CHAPTER THREE

Brd2 Gene Disruption Causes "Metabolically Healthy" Obesity: Epigenetic and Chromatin-Based Mechanisms that Uncouple Obesity from Type 2 Diabetes

Fangnian Wang[*], **Jude T. Deeney**[†], **Gerald V. Denis**[‡,§,¶,‖,1]
[*]IQuum Inc, Marlborough, MA 01752, USA
[†]Department of Medicine, Boston University School of Medicine, Boston, Massachusetts, USA
[‡]Flow Cytometry Core Facility, Boston University School of Medicine, Boston, Massachusetts, USA
[§]Nutrition Obesity Research Center, Boston University School of Medicine, Boston, Massachusetts, USA
[¶]Cancer Research Center, Boston University School of Medicine, Boston, Massachusetts, USA
[‖]Department of Pharmacology and Experimental Therapeutics, Boston University School of Medicine, Boston, Massachusetts, USA
[1]Corresponding author: e-mail address: gdenis@bu.edu

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Abstract

Disturbed body energy balance can lead to obesity and obesity-driven diseases such as Type 2 diabetes, which have reached an epidemic level. Evidence indicates that obesity-induced inflammation is a major cause of insulin resistance and Type 2 diabetes. Environmental factors, such as nutrients, affect body energy balance through epigenetic or chromatin-based mechanisms. As a bromodomain and external domain family

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transcription regulator, Brd2 regulates expression of many genes through interpretation of chromatin codes and participates in the regulation of body energy balance and immune function. In the severely obese state, Brd2 knockdown in mice prevented obesity-induced inflammatory responses, protected animals from insulin resistance, glucose intolerance and pancreatic beta cell dysfunction, and thus uncoupled obesity from diabetes. Brd2 provides an important model for investigation of the function of transcription regulators and the development of obesity and diabetes; it also provides a possible, innovative target to treat obesity and diabetes through modulation of the function of a chromatin code reader.

ABBREVIATIONS

BET bromodomain and extraterminal domain
BMI body mass index
HOMA homeostatic metabolic assessment
MHC major histocompatibility complex
PPAR peroxisome proliferator-activated receptor
SNP single nucleotide polymorphisms
TNF tumor necrosis factor
TWEAK TNF-related weak inducer of apoptosis
TZD thiazolidinedione

1. INTRODUCTION: THE PROBLEM OF OBESITY AND ITS COMPLICATIONS

Over the course of recorded history, many different diseases and risks have threatened human survival on a wide scale, including epidemics of infectious organisms, war, starvation, famine, sudden climate change, and ethnic cleansing. As recently as the end of the Second World War, a chronic lack of calories in Europe and Japan was widely acknowledged as a major contributor to poor health, elevated infant mortality, increased disease susceptibility (particularly to tuberculosis), and its attendant consequences of economic and political instability. The end of that war brought the benefits of modern peace: a dramatic transformation of the international economy, accompanied by huge increases in industrial and agricultural output, vast international trade in manufactured goods, raw materials and foods (both processed and unprocessed), and a pronounced shift from manual to sedentary labor. These tremendous structural and economic changes, combined with increased life expectancy and the rise of agribusiness, advertising, and a hugely successful international food industry, have contributed to the transformation of obesity from a quaint feature of royal families in former

times to an alarming, international public health worry that affects all socioeconomic classes at present. Thus, of the serious and widespread menaces to public health in human history, obesity is the newest challenge.

Obesity is defined as a body mass index (BMI) of $\geq$30, calculated as body weight in kilograms divided by the square of height in centimeters. Lean individuals are defined as BMI $<$25 and individuals with intermediate BMI ($\geq$25 to $<$30) are classified as overweight. Morbid obesity is defined as BMI $\geq$40 and super obesity is defined as BMI $\geq$50. These higher categories of obesity are used to be extremely rare, and they still are relatively rare. However, as the U.S. population distribution shifts to higher mean BMI, these extreme forms of obesity are increasing in prevalence at a greater rate than the mean. According to the most recent statistics from the U.S. Centers for Disease Control (CDC, 2010 data), all U.S. states now report at least 20% prevalence of obesity among adults. Mississippi, West Virginia, and Alabama currently report the highest rates (34.0%, 32.5%, and 32.2%, respectively), but several other states are not far behind. Worldwide, 1.7 billion people are classified as overweight (Haslam & James, 2009). These statistics pose fundamental and novel problems for health maintenance and delivery systems.

The speed with which overweight and obesity have become a public health crisis is breathtaking http://www.cdc.gov/obesity/data/adult.html. For a useful meta-analysis, see Finucane et al. (2011). Obesity in the United States is associated with a dramatic increase in the prevalence of obesity-associated diseases, particularly cardiovascular disease and Type 2 diabetes, as well as hypertension, stroke, metabolic syndrome and insulin resistance, nonalcoholic fatty liver disease, and some forms of cancer, notably breast cancer in postmenopausal women and adult colorectal cancer in both men and women. About 90% of Type 2 diabetes is attributable to excess weight (Hossain, Kawar, & El Nahas, 2007). The incidence of Type 2 diabetes, in particular, has now reached epidemic proportions in the United States and internationally, affecting about 12% of U.S. adults and more than 25% of those over the age of 65. Type 2 diabetes is thought to be responsible for 4.6 million deaths worldwide each year. An oft-cited early estimate of the incidence of Type 2 diabetes was 366 million worldwide by 2030 (Wild, Roglic, Green, Sicree, & King, 2004). However, more recent estimates have pegged the anticipated number of diabetic individuals to be 439 million by 2030 (Shaw, Sicree, & Zimmet, 2010), almost entirely due to obesity-driven metabolic complications. There is reason to worry that this prediction will soon have to be revised upward yet again.

