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# *In Vivo* Studies on the Transcriptional and Posttranslational Regulation of the CCAAT/Enhancer Binding Protein β

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# Abstract


## *In Vivo* Studies on the Transcriptional and Posttranslational Regulation of the CCAAT/Enhancer Binding Protein β

The transcription factor CCAAT/enhancer binding protein β (C/EBPβ) gene has CREB responsive elements (CRE) in its promoter, and its transcription is regulated by CREB during adipogenesis. We have generated a mouse line with a deletion of the CRE elements on the C/EBPβ promoter and studied the role of these elements in macrophages. We show that the CREs are important for the induction of C/EBPβ expression following treatment of the macrophages with IFNγ/LPS. Moreover, we found two novel targets for C/EBPβ transcription in macrophages, that are macrophage scavenger receptor 1 (Msr1) and interleukin 13 receptor α1 (IL13α1). We also show that the well-known regulation of the arginase 1 gene by C/EBPβ is dependent on the ability of CREB to upregulate C/EBPβ. FACS analyses on our bone marrow-derived macrophage population, showed that the cells are Mac1(+), F4/80(+) and Gr1(+), typical markers of Natural Suppressor macrophages. Taken together, the C/EBPβ target genes found in the macrophage and the cell surface markers, suggest an immunosuppressive phenotype. We propose a novel role for C/EBPβ in mediating the molecular switch from inflammatory to immunosuppressive macrophages.

In a separate project, we study the role of the Thr188 and Ser176, Ser180 and Ser184 phopshorylation sites, which are located in the regulatory domain of the C/EBPβ protein. Thr188 is a known MAPK phosphorylation site, whereas the three serines, whether all or some, were recently shown to be targets for GSK3β phosphorylation. We created two mouse lines in which either Thr188, or the three serines were mutated to alanines. We analyzed the expression of the mutant C/EBPβ in various tissues, as well as the expression of C/EBPβ target genes in primary macrophages from both the mouse lines. We found that the three serines have a role in modulating C/EBPβ's autoregulatory loop as well as in reducing the transcription factor's transactivational activity. Moreover, based on the migration pattern of the mutant C/EBPβ proteins, we propose a model suggesting cooperativity between the MAPK and GSK3β phosphorylation sites. We conclude that the phosphorylation sites in question are implicated, whether directly or indirectly, in the modulation of the transcription factor's activity.

# Zusammenfassung

## *In Vivo* Studien zur transkriptionalen und posttranslationalen Regulation des CCAAT/*Enhancer Binding* Proteins β

Das Gen des Transkriptionsfaktors CCAAT/*enhancer binding* Protein β* (C/EBPβ) besitzt CREB sensitive Elemente (CRE) in der Promotorregion. Die Transkription dieses Gens wird in der Adipogenese durch CREB reguliert. Es wurde ein Mausstamm generiert, bei dem die CRE Elemente des C/EBPβ-Promotors entfernt wurden. Die Rolle dieser Elemente wurde in Makrophagen untersucht. Es wird gezeigt, dass diese CREs wichtig sind für die Induktion der C/EBPβ-Expression nach Stimulierung von Makrophagen mit IFNγ/LPS. Darüber hinaus wurden zwei neue Gene gefunden, deren Transkription von C/EBPβ reguliert wird, der Makrophagen *scavenger receptor* 1 (Msr1) und Interleukin 13 Rezeptor α1 (IL13α1). Des weiteren wird gezeigt, dass die bereits bekannte Regulation des Arginase 1 Gens durch C/EBPβ von einer CREB induzierten Aktivierung der C/EBPβ Transkription abhängig ist. FACS Analyse zeigte, dass aus Knochenmark gewonnene Makrophagen Populationen positiv für typische Marker der *Natural Suppressor* (NS) Makrophagen (Mac1, F4/80 und Gr1) waren. Die gefundenen C/EBPβ-regulierten Gene in Makrophagen und die Zelloberflächenmarker legen nahe, dass es sich um einen Mausphänotyp mit eingeschränktem Immunsystem handelt.

