is calculated as number of recombination events/number of total meioses × 100.

Although the probability of recombination varies along the genome, 1 cM corresponds on average to one million base pairs on the mammalian genome.

2. Physical mapping. Once that a sufficiently narrow genetic distance between two markers has been established the DNA region can be physically collected using appropriate vectors.
3. Gene identification. Different techniques can be used to identify and characterize the coding candidate genes contained in a given physical DNA region. The presence of mutations in the affected individuals constitutes the ultimate proof that a candidate is the gene responsible for the phenotype under investigation.

Genetic Mapping

Effective genetic mapping implies the existence of several and close polymorphic markers, an issue not so easy to address before the completion of the genome project. The first drafts of the human and mouse genomes were released in 2000 and 2002, respectively. In the early 1990s researchers could only use RFLPs associated with known loci. The probes used to map the *ob* gene were T cell receptor beta (*Tcrb*), carboxypetidase A (*cpa*), met oncogene (*met*), and alpha2 procollagen (*Cola-2*), all having been mapped on chromosome 6. To maximize the variability of these markers Friedman and colleagues established a series of intraspecific and interspecific crosses, a simplification of which is represented in Fig. 1.3a. It is worth mentioning that things were further complicated by sterility of *ob/ob* mice, the impossibility of phenotypically distinguishing between heretozygous +/*ob* and +/+ mice and, last but not least, difficulties in unambiguously assigning the genotype due to the high variance in body weight, fatness and development of type 2 diabetes, depending on the genetic background.

The investigators initially used ovarian transplant to an agouty [30] recipient to set an interspecific cross between *Mus musculus* C57Bl/6J *ob/ob* female and *Mus spretus* male mice and an intraspecific cross between C57Bl/6J *ob/ob* female and DBA/2J +/+mice. The F1 progeny of the two crosses were intercrossed and the distance between a given RFLP and *ob* was determined by scoring the number of obese (*ob/ob*) F2 animals carrying either the *spretus* or the DBA allele. The use of around 750 informative meioses so obtained allowed to position *ob* 11.8 cM proximal to *Tcrb* and 2–4 cM distal to *Met*. Zero (0) recombinants were found for *Cpa* out of 123 scorable animals: the conclusion was that this locus was less than 2.3 cM from *ob* [31]. These same markers were mapped on human chromosome 7q31-34, the region postulated to contain the human *ob* gene [31].

A similar approach was employed in the case of the *db* mutation and permitted to build a genetic map spanning a region of 8 cM [27]. This map places *db* 3 cM distal from *Cjun* and *Ifa* and 5 cM proximal to D4Rp1. The interval defined for *ob* was about 7–8 cM. Physical mapping required a region not larger than 1 cM and this could not be realized without a denser physical map and thus a higher number of markers. To identify additional RFLPs in the *ob* region, the Rockefeller group made use of chromosome microdissection: 19 novel RFLPs were identified one of which did not show any recombinant event across 831 informative meiosis: this marker, named D6Rck13 was used as an entry point for physical mapping of the *ob* mutation [32] together with *Pax4* that was found to be tightly linked to *ob* [33]. Chromosomal microdissection was used in the same way to more finely map the *db* gene [34].

Physical Mapping of the *ob* Gene

So far the discovery of the *ob* and the *db* genes has been treated in parallel. The following paragraphs will be dedicated to the final steps that led to *ob* identification. In contrast to genetic maps, physical maps relate genome positions to each other, using physical distances measured along the DNA helix and expressed in base pairs (bp). Physical mapping make use of intentional fragmentation of genomes that are subsequently