Unless reversed, the rapidly worsening problem of obesity predicts an epidemic of comorbidities that will strain or break many health care delivery

systems, both in the United States and internationally. In the absence of national agreement about the proper role of U.S. health care delivery systems with controlled costs, or political will to deliver well-designed solutions, the U.S. economy is uniquely vulnerable to this expected impact. Thus, obesity poses a critical challenge of overarching importance for U.S. public health, particularly pediatric health: many American children are already being swept along with this national wave of progressive metabolic dysfunction in obesity. For the purpose of this chapter, some of the genes and epigenetic forces that drive obesity and metabolic dysfunction will be examined.

2. COMPLEX POLYGENIC INTERACTIONS WITH THE ENVIRONMENT AND EPIGENETICS IN OBESITY

We live in an era of genetic determinism. Major effort has been invested to identify "obesity-associated genes" with the translational goal of isolating the "drivers" from the "passengers," much the same way that oncogenes have been identified, in order to develop novel therapeutic targets. Evidence for a genetic component that influences risk for obesity controlling for environment has been developed through studies of adoption and twins (Maes, Neale, & Eaves, 1997, Silventoinen, Rokholm, Kaprio, & Sørensen, 2010). Genetic factors appear to explain as much as 90% of the variance in BMI among certain monozygotic twins. Furthermore, several recent genome-wide association studies have established convergent validity in the discovery of genes that are strongly associated with obesity throughout the human lifespan. The first robust association was reported for *FTO* (fat mass and obesity associated) (Frayling et al., 2007). This association was joined by insulin-induced gene 2 (*INSIG2*), melanocortin 4 receptor (*MC4R*), transmembrane protein 18 (*TMEM18*), glucosamine-6-phosphate deaminase 2 (*GNPDA2*), neuronal growth regulator 1 (*NEGR1*), brain-derived neurotrophic factor (*BDNF*), and potassium channel tetramerization domain containing 15 (*KCTD15*), as associated with pediatric obesity (den Hoed et al., 2010; Zhao et al., 2009). Intensive research in molecular genetics continues on other well-established loci of obesity-associated genes, including those that encode the uncoupling proteins (Oppert et al., 1994; Ricquier, Casteilla, & Bouillaud, 1991), peroxisome proliferator-activated receptor (PPAR)γ (Lefebvre et al., 1998; Vidal-Puig et al., 1997), low-density lipoprotein receptor (Morris, Zee, & Robinson, 1994), hormone-sensitive lipase (Klannemark et al., 1998; Stich et al., 1997), beta adrenergic receptors (Reynisdottir, Ellerfeldt,

Wahrenberg, Lithell, & Arner, 1994; Walston et al., 1995), and inflammatory cytokine genes such as tumor necrosis factor (TNF)-α (Saghizadeh, Ong, Garvey, Henry, & Kern, 1996) and interleukin-6 (Kern, 1997; Kern, Ranganathan, Li, Wood, & Ranganathan, 2001). The hope of these studies has been that, through identification and characterization of candidate genes for obesity and metabolic syndrome, potent new drugs will be developed that specifically target these proteins or pathways and will have therapeutic value for prediabetic or diabetic obese patients.

Although more than 100 genes have been identified that influence body weight (Leibel, 2008), the usual patterns and rules of Mendelian inheritance have proven inadequate to understand the inheritance of predisposition to metabolic syndrome and Type 2 diabetes. At least 18 genes have been directly associated with Type 2 diabetes (Florez, 2008; Ridderstråle & Groop, 2009). This fact indicates that, unlike inborn errors of metabolism, such as Tay–Sachs disease, for which a straightforward causal connection exists between a mutant allele and a well-characterized dysfunction (Schneck et al., 1970), the genetic mechanisms that promote increased risks in obesity for Type 2 diabetes, cardiometabolic diseases, and obesity-associated cancers are not so straightforward.

It is also clear that the interaction of genes alone is insufficient to explain the contemporary problem of obesity. In view of the new international environment of widespread overnutrition, it also seems important to ask how genes respond to weight gain or weight loss, not just how specific genes make us fat or thin. Dietary caloric excess that is totally misaligned with physical energy expenditure characterizes this environment. Epidemiologists and U.S. public health officials have taken note of critical features of the current American environment, particularly easy access to cheap calories, the profligate use of sugar sweeteners like "high fructose corn syrup" in the food industry (Duffey & Popkin, 2008; Popkin, 2007; Popkin & Nielsen, 2003) and widespread overconsumption of fried, fatty, "fast" foods.

Ten thousand years of human evolution have tended to produce a lean physical phenotype. Indeed, severe pediatric obesity of genetic origin is observed in less than 0.01% of the population (Farooqi & O'Rahilly, 2006). Thus, the dramatically increased prevalence of overweight, obese, and morbidly obese phenotypes in the past decades of the twentieth century and first decade of the twenty-first century must be attributable primarily to environmental and consumption changes, i.e., environmental obesogenic pollutants, reduced energy expenditure and diet-induced obesity. Strong evidence for the role of the environment when genetic factors are relatively well controlled

was obtained from studies of the Pima Indians, an aboriginal population that lives in the desert southwest of the United States and in an area of northern Mexico with a similar climate. The genetic variation between the two populations is relatively small. Pima Indians in the United States are on average 25 kg heavier than Pima Indians in Mexico (Ravussin, 1995), with corresponding differences in cardiometabolic risk. These differences have been attributed primarily to diet. Alternatively, population patterns of obesity may be attributed in part to epigenetic shifts, not to mutation in human DNA, because the timescale on which these changes in public health have occurred are far too rapid to be due primarily to evolution.