In einem weiteren Projekt wurde die Rolle der Kinasesubstrate Ser176, Ser180, Ser 184 und Thr188 untersucht. Diese Aminosäuren befinden sich in der regulatorischen Untereinheit des C/EBPβ Proteins. Thr188 ist eine bekannte Phosphorylierungsstelle für MAPK, während die drei Serine zumindest teilweise von GSK3β phosphoryliert werden. Es wurden Mausstämme generiert, bei denen entweder Thr188 oder die drei Serine zu Alaninen mutiert wurden. Die Expression der C/EBPβ Mutanten wurde in verschiedenen Geweben und die Expression von C/EBPβ-regulierten Genen in primären Makrophagen der beiden Mausstämme untersucht. Die drei Serine spielen eine Rolle sowohl bei der Modulation der autoregulatorischen Schleife, als auch bei der Verringerung der Aktivität des Transkriptionsfaktors. Das Migrationsmuster der mutierten C/EBPβ Proteine legte einen synergistischen Effekt der Phosphorylierung durch MAPK und GSK3β nahe. Aus den Ergebnissen kann geschlossen werden, dass die Phosphorylierungsstellen entweder direkt oder indirekt die Aktivität des Transkriptionsfaktors modulieren.

* Die Namen der Proteine werden aus dem Englischen übernommen (kursiv), um die allgemein verwendeten Abkürzungen beibehalten zu können und um Unklarheiten in bezug auf die englische Originalliteratur zu vermeiden.

# 1. INTRODUCTION

Transcription factors are versatile proteins that are able to interpret environmental signals and convert them into specific changes in gene expression. The target genes for one transcription factor are multiple, and the circumstances in which they are turned on can vary and in certain cases appear to be contrasting. A transcription factor has to know which of its targets it must switch on in a particular moment, as well as to what degree the transcription must take place and when it must stop. It is evident that transcription factors are highly specialized in their functions, and therefore must be studied in a circumscribed environment and under limited conditions at a time.

This thesis is a study on the transcriptional regulation of the C/EBPβ transcription factor, and how certain posttranslational modifications of the C/EBPβ protein can modulate its function. Most of the work was carried out in macrophages, a choice that led us to new discoveries on the role of this transcription factor in immunity. For the sake of clarity, I will first give an overview of the immune system, with a focus on macrophages, and then I will describe C/EBPβ *per se*, as well as in the context of the macrophage.

## 1.1 The Hematopoietic System

All the cellular elements of the blood derive from the same progenitor, the hematopoietic stem cells in the bone marrow (Orkin, 1995). Hematopoietic stem cells initially give rise to stem cells of more limited potential, called multipotent progenitors. The multipotent progenitor generates the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP), which proliferate and differentiate into the immature, and finally mature, cells of the blood and the immune system. Hematopoietic cells include at least nine mature cell types that are distinct in both morphology and function (figure 1.1).

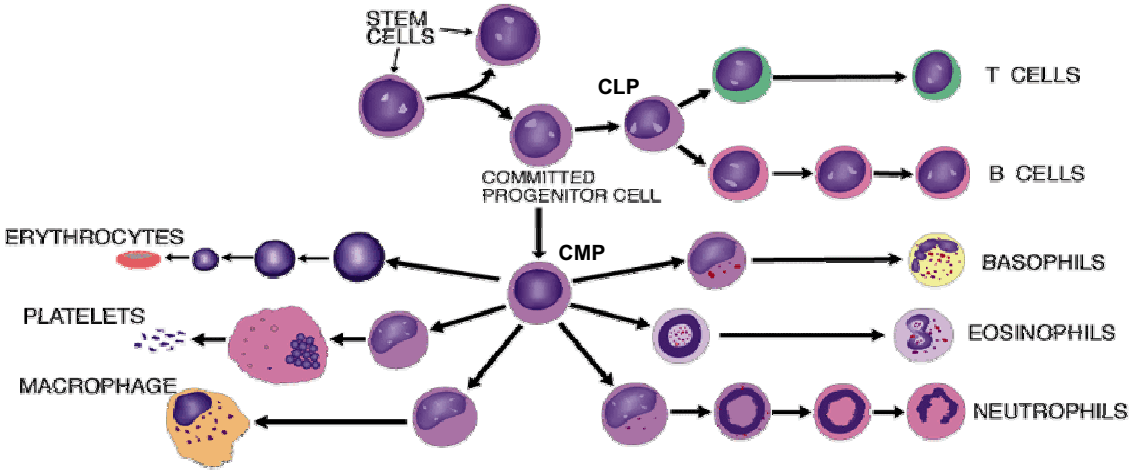



**Figure 1.1: Schematic representation of hematopoiesis.** Hematopoietic stem cells in the bone marrow can either self-renew, or differentiate into progenitors that generate precursors of the myeloid or the lymphoid lineage. The commitment process is characterized by massive cell proliferation in the early phase followed by successive restriction to distinct cell lineages and to cell differentiation.