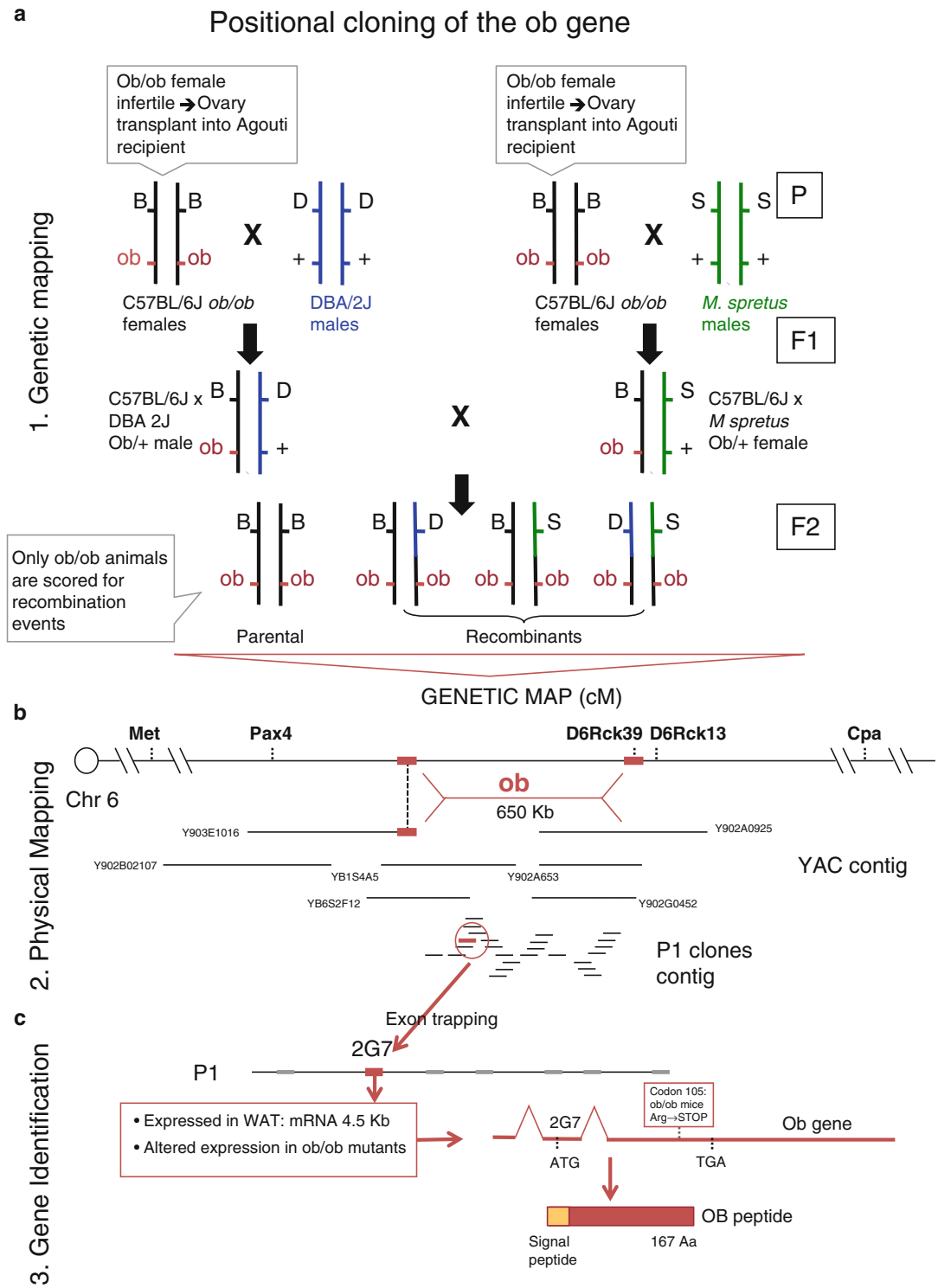

Fig. 1.3 Positional cloning of the *ob* gene is represented following the three phases of its experimental design. The key reagents and steps are represented in *red*. (**a**) Genetic mapping: a representative type of cross (interspecific intercross) is represented. *Mus spretus* (*green allele*) males were mated to C57Bl/6J *ob/ob* ovarian transplants.

cloned into appropriate vectors to build a contig, a contiguous array of overlapping clones. Yeast Artificial Chromosomes (YACs) have been widely used to this purpose since they allow to clone fragments as big as 1,400 Kb. Once that the two entry points (distal and proximal with respect to the centromere) of the gene of interest are isolated, YAC rescued ends are used to walk along the genome by identifying new YACs to cover the whole contig. Importantly YAC ends and YAC subclones sequencing generate new polymorphic markers that are used to narrow down the DNA interval of interest.