There is new interest in the maternal–fetal environment and an effort to understand a potential role for epigenetic mechanisms in the transmission of metabolic risk factors. The epigenetic modifications of nucleosomal histones and DNA that underlie these phenomena are poorly understood. These modifications include DNA methylation and histone acetylation or phosphorylation and are critical for proper control of the response of promoters to signal transduction in the differentiated cells of the adult (Bernstein, Meissner, & Lander, 2007; Li, Carey, & Workman, 2007), as well as for embryonic and fetal development (Bernstein et al., 2006). Indeed, some of the most interesting work on epigenetic and developmental mechanisms has been conducted in rat dams fed a high-fat diet. Investigators have identified alterations in homeobox gene expression patterns, such as HoxA10, that likely influence stem cell fate choice in osteogenic and adipogenic cell lineages (Chen et al., 2012). These alterations of transcriptional programming depend critically on patterns of acetylation and methylation of histone lysines in nucleosomal chromatin at key target genes. This concept suggests that patterns of obesity and metabolic dysfunction may indeed run in families, but not in the same way or subject to the same mechanisms as patterns of familial cancers.

One famous and well-studied case that strongly supports an epigenetic mechanism for cardiometabolic risk is the Dutch "Hunger Winter" of 1944–1945. This wartime event, when severe starvation affected the western Netherlands, revealed in detail for the first time that epigenetic mechanisms play a critical role in human BMI and insulin sensitivity. Specifically, maternal hunger created by cold, harsh living conditions, and caloric deprivation of pregnant mothers was linked to insulin resistance, obesity, an atherogenic lipid profile, and elevated cardiovascular risk in the surviving children as they aged (Kyle & Pichard, 2006). Of particular interest, children born to the same mothers in times of less severe deprivation did not show the same risk patterns. More recent work has shown that

infants born to obese, overweight, and Type 2 diabetic mothers display increased adiposity and elevated risk for later metabolic disease (Heerwagen, Miller, Barbour, & Friedman, 2010). In addition, a UK study found that males who were most underweight at birth were seven times more likely to develop metabolic dysfunction and Type 2 diabetes later in life than males who were heaviest at birth (Hales et al., 1991). There is strong support for an inverse relationship between birth weight and hypertension in the adult, for both men and women, in which low birth weight predicts risk among the highest weight adults (Barker, Osmond, Golding, Kuh, & Wadsworth, 1989). These observations suggest that maternal uterine environments vary in ways that create stable and lasting consequences for the metabolic patterns of offspring exposed to that environment during gestation (Barker, 1995). Many of these patterns have been ascribed to epigenetic modification of key genes that regulate energy metabolism. Such mechanisms have been and continue to be explored and validated in rodent models (Levin & Govek, 1998; Nathanielsz, Poston, & Taylor, 2007; Samuelsson et al., 2008; Shankar et al., 2008). Thus, new research focusing on the specific modifications of nucleosomal histones in chromatin and DNA is worthy of attention.

Interesting work has shown that manipulation of DNA methylation pathways by metabolic supplementation can alter the intergenerational, epigenetic patterns of obesity (Waterland, Travisano, Tahiliani, Rached, & Mirza, 2008), which directly addresses mechanisms that may be relevant to the Hunger Winter offspring. Such approaches, if validated with animal models and in clinical trials, offer the possibility of a "personalized medicine" approach to obesity therapeutics (Martínez, Cordero, Campión, & Milagro, 2012). Innovative clinical investigations to measure how an altered maternal uterine environment during gestation affects DNA methylation patterns have already been reported (Cooper et al., 2012).

Although chromatin modifications are well studied in embryogenesis and cancer (Sharma, Kelly, & Jones, 2010), much less is known about their role in metabolic diseases. Intense research is now focused on how DNA sequence-specific transcription factors such as PPARγ and transcription coregulators such as PGC-1α (Puigserver et al., 1998) interact with histones and nucleosome remodelers (Pedersen, Kowenz-Leutz, Leutz, & Nerlov, 2001) to alter chromatin and reprogram gene expression networks (Jaenisch & Bird, 2003). These transcriptional coregulator functions are also important in inflammation (Freund, Orjalo, Desprez, & Campisi, 2010; Orjalo, Bhaumik, Gengler, Scott, & Campisi, 2009), which plays a critical

role in the development of insulin resistance in obesity (Bastard et al., 2006; Shoelson, Herrero, & Naaz, 2007).

Histone acetylation is also being considered as a relevant target for epigenetic manipulation to affect energy metabolism. For example, genome-wide patterns of histone H3 acetylation at lysine 9 and 18 have been reported in INS-1 cells, a rat model for the pancreatic β-cell, upon exposure to peptide incretin hormones (Kim, Nian, & McIntosh, 2009). These modifications alter transcriptional coregulator function which likely coordinates reprogramming of transcriptional networks that respond to glucose signal transduction in the β-cell. This approach may have translational significance for obese, insulin-resistant patients, because these peptide hormones potentiate glucose-stimulated insulin secretion in the islet, among other effects. On the other hand, increased histone acetylation has been widely appreciated for decades as a mark of transcriptional activation at numerous loci. Absent a more specific, promoter-defined, or β-cell-specific signature of histone acetylation that is unique and resolvable from the general somatic pattern, these insights have relatively modest utility and present no new therapeutic targets for Type 2 diabetes. The emerging practicality of manipulation of chromatin-controlled transcriptional programs for therapeutic benefit is potentially useful in view of the new availability of a class of bromodomain protein-directed, small-molecule inhibitors (Belkina & Denis, 2012; Filippakopoulos et al., 2010; Muller et al., 2011; Nicodeme et al., 2010). If specific, functional targets of histone acetylation could be identified, small-molecule therapeutics for aberrant histone acetylation/transcriptional coactivation may develop an experimental basis for animal model experiments. However, the field has not yet reached this stage of development.