### 1.1.1 The Lymphoid Lineage

The CLP is capable of differentiating into Natural Killer (NK) cells, B lymphocytes, and T lymphocytes, depending on the compartment in which differentiation takes place. NK cells originate in the bone marrow and then emigrate to the peripheral blood. These lymphoid cells lack antigen specific receptors and are part of the innate immune system (Blach-Olszewska, 2005). They are important in the killing of cellular targets, such as tumor cells. An NK cell kills a target cell either by releasing perforin, which damages the target cell membrane leading to death, or by inducing apoptosis. B lymphocytes develop in the bone marrow, and are able to rearrange genes encoding for immunoglobulins, which they express on the cell surface, in order to obtain antigen specificity. When B lymphocytes are activated, they differentiate into plasma cells and secrete antibodies. Although B cells are important mediators of immunity, for the scope of this thesis, I will concentrate on T lymphocytes as far as the lymphoid lineage is concerned.

In the thymus, the CLP differentiates into CD4(+)CD8(+) T cells. CDs are hematopoietic cell surface markers, and in particular CD4 and CD8 are receptors expressed on the T cell membrane. Thymocytes that recognize self antigens are eliminated by apoptosis, ensuring the selection of T cells that recognize a wide variety of foreign antigens in conjunction with the major histocompatibility complex (MHC) (Robey and Fowlkes, 1994). Finally, the T cell matures by downregulating the expression of either the CD4 or the CD8 coreceptors, upregulating the expression of CD3, and

leaving the thymus to populate peripheral lymphoid organs (Scollay, 1991). Peripheral T cells circulate in a quiescent state until they encounter an antigen-presenting cell (APC) bearing a cognate antigenic peptide bound to an appropriate MHC molecule (Crabtree, 1989). Engagement of the T cell receptor complex by the peptide-MHC complex results in T cell activation. CD8(+) or cytotoxic T cells are capable of killing cells infected with viruses. So called CD4(+) T helper lymphocytes differentiate into two distinct subsets upon T cell receptor engagement, named T helper 1 (Th1) and T helper 2 (Th2) (Mossman *et al*., 1986). Th1 cells are responsible for cell-mediated, inflammatory immunity, including the activation of inflammatory macrophages, while Th2 cells contribute to humoral responses, activate mast cells and eosinophils, and often exhibit antiinflammatory properties. The two subsets of T helper cells can be distinguished by different patterns of secreted cytokines. In general, Th1 cells produce IL2 (interleukin 2), IFNγ (interferon γ) and TNFβ (tumor necrosis factor β), whereas Th2 cells produce the interleukins IL4, IL5, IL10 and IL13.

T cells are responsible for activating other cells of the immune system, such as the macrophages. Th1 and Th2 T cells can elicit very different responses from macrophages, and this property will be described further on.

### 1.1.2 The Myeloid Lineage

The common myeloid progenitors give rise to either megakaryocyte/erythrocyte progenitors (MEP) or granulocyte/monocyte progenitors (GMP) (Akashi *et al*., 2000). The transcription factors PU.1 and C/EBPα are responsible for normal myeloid differentiation from stem cells to monocytes or granulocytes (Behre *et al*., 1999). In particular, PU.1 induces expression of the MCSF (macrophage colony stimulating factor) receptor and the development of monocytes, whereas C/EBPα increases the expression of the GCSF (granulocyte colony stimulating factor) receptor and leads to mature granulocytes.

Megakaryocytes are the precursors of platelets, which play a fundamental role in blood clotting and wound healing. Erythrocytes are enucleated red blood cells, which contain haemoglobin, and are essential for delivering oxygen to tissues, in exchange for $CO_2$, which is discharged in the lungs.

Granulocytes have densely staining granules in their cytoplasm and they are sometimes called polymorphonuclear leukocytes, because of their oddly shaped nuclei. There are three types of granulocytes and they are called neutrophils, eosinophils and basophils. Neutrophils are phagocytic cells and they are the most numerous cellular component of the innate immune system. If on one hand C/EBPα is important for granulocyte maturation, other C/EBP family members (i.e. C/EBPβ, -δ, and -ζ) increase at the stage where proliferation ceases in neutrophils (Bjerregaard *et al*., 2003).

Moreover, C/EBPε is involved in the expression of specific granules in neutrophils. Eosinophils are thought to be important in defense against parasitic infections. The function of basophils is still largely unclear, but it is probably complementary to that of eosinophils.