In the case of the *ob* gene the entry points to start genome walking were 2 YACs, respectively containing Pax 4 and D6Rck13 (Fig. 1.3b), The strategy explained above allowed to cover the entire region and build a contig with 6 YACs and a series of P1 mouse bacteriophage clones: the handling and amplification of P1s is easier and they can host fragments as large as 100–200 Kb. Animals typing with new polymorphic sequences markers permitted to establish the position of the *ob* gene in an estimated interval of 650 Kb, defined by two markers, each scoring a single recombination event out of 1,606 meioses: one was a novel single strand length polymorphism named D6Rck39 and mapping near named D6Rck13; the other was the distal end of a YAC containing *Pax4*. To facilitate genes identification in the critical 650 Kb region, this was also covered with 24 P1s isolated using YAC ends. In retrospective things were further complicated by the very low rate of recombination (nearly threefold lower than the average for mouse genome) that characterizes the 2.2 Mb critical region where *ob* had been mapped. Indeed, out of 1,606 meioses there were only six recombination events: so that a genetic distance of 1 cM corresponds to about 5.8 Mb in this case [35].

Ob Gene Identification

I joined Friedman's laboratory in the fall of 1993, when the physical map of the *ob* gene had been almost completed: The reader will excuse me if from now on the chapter is written with the addition of some biographical/personal notes. I had just obtained my PhD in Italy with a thesis on population genetics in ancient populations and I was fascinated by the potential that linkage analysis and positional cloning were disclosing: from a disease to its genetic cause. I will never forget my interview with Jeffrey Friedman in his office at the sixth floor of the Rockefeller building. I remember nervously waiting for him and admiring that astonishing view on the East River. He entered and everything was very quick and unusual: Jeff talked fast and for me it was at times hard to understand. Nevertheless he was gentle and friendly; he briefly asked of my PhD work and then started to tell a story of genetically obese mice that had been parabiosed: a word that I learnt that day. He used a precious Montblanc fountain pen to sketch schemes and notes while talking and in front of me pairs of "parabiosed" lean and obese mice were shaped: the Coleman experiment. Then he unexpectedly asked: "So, what do you think?" In rudimentary and uncertain English I answered that it looked like there was a defect in a common signaling pathway, affecting *db/db* and *ob/ob* mice. He seemed satisfied with that

Fig. 1.3 (continued) F1 females (B6 spretus *ob*/+) from this cross were bred to F1 males (B6D2 *ob*/+) generated from a C57Bl/6J *ob/ob* × DBA 2J (*blue allele*) +/+ mating. Only obese animals were used for genetic mapping. Modified from [29] with permission. The *ob* gene was then mapped between *Pax4* and the polymorphic marker D6Rck13 that provided entry points to start the Physical mapping (**b**) of the *ob* gene: a physical contig was built using Yeast Artificial Chromosomes (YACs) and P1 clones. This permitted to isolate novel markers (*in red* the position of the key ones) and to further narrow down the interval to 650 Kb, between the end of YAC Y903E1016 and the marker D6Rck39 (Modified from Zhang et al., 1994). (**c**) Exon trapping on a P1 clone permitted to isolate among others (*in grey*) the 2G7 candidate (*in red*) that proved to be part of the *ob* gene, based on the following evidences: 2G7 is specifically expressed by WAT, its expression is altered in *ob/ob* mice, 2G7 belongs to an open reading frame of a 3 exons gene coding for a 167-Aa peptide that presents a point mutation at codon 105 (Arg → STOP) in C57BL/6J *ob/ob* mice

response, then he asked what my feeling regarding team work was. One week later he offered me a postdoctoral position in his laboratory. I became part of the *ob* team and started to collaborate closely with Yiying Zhang and Ricardo Proenca: my role in the project was to characterize the expression of candidates isolated from the contig by means of exon trapping. This technique, now supplanted by extensive DNA sequencing and bioinformatics tools, allows isolation of exons from large genome DNA fragments and relies on the conservation of sequence at intron–exon boundaries in all eukaryotic species. By cloning a genomic fragment into the intron of an expression vector (pSPL3 in the case of the *ob* gene), exons (if any) encoded in the genomic fragment are spliced into the transcript encoded on the plasmid. Reverse transcriptase polymerase chain reaction (RT-PCR) using primers specific for the vector transcript provides a product for subsequent analysis [36].