Activators of PPARγ function, such as pioglitazone, also appear to play a role in transcriptional programming controlled by PPARγ in part through alteration of histone modifications. In the β-cell, increased methylation of histone H3 at lysine 4 in the promoter regions of *Ins1*, *Ins2*, and *Glut2* genes is maintained through the dimethyl-lysine regulating complex Set7/9 methyltransferase and is required for proper transcription of these loci (Deering, Ogihara, Trace, Maier, & Mirmira, 2009). Pioglitazone treatment of obese *db/db* (leptin receptor-deficient) mice or high-fat diet-fed wild-type mice, which is well known to improve metabolism, dramatically improves *in vivo* transcription of *Ins1*, *Ins2*, and *Glut2* genes. This improvement is associated with increased histone acetylation of these key target genes (Evans-Molina et al., 2009). Similar enrichment in methylation of histone H3 at lysine 4 has been reported in β-cell lines and islets, and parallel increases transcription of these same genes (Francis, Chakrabarti, Garmey, & Mirmira, 2005).

In normal human pancreatic islets, histone lysine methylation patterns have been identified that define transcriptionally "primed" as well as transcriptionally active promoters (Bhandare et al., 2010), but this vein of research is relatively underdeveloped, compared to the study of histone lysine methylation in cancer. Additional experiments to test mechanistic hypotheses are critically needed to deepen understanding of how manipulation of chromatin "readers" and "writers," and DNA methylation enzymes, might be mobilized therapeutically (Belkina & Denis, 2012) to improve β-cell dysfunction. Most of the relevant work to date has been no more than correlative.

3. THE "*brd2 lo*" MOUSE MODEL OF "METABOLICALLY HEALTHY" OBESITY

How are these engines of histone modification (the "writers" of the epigenome) and the transcriptional machines that respond to signal transduction to interpret these modifications (the "readers" of the epigenome) to be studied and understood in their regulation of diverse transcriptional networks? Animal models that enable manipulation of chromatin and epigenetic mechanisms will obviously become increasingly important as investigators seek to understand the relevant genes and signal transduction pathways at work in obesity. In particular, one model with an epigenetic basis for obesity has received attention recently and offers a novel interpretive tool: the Brd2-deficient model for "metabolically healthy" obesity. In both humans and mice, the *BRD2* gene encodes an unusual transcriptional coregulator that contains double bromodomains (Haynes et al., 1992; Horn & Peterson, 2001; Jeanmougin, Wurtz, Le Douarin, Chambon, & Losson, 1997; Winston & Allis, 1999). Brd2 belongs to the bromodomain and extraterminal domain (BET) family of transcriptional coregulators defined by two tandem, mutually related bromodomains at the amino-terminus of the protein that bind to acetylated lysines in nucleosomal chromatin (Kanno et al., 2004; Nakamura et al., 2007), particularly acetyl histone H4 (Umehara et al., 2010) and transcriptionally couple histone acetylation to gene activation (LeRoy, Rickards, & Flint, 2008). The amino-terminal dual bromodomains in this family are followed by an "extraterminal" domain that is involved in protein–protein interactions (Lin et al., 2008; Rahman et al., 2011), thus the "BET" family name. The bromodomain is the only protein structural motif (Dhalluin et al., 1999) that is capable of "reading" sites of histone acetylation in nucleosomal chromatin (Sanchez & Zhou, 2009). Brd2 studies have been important because they have revealed remarkable and unexpected roles for chromatin regulation in energy metabolism.

3.1. Gene targeting of the *Brd2* locus in mice

Targeted disruption of the *Brd2* gene, which is located in the class II major histocompatibility complex (MHC) near *Tnf*, causes extreme obesity with hyperinsulinemia, but also hypoglycemia, hyperadiponectinemia, and improved glucose tolerance quite distinct from other animal models of obesity (Wang et al., 2009). *Brd2* had no previously known link to obesity, insulin sensitivity, or energy metabolism (Belkina & Denis, 2010; Denis, Nikolajczyk, & Schnitzler, 2010). The gene was disrupted in mouse embryonic stem cells by insertion of a *lacZ* cassette that encodes β-galactosidase. The cells were engineered by BayGenomics at the University of California, Davis, which is an arm of the Mutant Mouse Regional Resource Centers, and which receives support from the National Institutes of Health (http://www.mmrrc.org/catalog/overview_BG.php). Two types of embryonic stem cells were developed, one with a *lacZ* insertion in the coding region of the gene (designated RRE050) and one with a *lacZ* insertion in the promoter region (designated RRT234). These latter cells were used to develop the *brd2 lo* mice (Wang et al., 2009). The phenotype could not have been predicted (Wang et al., 2009), yet holds out the possibility of a more unified, epigenetically-based mechanism that underlies several human comorbidities associated with obesity and inflammation (Denis, 2010).