### 1.1.3 Macrophages

Macrophages are the mature form of monocytes. Monocytes are generated in the bone marrow by a granulocyte/monocyte progenitor, which has gone through two stages of differentiation involving the formation of monoblasts and promonocytes. Newly produced monocytes are released into the blood where they circulate for 1-3 days and subsequently undergo differentiation into macrophages upon migration from the capillary bed to extravascular tissues (Volkman and Gowans, 1965). An increase in the expression of macrophage-associated antigens CD71 (i.e. the transferrin receptor), CD14 and CD11 (also called Mac1), after 3-7 days of culture *in vitro*, define a time-dependent differentiation of monocytes to macrophages (Gessani *et al*., 1993*)*. Antibodies against Mac1 (or CD11b), which is a granulocyte/macrophage-specific integrin molecule, are often used as markers for myeloid cells. F4/80 is another commonly used antibody, which detects a member of the epidermal growth factor (EGF)-transmembrane 7 (TM7) family (MacKnight and Gordon, 1998). Although the function of the molecule has remained elusive, it was recognized as a murine macrophage-specific surface marker.

### 1.1.4 Mechanisms of Phagocytosis in Macrophages

Macrophages are distributed widely in the body tissues, where they play a critical role in innate immunity, but also participate in acquired immunity. They are essential in the humoral immune response because, like neutrophils, they are phagocytic. In other words, a macrophage can bind a pathogenic particle to the surface of the cell, internalize it and destroy it (reviewed in Aderem and Underhill, 1999). This property is not only fundamental for triggering an inflammatory response upon microbial infections, but also for clearing out apoptotic cells and exerting anti-tumoral functions. Macrophages are often found to be involved in the infection of parasites such as helminths (MacDonald *et al*., 1999) and protozoa, such as Trypanosoma, Plasmodium, and many others. At first the macrophage engulfs a parasite to protect the organism, but often it can become the target for the infection by the parasite, who subverts typical macrophage antimicrobial functions and exploits the antiinflammatory effects (Denkers and Butcher, 2005).

Phagocytosis can involve different receptor molecules on the surface of the macrophage, defining two distinct immune response pathways. The innate immune response is mediated by a direct recognition of the pathogen by the macrophage and does not involve other immune cells. The acquired immune response involves phagocytosis of

particles via antibody receptors and the reciprocal activation with T cells mediated by antigen presentation. Examples are given below.

LPS (lipopolysaccharide) is the polysaccharide that coats gram-negative bacteria (Raetz *et al*., 1990). The lipid A domain (or endotoxin) of LPS is a glucosamine-based phospholipid that makes up the outer monolayer of the outer membrane of the bacteria. Endotoxin is recognized by a lipopolysaccharide binding protein (LBP), a 50kDa polypeptide synthesized in hepatocytes and released in a 60kDa glycosylated form in the plasma. LBP serves as an opsonin, which strongly enhances the recognition of LPS by macrophages. CD14 was identified as the receptor for LPS-LBP complexes (Wright *et al*., 1990), and can be considered a mediator of macrophage innate immunity. It was only in 1999 that Chow and coworkers demonstrated that TLR4 (Toll-like receptor 4) is involved in lipopolysaccharide signaling and serves as a cell-surface coreceptor for CD14. TLR4 was found to interact physically with MD2, an additional component of the LPS signaling pathway (Shimazu *et al*., 1999). TLR signaling relies on the function of the intracellular adaptor protein MyD88, which presumably acts in conjunction with other TLR-specific adaptor proteins, such as Tollip and Mal (Janssens *et al*., 2003) (figure 1.2).

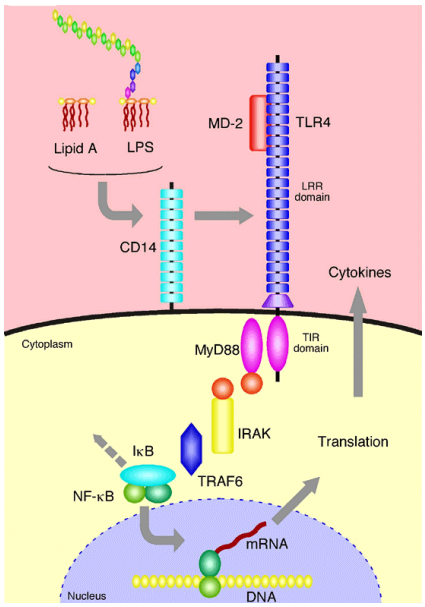



**Figure 1.2: Schematic representation of the LPS signaling pathway.** LPS is recognized by the CD14 receptor, which, in association with TLR4 and MD2, triggers the intracellular signal. In the cytoplasm, MyD88 phosphorylates IRAK and activates a pathway, which leads to IκB/NFκB activation, and transcription of target genes. Among the target genes are many cytokines. (Image taken from glycoforum website: www.glycoforum.gr.jp)

These adaptor proteins are necessary for the recruitment and activation of different IL1 receptor-associated kinase (IRAK) family proteins, which further transmit the signal. This leads to activation of the IκB kinase complex and mitogen-activated protein kinases (c-Jun N-terminal kinase/p38), which induce NFκB and AP-1-dependent gene transcription, respectively. Biophysical approaches used to study intramolecular interactions revealed that LPS is associated with non-TLR-related molecules as well, ranging from integrins such as CD11b/CD18 to chemokine receptors, scavenger

receptors, and many others (Triantafilou *et al*., 2002). Many of these receptors are clustered upon LPS triggering in lipid rafts, suggesting the formation of supramolecular LPS activation clusters.