Once the putative exons had been isolated from genomic DNA of P1 clones, each of them was sequenced and checked against all sequences present in Genbank. Then Northern blots and reverse transcription PCR were used to screen for the presence of corresponding RNA from a panel of mouse tissues (Tissue Blot) and to analyze candidate expression in *ob/ob* and wild type mice (Mutant Blot). Candidate names followed the cloning strategy code. I remember the first two exons I hybridized to a Northern Blot, named 27 and 28: in their case we were encouraged by some specific expression in the brain (a putative site for ob expression): the signal however was weak and appeared as a smeared band. One Friday (it was early May 1994) I hybridized on the Tissue Blot a ^{32}P labelled probe made out of a candidate named 2G7 that had been just isolated and sequenced, and for which no match in the database had been found. The next Saturday morning, when I developed the film, a sharp very intense and specific band with a size of around 4.5 Kb was visible only in the lane loaded with white adipose tissue (WAT) RNA. My first thought was: "I finally obtained a decent Northern experiment" and the second, "Jeff should see this." When I turned on my back Jeff was there, staring at the film and already very excited about the result. He said something about Coleman having predicted a signal originating in the white adipose tissue (depot of energy stores by definition) and in the minute after listed a series of experiments that needed to be done immediately: the first was the characterization of the 2G7 signal in the *ob/ob* mutants. I was invited to a dear friend's wedding that day, but that did not stop Jeff Friedman, who personally hybridized the same probe used for the Tissue Blot to a blot containing RNA from WAT of two different *ob* mutants. The result was quite clear: 2G7 mRNA was absent in the adipose tissue of SM/Ckc-+$^{Dac}ob^{2J}/ob^{2J}$, present in the lean littermate wild type (SM/Ckc-+^{Dac}+/+) and dramatically induced (about 20-fold) in the C57Bl/6 *ob/ob* mutant obese mice as compared to the corresponding lean control (C57Bl/6 +/+). These results were replicated also by RT-PCR [35]. By Sunday afternoon we were reasonably certain that 2G7 was an excellent candidate and the same probe was used to screen a mouse adipose cDNA library in search of the full length gene. 22 complementary clones were isolated and sequence analysis revealed a methionine initiation codon in the 2G7 exon with a 167 aminoacid open reading frame, followed by a long 3′ untranslated region for a total of over 2,800 bp. A database search did not identify any significant homology to any sequence in Genbank. The final confirmation that this sequence was that of the *ob* gene came from sequence analysis (automated and manual) of its open reading frame in cDNA obtained from WAT of C57Bl/6 *ob/ob* mice that overexpressed the transcript: the coding sequence was identical except for one C → T transition that transformed codon 105, coding for arginine, into a stop codon [35]. The hunt, started almost 50 years before had reached an happy end. That evening, before leaving, I left a note on Jeff's desk: "I have no words"; somebody else had left a rose in his office. The laboratory was unusually silent, we all shared a unique moment of intense excitement. I am not religious, but a very religious friend of mine coined a sentence that well describes these feelings: "A discovery is like looking into God's mind. It is why scientists accept to face frustration and failure most of their time."

In the following days we obtained further important and supporting evidences. Southern blot analysis using the 2G7 probe permitted to demonstrate that in SM/Ckc-+$^{Dac}ob^{2J}/ob^{2J}$ mutants absence of *ob* mRNA was associated with a different size of a BgII fragment of genomic DNA. This suggested that this mutation resulted from a deletion/insertion in a regulatory or intronic region. The hypothesis was later confirmed by Moon and Friedman [37] that reported the *ob2J* mutation to be the result of the insertion of a retroviral-like transposon containing several donor and acceptor sites in the first intron of the *ob* gene: this leads to the formation of several chimeric mRNAs, altered splicing and no synthesis of the correct mature *ob* mRNA [37].