3.2. Systemic, protective phenotypes in *brd2 lo* mice

Despite severe obesity approaching 100 g, *brd2 lo* animals show improved whole-body insulin sensitivity and do not develop insulin resistance or glucose intolerance. They show better whole-body insulin sensitivity than wild type, control animals on the C57Bl6/J background, despite dramatic obesity (Jornayvaz et al., unpublished data). Several whole-body mechanisms likely contribute to this protection (Wang et al., 2009). For example, although *brd2 lo* animals, both males and females, consume slightly more chow than age- and sex-matched wild-type controls, they burn slightly more calories as heat in isothermal housing. The animals carry more interscapular brown adipose tissue and their white adipose tissue expresses higher levels of uncoupling proteins, which provides a mitochondrial mechanism for increased heat production. This phenotype is known to be protective of metabolism in obesity. In addition, *brd2 lo* mice show a respiratory exchange ratio of oxygen consumption and carbon dioxide production that is suggestive of fat metabolism at all times of day and night, fed state or fasting state, rather than a shift between carbohydrate metabolism expected for the fed

state to fat metabolism expected for the fasted state (Wang et al., 2009). Continuous β-oxidation of fatty acid as the primary energy source might be expected to be metabolically protective, although the mitochondrial basis for this preferred mechanism of energy metabolism is not understood in these mice. Finally, production of high-molecular weight adiponectin, an insulin-sensitizing adipokine, was elevated almost to the serum levels seen in adiponectin transgenic mice on the *ob/ob* (leptin deficient) background (Kim et al., 2007). Elevated adiponectin confers yet another layer of metabolic protection in obesity. Yet it is important to point out here that the adiponectin promoter has not been directly genetically manipulated in the source embryonic stem cells; this result suggests that Brd2 coregulator function is ordinarily required for corepression of adiponectin transcription. This hypothesis has not yet been tested experimentally.

3.3. The *brd2 lo* phenotype protects adipose tissue

Remarkably, knocked-down expression of Brd2 in the 3T3-L1 differentiation model also protects cultured adipocytes from TNF-α-induced insulin resistance *in vitro*, probably by uncoupling TNF receptor signaling from transcription (Wang et al., 2009). The effect of reduced Brd2 levels also phenocopies the action of thiazolidinedione (TZD) drugs, such as rosiglitazone and pioglitazone, which are used as insulin sensitizers. Brd2 coactivator function opposes the action of PPARγ; Brd2 protein associates with PPARγ protein complexes and knockdown of Brd2 strongly stimulates PPARγ-dependent transcription and adipogenesis in 3T3-L1 cells (Wang et al., 2009).

3.4. Insulin resistant obesity is an inflammatory disease

Insulin resistance in the context of obesity is associated with a chronic state of subclinical inflammation (Bastard et al., 2006; Shoelson, Lee, & Goldfine, 2006; Weisberg et al., 2003; Xu et al., 2003), including increased serum concentrations of C-reactive protein, interleukin-6, interleukin-8, and TNF-α in patients and different animal models of obesity (Kahn et al., 2006). In insulin-resistant obesity, production of TNF-α in liver, fat, and muscle by infiltrating, proinflammatory (M1) adipose tissue macrophages promotes insulin resistance directly (Hotamisligil, Shargill, & Spiegelman, 1993). Here, M1 macrophages are distinguished from the more remodeling-purposed, alternatively polarized "M2" type of macrophage. Commonly, proinflammatory macrophages infiltrate white adipose tissue in patients and different animal models of obesity (Kahn et al., 2006), forming characteristic, histologically identifiable patterns

of leukocytes called "crown-like structures" (Apovian et al., 2008; Cancello et al., 2005, Cinti et al., 2005) that surround stressed, dead, and dying adipocytes, and are closely associated with metabolic risk. These structures have recently been shown also to contain B cells (McDonnell et al., 2012), and both T cells and B cells engage in crosstalk with peripheral blood monocytes to define the proinflammatory and anti-inflammatory balance of cytokines in the obese, insulin-resistant adult (Jagannathan et al., 2009, 2010). To a first approximation, the greater the proinflammatory balance, the greater the metabolic dysfunction and the more advanced the disease. However, much work remains to be done to understand the specific T cell, B cell, and macrophage subtypes that are required for disease progression, the anti-inflammatory and homeostatic mechanisms that oppose aggravated chronic inflammation, and the kinetics of entry onto and departure from the metabolic "theatres of action" of each of these cell types: the central and peripheral adipose depots, the liver, the pancreas, and the blood.

3.5. Metabolically protective phenotypes in the *brd2 lo* immune system

The *brd2 lo* mice exhibit broad-spectrum protection against the inflammatory complications of obesity (Wang et al., 2009). Inflammatory responses are mildly deficient in these animals (Belkina, Blanton, Wang, Liu, & Denis, 2010), which likely contributes to their protection against metabolic dysfunction. These observations lead to novel hypotheses about the interactions and crosstalk between adipocytes, macrophages, and T cells that control the inflammatory state of adipose tissue. White adipose tissue shows reduced inflammation: bone marrow-derived macrophages underproduce proinflammatory cytokines (Belkina et al., 2010, 2013), T cell migration is ablated (G. V. Denis et al., unpublished observations), and regulatory T cells are expanded (G. V. Denis et al., unpublished observations). Furthermore, because polarization of macrophages to the anti-inflammatory, proremodeling (M2) state requires PPARγ (Odegaard et al., 2007), increased PPARγ activity in *brd2 lo* macrophages and elevated serum adiponectin (Ohashi et al., 2009) is likely to promote M2 polarization, also protecting against inflammation-driven insulin resistance. By transcriptionally uncoupling obesity from insulin resistance, the mild hypo-inflammatory phenotype of the *brd2 lo* model focuses attention on T cell and adipose tissue macrophage mechanisms that likely protect "metabolically healthy" obese individuals and might be harnessed to protect the larger population of obese patients with insulin resistance. Consistent with the inflammatory mechanism, a report in *Nature*

Genetics has identified human single nucleotide polymorphisms (SNPs) in the *BRD2* locus that are significantly associated with rheumatoid arthritis, which is driven by autoimmune and inflammatory processes (Mahdi et al., 2009). Furthermore, *BRD2* variants may be associated with BMI in Pima Indians (Muller, Abdussamad, et al., 2011). Upregulation of proinflammatory cytokine genes has long been appreciated to promote insulin resistance and glucose intolerance, which precede serious metabolic dysregulation that leads to Type 2 diabetes (Pickup & Crook, 1998).