The polysaccharide capsules of bacterial pathogens sometimes allow them to resist direct engulfment by phagocytes. These bacteria become susceptible to phagocytosis, however, when they are coated with immunoglobulins (a process called opsonization) that engages the Fcγ receptors on macrophages, triggering the uptake and destruction of the bacteria (Indik *et al*., 1995). The release of specific immunoglobulins comes from B cells. Because of the involvement of other immune cells, which have produced antibodies specific for the pathogen, this is an example of acquired immunity. Endocytosis of a particle by the macrophage leads to its enclosure in an acidified cytoplasmic vesicle, called phagosome. The phagosome then fuses with one or more lysosomes to generate a phagolysosome, releasing the lysosomal enzymes into the phagosome interior where they destroy the bacterium.

### 1.1.5 The Activation of Macrophages

Macrophages can be activated *in vitro* by the addition of LPS to the culture medium. LPS stimulates phagocytes to synthesize cytokines such as TNFα, IL1, and IL6, which play a role in inflammatory reactions and activation of immune responses (Hermann *et al*., 1991). IFNγ, a cytokine produced by T cells during the inflammatory response, is often used *in vitro* in combination with LPS to activate macrophages. Through the use of neutralizing IFNγ-specific monoclonal antibodies and gene-targeted mice, it has been possible to establish unequivocally the predominant role played by IFNγ in generating activated macrophages, both *in vitro* and *in vivo* (Dalton *et al*., 1993). On the surface of the macrophage are receptors specific for IFNγ, called IFNGR1 and 2 (reviewed in Stark *et al*., 1998). In unstimulated cells, IFNGR1 associates with Janus kinase (JAK)-1, and IFNGR2 associates with JAK2. IFNγ induces oligomerization of the IFNγ receptor subunits, which leads to the transphosphorylation and activation of JAK1 and JAK2. The activated JAKs then phosphorylate Tyr440 of IFNGR1, creating a docking site for signal transducer and activator of transcription (STAT) 1. While bound to the receptor, STAT1 is phosphorylated on Tyr701 and is released from the receptor, forming a homodimer that translocates to the nucleus. The STAT1 homodimer binds to IFNγ-activated site (GAS) elements present on the promoters of IFNγ-regulated genes, such as IFN γ-regulated factors (IRFs), to initiate transcription.

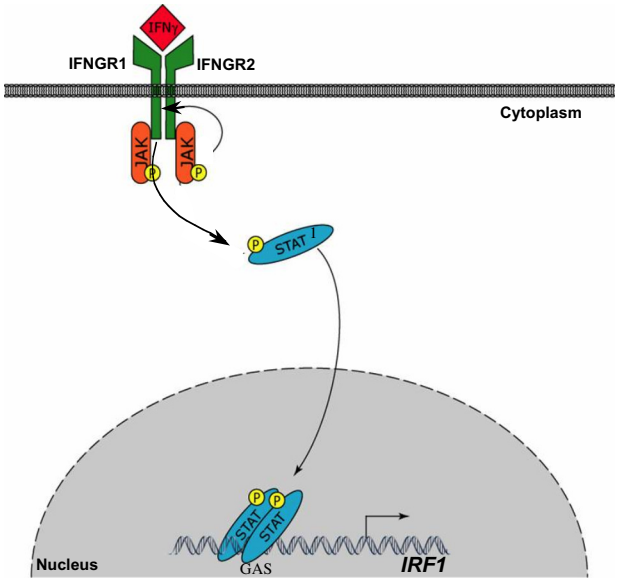



**Figure 1.3: Schematic representation of the JAK/STAT signaling pathway.** IFNγ binds to its heterodimeric receptor, which induces reciprocal phosphorylation of the JAKs. JAKs in turn mediate phosphorylation of IFNGR1, which becomes a docking site for STAT1. On the receptor STAT1 is phosphorylated and released. The transcription factor then forms a homodimer, translocates to the nucleus, and recognizes GAS elements in the promoters of its target genes (such as IRF1). (Image taken and readapted from Science Nerd Depot website: www.sciencenerddepot.com)