The human *ob* gene was cloned by screening an adipose tissue phage cDNA library: as in the case of mouse it predicted a 167-amino-acid open reading frame that was 84 % identical to the murine OB peptide. A high level of evolutionary conservation was revealed also by hybridization of the *ob* cDNA to the genomic DNA of several vertebrates including rat, pig, sheep, cow, cat, chicken, and eel. A more close analysis of both human and mouse OB amino acid sequence revealed the presence of a signal peptide at its N terminus, suggesting that these could be secreted proteins. In vitro transcription and translation experiments in the presence of microsomal membranes confirmed the in silico prediction: the protein coded by the OB gene is a precursor of about 18 kDa that is truncated by 2 kDa and translocated when exposed to a posttranslational processing system. The ob protein fulfilled then one of the most important characteristics of the satiety signal postulated by Kennedy, Hervey, and Coleman: it had the potential to be a circulating peptide, a hormone. These results are represented in Fig. 1.3c.

Elucidation of ob Gene Expression and Bioactivity

All these findings were presented in the first original papers and can be summarized as follows: the murine *ob* gene is expressed by the white adipose tissue and it codes for a 167-amino-acid secreted protein. It is mutated in two independent strains of *ob/ob* mutants and there is a human homologue.

Some relevant questions remained to be addressed and are here briefly outlined with their outcome:

- *Is ob gene expression specifically associated with mature adipocytes and with their terminal differentiation*? The white adipose is composed of two main fractions, namely, a stromal vascular fraction containing preadipocytes, endothelial cells, inflammatory cells and fully mature adipocytes. The use of an established preadipocytes cell line able to undergo adipose conversion (F442A) permitted to positively answer this question. Ob gene expression was in fact typical of the mature adipocyte [38]. This finding by itself paved the way to the concept of adipokine, a molecule secreted by adipocytes, and further, it changed the concept of adipose tissue, not any longer a passive depot of energy storage, but an active endocrine organ [38].
- *What is ob gene regulation in satiety signal resistant mice*? From Hervey's and Coleman's parabiosis experiments we knew that some obese models (*db/db* and VMH lesioned) are resistant to the satiety signal. VMH lesions can be obtained by one intraperitoneal injection of gold-thioglucose (GTG): this causes hyperphagia and in 4 weeks mice double their initial body weight. This model was in place in Friedman's laboratory. Gene expression studies revealed that in both *db/db* and GTG treated mice ob gene expression was upregulated by 20-fold [38]. The data were discussed as follows: "(they) position the *db* gene and the hypothalamus downstream of ob in the pathway that controls body weight…and are consistent with the hypothesis that the ob receptor is encoded by the *db* locus" and further more the increase of ob RNA in GTG mice suggested a non-cell autonomous function of the ob gene product in fat cells, since the overexpression of the wt protein did not prevent obesity in the absence of an intact hypothalamus.
- *Is the ob protein circulating in blood*? Antibodies raised against the recombinant OB

protein allowed to detect a 16 kDa band in the plasma of wild type mice, but not in that of C57Bl/6J *ob/ob* mutants, suggesting that the point mutation caused an unstable truncated protein. The signal was more intense in the lane loaded with the *db/db* plasma [39]. These results established that the OB protein, as postulated by the existence of a signal peptide sequence at its N terminus, was a secreted protein and possibly a hormone.