Furthermore, reduced inflammation protects against insulin resistance and characterizes human "metabolically healthy" obese populations. These human subjects display attenuation of each of: glucose intolerance, dyslipidemia, hyperuricemia, and hypertension (Bonora et al., 1991; Wildman et al., 2008). Therefore, control of adipose tissue-infiltrating inflammatory cells and their chromatin-regulated gene expression holds promise for insulin-resistance patients. In obesity, macrophages, T cells, and adipocytes produce proinflammatory and anti-inflammatory cytokines, chemokines, and adipokines. Given that these factors synergize with, antagonize, and regulate each other, there are many unanswered questions about what happens during the adipocyte–macrophage–T cell interactions (Kintscher et al., 2008), and how and when insulin resistance develops. We have proposed that these animals offer a novel model for human "metabolically healthy" obesity. The model motivates interesting and informative hypotheses to test the links among leukocyte migration, proinflammatory cytokine production, and insulin resistance in obesity.

4. WHO ARE "METABOLICALLY HEALTHY" OBESE HUMANS?

In most humans, the main effect of increasing BMI is a nonlinear deterioration in metabolic and cardiovascular health. This process has been described as a gradual clustering of traits that include visceral obesity (as distinct from elevated BMI), insulin resistance, dyslipidemia, hypercholesterolemia, and hypertension. In the clinic, insulin resistance status is often determined by the homeostatic metabolic assessment (HOMA), which is assessed using the formula: fasting insulin (μU/mL) × fasting glucose (mg/dL)/405 (Ditschuneit, Flechtner-Mors, Johnson, & Adler, 1999; Matthews et al., 1985). As a stringent measure of insulin resistance, a value of HOMA >2 is often applied. According to the original Adults Treatment Panel III of 2001, a metabolic syndrome diagnosis is rendered when three or more of these five criteria

are fulfilled: fasting plasma glucose concentration of at least 110 mg/dL, waist circumference ≥88 cm for women and 102 cm for men, serum high-density lipoprotein concentration <50 mg/dL for women and <40 for men, blood pressure of ≥130/85 mmHg, and serum triglyceride concentration of ≥150 mg/dL (Grundy et al., 2005). The International Diabetes Federation/American Heart Association/National Heart Lung and Blood Institute unified criteria are similar and require three of the above criteria, including the above cut points for triglycerides and blood pressure, and a cut point for fasting glucose more stringent than ATP III (>100 mg/dL) (Alberti et al., 2009) with the added criteria of central obesity, drug treatment for diabetes, elevated triglycerides, low levels of high-density lipoprotein, or hypertension. Nevertheless, there is no universally accepted definition of metabolic syndrome. There has been disagreement in the literature about the optimal criteria to use for metabolic dysfunction in obesity because risk in certain human populations, such as specific ethnic groups, for certain diseases such as cardiovascular disease, may be better predicted by some criteria, such as glucose intolerance, than by others, such as metabolic syndrome. Oral glucose tolerance tests and criteria for impaired fasting glucose and impaired glucose tolerance have been used to supplement the diagnosis of metabolic syndrome, but discussion of this literature is beyond the scope of this chapter.

However, it is pertinent that, as mentioned above, certain obese individuals are "metabolically healthy" and enjoy reduced risk for cardiovascular disease and Type 2 diabetes (Klöting et al., 2010; Sims, 2001; Succurro et al., 2008; Wildman et al., 2008). "Metabolically healthy" obese adults have been defined as abdominally obese (BMI ≥30) but lacking metabolic syndrome (Meigs et al., 2006). Such individuals comprise about 25% of the adult obese population in the United States, however, this prevalence depends on inclusion criteria, with some reported disagreement in prevalence, from a minimum of 11% of obese subjects in an Italian study (Calori et al., 2011) to 47.9% of obese subjects in a Korean study (Lee, 2009). The "metabolically healthy" obese phenotype is best conceptualized as a continuous distribution of preserved insulin sensitivity as a function of increasing BMI (Blüher, 2010). Some of these individuals show protective, elevated levels of adiponectin (Aguilar-Salinas et al., 2008) and maintain normal glucose tolerance despite startlingly high BMI.

Factors that couple obesity to insulin resistance and metabolic syndrome are of great medical interest, because they underlie the etiology of obesity-driven Type 2 diabetes. Thus, the "metabolically healthy" obese individual is likely to provide a goldmine of information. The study of this population

for genes and pathways that couple obesity to insulin resistance has the potential to identify novel, "druggable" targets to help unhealthy obese patients avoid the worst comorbidities of their condition. Significantly, the "metabolically healthy" obese phenotype is associated with a reduced inflammatory profile (Karelis et al., 2005; Romano et al., 2003). Thus, it is likely that inflammatory functions of the innate and adaptive immune system are essential to link obesity to insulin resistance, cardiometabolic risk, and Type 2 diabetes. Greater detail concerning the immune cell subtypes, their cytokine production profiles, and kinetics of mobilization in the insulin-resistant obese subject, and how these differ in critical ways form the "metabolically healthy" obese subject is urgently required.

5. OTHER ANIMAL MODELS OF "METABOLICALLY HEALTHY" OBESITY

Additional mechanistic understanding of how this population of humans is protected from obesity-driven comorbidity will be achieved through hypothesis building and testing in animal models. Beyond the example of *brd2 lo* mice, there are other, fundamentally different types of animal models available, some that are primarily immunological, while others are adipose tissue directed. These different molecular and cellular perturbations or deficiencies will enable more precise mechanistic exploration of the relevant pathways that couple obesity to insulin resistance.