In macrophages LPS and IFNγ synergize in inducing mechanisms to kill microbial targets. Two of the most important mechanisms involve the production of reactive oxygen and reactive nitrogen intermediates, during a process called "respiratory burst" (Bogdan *et al*., 2000). The production of reactive oxygen intermediates is initiated by NADPH oxidase with the release of superoxide in the cell, which can be converted to $H_2O_2$ or hydroxyl radicals and hydroxyl anions. Most of these intermediates are toxic to bacteria. Nitric oxide (NO) is produced by inducible nitric oxide synthase (iNOS) in macrophages. iNOS converts L-arginine and molecular oxygen to L-citrulline and NO (or NO radicals and subsequent intermediates). The *iNOS* gene can be activated via several signal transduction pathways and molecules, including JAK1/STAT1α/IRF1, IκB/NFκB and MAPK. The production of NO is also subject to the availability of L-arginine as a substrate. NO can be toxic to several pathogens, including viruses (Saura *et al*., 1999), and their host cells. In addition, both LPS and IFNγ are able to induce IFNβ secretion and an antiviral state in murine peritoneal macrophages (Gessani *et al*., 1989).

Activated macrophages express a whole series of phenotype markers essential for specific effector functions, such as the class II major histocompatibility molecule (MHC II), macrophage colony-stimulating factor (M-CSF) receptor and Fcγ receptors. MHC II molecules are heterodimers made of 34 and 28 kDa chains found mainly on the surfaces of macrophages, B cells, dendritic cells, the Langerhans cells of the skin and lymphoid organs. Cells expressing MHC II molecules are called antigen-presenting cells (APC). Once a macrophage has phagocytosed an antigen, it will unfold the proteins and break them down by partial proteolysis. This process will uncover epitopes which are recognized by MHC II molecules, which in turn will expose the peptides on the outer membrane of the macrophage. CD4(+)T-helper cells will recognize the peptides

presented by APCs and initiate the diverse cellular interactions that result in B cell activation, development of inflammatory reactions, and activation of CD8(+) cells to become active killer cells. Interferon γ (IFNγ) is produced by T cells during antigen presentation and binds to macrophages inducing *de novo* expression of MHC II molecules. The whole process subsides as the antigen is eliminated (reviewed in Unanue and Allen, 1987).

### 1.1.6 Antiinflammatory Macrophages

The macrophage activation just described is a typical inflammatory response to stimuli received by Th1 T cells. In general, macrophages induced in Th1-dominated immune responses, secrete inflammatory cytokines, such as IL1, IL6 and TNFα, and are called inflammatory macrophages or M1 macrophages (Mills *et al*., 2000). M1 macrophages possess cytotoxic and antimicrobial effector functions based on their ability to produce NO (MacMicking *et al*., 1997). On the contrary, M2 macrophages are elicited by the Th2 immune response and display antiinflammatory properties. These macrophages are particularly important in the case of injury, when excessive inflammation could impair tissue regeneration.

M2 macrophages produce arginase 1, which is an enzyme that converts L-arginine to L-ornithine and urea (the nitrogen elimination step of the Krebs urea cycle). L-ornithine can be further processed into polyamines, which nourish cell growth and promote tissue repair (Jenkinson *et al*., 1996). M2 cells are believed to attenuate Th1 responses and induce peripheral tolerance, possibly also to prevent auto-immunity. However, because of this permissive feature, M2 macrophages are often associated with parasite infections and tumor progression (Baetselier *et al*., 2001; Mills *et al*., 1992).

Arginase 1 and iNOS compete for the same substrate for their reactions, L-arginine (Modolell *et al*., 1995). In a way, this bestows on arginase 1 the additional property of limiting NO synthesis and mediating part of the passage from M1-type to M2-type macrophage.

### 1.1.7 Specialized Macrophages

Resident macrophages are distributed constitutively throughout the organism in the absence of any inflammatory signal and display regional heterogeneity. Functional, morphological and phenotypic heterogeneity may reflect the acquired environments of these cells. Only a few examples of specialized macrophages will be given.

The resident macrophages of the liver are called Kupffer cells. Kupffer cells' responses to LPS and other gut-derived stimuli may be important in their interactions with hepatocytes (Decker, 1998). These cells could be at least partly responsible for regulating the acute phase response in injury and malignancy.

In the spleen there is a heterogeneous population of macrophages (Buckley *et al*., 1987), which have different functions according to their localization. They trap and process antigens in the marginal zone, interact with T and B lymphocytes in the lymphoid areas (the white pulp), and phagocytose senescent erythrocytes in the red pulp.