- *Is the ob protein able to induce satiety and weight loss*? This was obviously one of the most relevant questions. In collaboration with the laboratory of Molecular Biophysics headed by Stephen Burley the OB protein was expressed and renatured. Then administered to *ob/ob*, wild type and *db/db* mice by daily injections. The results obtained in *ob/ob* mice were striking. After 33 days of treatment they had lost 40 % of their initial body weight and had reduced their food intake by 40 % becoming similar to normal wild type animals: importantly their adipose mass was reduced by 50 %, whereas the lean mass was not significantly changed. None of these changes was observed in PBS treated controls. Of note, *ob/ob* mice not treated with the OB protein, but eating the same amount of food intake (pair-fed) lost significantly less weight than animals receiving the recombinant protein, indicating that the input component per se was not the only parameter determining a negative energy balance. Indeed Pelleymounter and colleagues demonstrated that the OB protein is able to increase metabolic rate and locomotor activity [40] and its role in the induction of energy expenditure is now widely acknowledged [41]. Wild type animals treated with the OB protein reduced their food intake and body weight significantly and stabilized both parameters at levels that were respectively 92 % and 88 % those of untreated controls. An impressive reduction in fat mass was observed also in this case with adipose mass accounting for only 0.67 % of total body weight as compared to the 12 % observed in not treated animals. In *db/db* mice no effect was observed, this being consistent with the postulated defect in OB protein receptor [39]. The first "public presentation" of these results was during a lab meeting given by Jeffrey Halaas. In that occasion Friedman announced that we had to pick a name for this novel protein that made mice become thin. He proposed to use the Greek root for thin: leptós. We all agreed to leptin and this name was used for the first time in the Halaas et al. [39] paper published in Science. In the same issue two other important contributions highlighting further aspects of leptin action appeared: weight loss following brain direct administration of very low dosage of the protein suggested that it could act directly on neuronal networks that control feeding and energy balance [42]; Pelleymounter and coworkers demonstrated that the OB protein lowered blood glucose and insulin levels in *ob/ob* mice and that it acted to increase energy expenditure [40].
- *Do leptin levels in plasma reflect the degree of obesity*? We knew that the ob gene mRNA was increased in the WAT of obese models in which the pathway had been interrupted (VMH lesioned *db/db* mice). The same was observed also in mice treated with high fat diet and in old overweight mice [38] but not in three classical models of murine obesity represented by the *Agouti* (A^y/+), the *tubby*, and the *fat* mouse. This was an indication that the transcription machinery in the WAT was not equally induced in all cases: what was important to establish however were the circulating levels of leptin, since all evidences that had been accumulating over time pointed to a hormonal-like biological action with important targets situated in the hypothalamus. The availability of good antibodies permitted first to use a titration method that employed known concentration of the recombinant protein loaded on a Western Blot, and then to develop an ELISA assay (in collaboration with Roger Lallone in Birmingham) for an accurate measurement of plasma leptin in mouse and in humans. The results were very clear: plasma leptin levels were much higher in obese individuals: all rodent models of obesity exhibited dramatically increased level of the circulating

protein as compared to lean controls including also the *Agouti*, *tubby*, and *fat* mouse, for which ob mRNA did not change (see above). In both humans and mouse a positive and highly significant correlation existed between body mass index and plasma leptin, although there was high variability of the circulating protein at each BMI. Consistently weight loss due to food restriction was associated with a decrease in plasma leptin in both humans and mouse [38].

Conclusions

Altogether these results establish that all the characteristics of the OB protein postulated by Coleman were true:

1. It was a circulating signal originating from energy stores (WAT) with the features of a hormone and could thus be exchanged between parabiosed animals.
2. It was a bioactive molecule with a potent anorectic effect and this explained the weight loss in *ob/ob* mice parabiosed to wild type or *db/db* mice and in wild type parabiosed to *db/db*.
3. Its levels reflected the amount of energy stores, now better defined as the white adipose tissue, and thus animals parabiosed to obese models (*db/db* or VMH lesioned) starved to death because they had been receiving very high concentration of the satiety signal.
4. It signals to the hypothalamus. Although *db* subsequent cloning and localization [43, 44] provided the definite confirmation of this hypothesis, at this stage we were already reasonably confident that at least one of leptin targets was in the hypothalamus, based on the following evidences: ob transcriptional levels were increased in mice with VMH lesions, suggesting that a feedback loop is interrupted in this model; leptin direct administration in the brain results in food intake reduction and weight loss even at very low dosage.

In my view a phase of studies on energy balance is concluded by these findings and another is disclosed. Leptin has changed the perspective on weight control problem, by permitting the energy homeostasis field to enter a molecular era. It has been a formidable tool and the heart of the matter to start dissecting the complex mechanisms that regulate feeding behavior and body weight.

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