1. Low-inflammatory models include
   - **a.** *Interleukin-1 Receptor 1 knockout*: A reduced inflammatory profile, particularly lower levels of TNF and interleukin-6, appears to protect these animals from high-fat diet-induced insulin resistance and glucose intolerance (McGillicuddy et al., 2011).
   - **b.** *Inducible nitric oxide synthase knockout*: Deficient polarization of macrophages as a result of knockout of *Nos2* protects against obesity-induced skeletal muscle insulin resistance, and this is associated with improved phosphoinositide 3-kinase/Akt activity (Perreault & Marette, 2001).
   - **c.** *Ablation of TNF*: Antibody against TNF improves insulin resistance in obesity (Hotamisligil et al., 1993), and mice deficient in TNF signaling are protected from insulin resistance in obesity (Uysal, Wiesbrock, Marino, & Hotamisligil, 1997).
   - **d.** *TWEAK knockout*: TNF-related weak inducer of apoptosis (TWEAK), a cytokine of the TNF superfamily, is important not only

for tissue remodeling after injury (Burkly, Michaelson, Hahm, Jakubowski, & Zheng, 2007) but also for remodeling of adipose tissue to accommodate increased storage in obesity (Li et al., 2009). The TWEAK pathway is activated in obese Type 2 diabetic patients (Chacón et al., 2006). TWEAK deficiency (Campbell et al., 2006) shifts macrophage polarization to the alternatively-activated "low-inflammatory" phenotype, increases collagen turnover, and decreases JNK activation in gonadal adipose tissue, conferring metabolic protection in obesity (M.S. Obin, Tufts University, School of Medicine, personal communication).

e. *IκBα superrepressor*: Expression in the liver of the repressor of NF-κB signaling protects against high-fat diet-induced and low level NF-κB-induced insulin resistance (Cai et al., 2005).

2. Adipose tissue models include

a. *Collagen 6 knockout*: Increased capability of adipose depots to remodel and accept increased storage in obesity appears to reduce adipocyte stress and apoptosis (Khan et al., 2009). This increased capacity depends on loss of collagen 6, and improves fasting glucose and glucose tolerance perhaps by relaxing physical, steric constraints on adipose depots.

b. *Adiponectin transgenic*: Severe obesity with insulin resistance on an *ob/ob* (leptin deficient) background can be ameliorated in a dramatic fashion by transgenic expression of adiponectin (Kim et al., 2007), a factor that sensitizes cells to insulin signaling.

Such mouse models will be useful to reveal different kinds of metabolic protection and how crosstalk may also protect organ systems from comorbidities (Denis, 2010).

6. DEREPRESSION OF INSULIN TRANSCRIPTION IN THE "*brd2 lo*" ENVIRONMENT

A deeper understanding of the mechanisms that control maternal–fetal transmission of increased risk for Type 2 diabetes is critical at this stage of the obesity epidemic. There is significant evidence, obtained initially from studies of the Pima Indians, that mothers with Type 2 diabetes can confer elevated diabetes risk to offspring (Dabelea, 2007; Dabelea & Pettitt, 2001; Dabelea et al., 2000, 2008). The development of Type 2 diabetes is dependent on both the gradually decreasing metabolic health of the obese individual as insulin resistance and inflammation increases, as well as strain

on β-cell production of insulin that eventually leads to β-cell failure. The question of how epigenetic mechanisms influence the distinct but related risks to declining insulin action and insulin production has not been well studied.

Increased β-cell proliferation and differentiation, as well as increased insulin transcription and release from pancreatic β-cells *in vivo*, undoubtedly protects obese *brd2 lo* animals from progression to glucose intolerance and β-cell failure. Islets show no signs of apoptosis or stress but are expanded from an early age, likely as an early perturbation to islet homeostasis that is directly attributable to Brd2 reduction, not to insulin resistance in the periphery (Wang et al., 2009).

We explored the potential significance of reduced Brd2 expression during development. We wanted to know if reduced Brd2 expression in the embryonic stem cells of RRE050 or RRT234 origin (Wang et al., 2009) had altered biology relevant to energy metabolism. Based on published results of increased *insulin 1* gene transcription in β-cell lines, we hypothesized that insulin transcription would be potentiated in the RRE050 or RRT234 cells. Accordingly, we transfected RRE050 embryonic stem cells with an eGFP reporter construct for the *Ins1* promoter, obtained as a generous gift from Dr. Manami Hara, then selected the cells under hygromycin as shown in Fig. 3.1.

Remarkably, this result shows that reduced levels of Brd2 protein potentiate transcription of the insulin gene extremely early in mouse development, even before the embryonic stem cell has lost totipotent characteristics during the course of *in vitro* differentiation. One potential implication of these data is that targeted inhibition of Brd2 or genetic modification of embryonic stem cells could provide a therapeutic strategy for β-cell failure through regeneration of β-cell mass, or for gene therapy for Type 1 diabetes. Epigenetically based therapeutics for metabolic dysfunction are therefore feasible, although detailed mechanistic studies are obviously now required.