Some macrophages penetrate the blood-brain barrier, entering the nervous system during embryonic development, and are called microglia. Microglia play a role in removing dying neurons in the developing central nervous system (CNS)(Perry *et al*., 1985). Indeed, naturally occurring cell death is a major event in the development of the CNS, and as much as 50% of the original neuronal population generated in the embryo may degenerate before maturity. However, the role of microglia in the adult brain is still a matter of investigation.

Furthermore, there are populations of myeloid cells, which are similar in many ways to macrophages, but are considered to derive from different differentiation pathways. Among these are dendritic cells (DC), Langerhans cells and mast cells.

Dendritic cells are characterized by the presence of polarized lamellipodia and long spiny processes continuously extended and retracted, allowing these cells to be extremely motile. Motility allows DCs to move from the blood to peripheral tissues and from these tissues to lymphoid organs, to meet antigen-specific T cells (Banchereau and Steinman, 1998). Immature DCs can internalize efficiently a diverse array of antigens for processing and loading onto MHC molecules, as a consequence of high endocytic activity (Cella *et al*., 1997). As a result, DCs start to mature by accumulating MHC II complexes in distinctive non-lysosomal vesicles. Finally, in the mature DCs peptide-MHC II complexes are present stably on the plasma membrane, allowing selection of even rare antigen-specific T cells, a feature which has earned them the name of professional antigen presenting cells (APC).

Langerhans cells are the specialized dendritic cells found in the suprabasal layer of the epidermis (Thorbecke *et al*., 1980). Like DCs, they are professional APCs, and they are characterized by a high motility, which enables them to reach local lymph nodes. The most typical organelles of Langerhans cells are Birbeck granules, which are tennis racket-shaped bodies that seem to have a role in the transfer of molecules entering the cell via receptor-mediated endocytosis.

Mast cells reside mainly near small blood vessels and surfaces exposed to the environment, where pathogens and allergens are frequently encountered (Galli *et al*., 2005). They release substances that affect vascular permeability when activated, and are known for their role in orchestrating allergic responses.

### 1.1.8 The Role of Macrophages in Atherosclerosis

Macrophages express scavenger receptors on their surface, which recognize modified (i.e. oxidized or acetylated) low-density lipoproteins (LDL) (Freeman, 1997). These

receptors are involved in lowering the cholesterol levels in the blood, an event that mostly occurs in the liver with the help of Kupffer cells (Van Berkel *et al*., 1991). However, in some cases the uptake of lipid by macrophages may lead to disease.

The earliest event in the development of atherosclerosis is the adhesion of circulating monocytes to regions of the lumenal surface of the endothelium of a blood vessel (Ross, 1993). The adhesion occurs through the vascular cell adhesion molecule-1 (VCAM-1). Signals that induce VCAM-1 expression are unknown, but could be the altered generation of inflammatory mediators. Once macrophages have been recruited, they can secrete chemotactic factors to increase the response (Jessup *et al*., 2002). Adherent macrophages migrate across the endothelium and reside in the intima. Most of the intimal macrophages accumulate large intracellular deposits of lipid, which locate in cytoplasmic fat droplets, giving the cells a foamy appearance. The increasing number of macrophages that accumulate in the lesion around the lipid core eventually develop into atherosclerotic plaques. Cellular debris, extracellular lipids and calcium deposits that become part of the plaque, can make the lesion susceptible to rupture, thereby stimulating platelet deposition and formation of thrombi.

## 1.2 The C/EBP Family of Transcription Factors

The first C/EBP protein was identified as a heat-stable factor in rat liver nuclei capable of interacting with the CCAAT box motif in several gene promoters. The C/EBP gene was cloned in 1988 (Landschulz *et al*., 1988) and discovered to possess a basic-leucine zipper (bZIP) necessary for DNA binding and dimerization, a feature that today identifies a whole class of transcription factors. In the following years, five other members of the C/EBP family were identified (reviewed in Ramji and Foka, 2002), and were named with Greek letters from C/EBPα to -ζ (Table 1).