7. TRANSLATIONAL IMPLICATIONS OF EPIGENETIC REPROGRAMMING: CONCLUSIONS

Apart from the maternal–fetal transmission of increased cardiometabolic risk in the Hunger Winter case, other processes with a potential epigenetic component are likely at work in obesity. For example, weight regain after bariatric surgery has emerged as a worrisome problem for clinicians (Magro et al., 2008). Over the long term, a significant fraction of bariatric patients (20.4% for morbidly obese patients and 34.9% for super obese

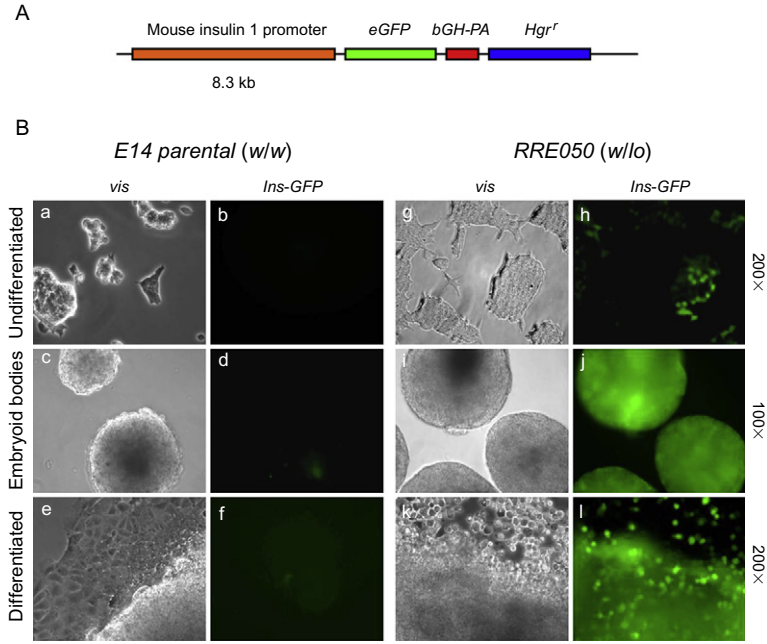

Figure 3.1 Reduced expression of Brd2 potentiates insulin transcription in embryonic stem cells. (A) Schematic of mouse insulin 1 promoter that drives expression of eGFP (*eGFP*). Promoter elements are also shown: bovine growth hormone poly A sequence (*bGH-PA*) and hygromycin resistance (Hgr^r). (B) Visual (*vis*) and fluorescence (*Ins-GFP*) micrographs of ES cells transfected with the construct in (A) and permitted to undergo differentiation. Undifferentiated cells were cultured on gelatin-coated tissue culture plastic in the presence of leukemia inhibitory factor (LIF) (undifferentiated), then LIF was withdrawn to permit the formation of embryoid bodies for 2 weeks (embryoid bodies), and culture was continued for an additional 2 weeks to permit additional differentiation (differentiated). Magnification is shown at the right-hand edge of the figure. The parental ES cells (*E14 parental*; *w/w*) were compared to Brd2 KO ES cells (*RRE050*; *w/lo*) as described in Wang et al. (2009), at the same stage of differentiation.

patients in one study) (Christou, Look, & MacLean, 2006) regain significant weight. For the fraction of patients for whom regain is unrelated to surgical failure, it is possible that epigenetic factors play a role in the difficulty with maintaining healthy weight in the 10-year-period that follows surgery. Likewise, diet and lifestyle modification for the less-morbidly obese also shows only mixed success.

Important, outstanding questions remain. It is unclear whether there is an epigenetic component to the "metabolically healthy" obese phenotype, either in humans or in mice. Studies to measure a maternal genetic

contribution to Brd2-regulated metabolism have yet to be performed in *brd2 lo* mice. There is some evidence for maternally defined DNA methylation patterns in *brd2 lo* mice (F. Wang, unpublished observations), but additional experiments are required to address this issue. Because *Brd2* is homologous to a known "maternal effect" gene in *Drosophila*, called *female sterile (1) homeotic* (Beck, Hanson, Kelly, Pappin, & Trowsdale, 1992; Denis & Green, 1996; Digan et al., 1986), we have proposed that Brd2-regulated metabolism may have a maternal effect in humans and mice (Belkina & Denis, 2012; Denis, 2010). Finally, we speculate that epigenetic modifications that attenuate expression of the *BRD2* locus in humans, nearby *TNF*, or other genes that encode proinflammatory factors, will be present in "metabolically healthy" obese patients, but modifications that exacerbate expression of such genes will be present in at-risk, "pro-inflammatory" obese populations. In support of this idea, recent work identifies increased inflammatory signatures in the uterine environment of obese female rats, which leads to weight gain and metabolic consequences for offspring (Shankar et al., 2011). The details of maternal effect or epigenetic contribution to body composition and fat distribution in humans are not well understood and will require further investigation in clinical studies and animal model systems.

More urgently, based on our published and preliminary data and other published data discussed here, we speculate that surgical, dietary, and exercise interventions may be insufficient to ensure sustained weight loss for obese, morbidly obese, and super obese patients if key genes important for energy metabolism have become epigenetically modified or reprogrammed in a way that resists whole-body return to healthy BMI. Such a mechanism has not been studied in patients or animal models after weight loss, but offers a compelling possible explanation for the persistent failure of these therapeutic interventions among many patients. Epigenetic "correction" might be possible with drugs that directly affect "writers" of histone methylation and acetylation marks such as histone deacetylase inhibitors, or "readers," such as the small-molecule BET protein inhibitors. Re-randomization of certain epigenetic marks of histone acetylation, methylation, or of DNA methylation, might help patients to lose weight by encouraging key tissues of the body to "forget" the unfortunate metabolic history of chronic obesity. Thus, metabolic "set-point" could be reset to a healthy orexigenic state and BMI would be easier to normalize after weight loss. Without this kind of drug intervention to correct the epigenetic status quo of chronic obesity, successful and sustained, long-term weight loss, even after bariatric surgery, may well be impossible for most patients.

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