**Table 1:** Cloned C/EBP genes and phenotypic characterization of knockout models. (Taken from Lekstrom-Himes and Xanthopoulos, 1998)

| | | | | Phenotypic abnormalities | | | |
|---|---|---|---|---|---|---|---|
| **Name** | **Alternative name** | **Expression pattern** | **KO model** | **Hepatic** | **Metabolic** | **Hematologic** | **Other** |
| C/EBPα | C/EBP | Liver, adipose, lung, adrenal gland, placenta, ovary, peripheral blood mononuclear cells | yes | Hepatocyte prolifera-tion, perinatal lethal | Defective lipid storage, defective carbohydrate metabolism | Myeloid maturation block at myeloblast stage | |
| C/EBPβ | NF-IL6, LAP, CRP2, AGP/EBP, NF-M | Liver, intestine, adipose, lung, skin, myeloid lineage, ovary, neurons | yes | Hypo-glycemia | Defective lipid storage, defective carbohydrate metabolism (synergistic with C/EBPδ) | Immunodeficient, defective Th1 response, macrophage phagosome defect | Female sterility |
| C/EBPγ | Ig/EBP | ubiquitous | No | | | | |
| C/EBPδ | CELF, CRP3, NF-IL6b, RcC/EBP2 | Liver, lung, adipose, intestine, macrophages | yes | None detected | Defective lipid storage (synergistic with C/EBPβ) | None detected | Neuro-logic defects |
| C/EBPε | CRP1 | Myeloid and lymphoid lineages | yes | None detected | None detected | Immunodeficient, granulocyte defects, myeloid proliferation | |
| C/EBPζ | CHOP, Gadd 153 | ubiquitous | yes | | | | |

All C/EBP isoforms share substantial sequence identity in the C-terminal region, which contains the bZIP domain. This domain consists of a basic amino acid rich DNA binding region followed by a dimerization motif called “leucine zipper”. The leucine zipper is a heptad repeat of four or five leucine residues that assume an α-helical configuration. Two of these repeats interdigitate to form a coiled-coil structure at the site of dimerization. The dimer forms an inverted Y structure, in which each arm of the Y consists of a basic region capable of interacting with one half of the palindromic recognition sequence in the DNA major groove (Figure 1.4). Because the bZIP domain is highly conserved, the different C/EBP isoforms can form homo- or heterodimers with all the intrafamilial combinations. The optimal C/EBP DNA binding site has been shown *in vitro* to be a dyad symmetrical repeat A/G TTGCG C/T AA C/T, even though substantial variations are tolerated (Osada *et al*., 1996).

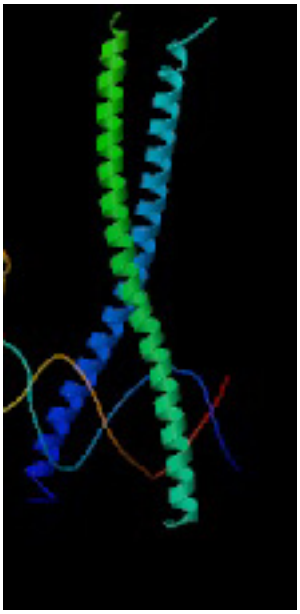


**Figure 1.4: Structure of the C/EBPβ basic region/leucine zipper domain bound to DNA**
The two α-helical basic regions (bottom) dimerize through the α-helical leucine zipper domain (top) to form an inverted Y shape structure. Each arm of the Y is formed by a single α-helix, one from each monomer, which binds to one half of the palindromic recognition sequence. (Rasmol structure obtained from the Protein Data Bank entry IH8A.)

At the N-terminus, C/EBP proteins are more divergent. This is where the trans-activation domain is situated. The interaction in this region with other transcriptional coactivators [e.g the SWI/SNF complex for C/EBPα and -β (Pedersen *et al*., 2001; Kowentz-Leutz and Leutz, 1999)] and with the basal transcriptional machinery, such as TBP and TFIID in C/EBPα (Nerlov and Ziff, 1995), will produce transcriptional activation in a more "isoform specific" manner.

Because of the structural similarity among all the C/EBP family isoforms, it is frequent to find functional redundancy between the family members, depending on their coexistence in a specific cell type. In this thesis I will focus on the C/EBPβ transcription factor, but it is important to keep in mind that the specific function of one family member can be easily masked by the presence of, or heterodimerization with another isoform.

### 1.2.1 The C/EBPβ Transcription Factor

The human isoform of C/EBPβ (also called NF-IL6) was first characterized as a protein that binds to the interleukin-1- responsive element in the promoter of the interleukin 6 gene and to similar sites in other cytokine genes (Akira *et al*., 1990). The C/EBPβ gene is intronless, but contains three different initiation codons that give rise to three C/EBPβ isoforms of different size from the same mRNA (Descombes and Schibler, 1991) (Figure 1.5). The largest protein, of 38kDa, is called LAP* (liver-enriched transcriptional-activator protein). The transactivation domain of this isoform contains four conserved regions, whereas the LAP isoform, 35kDa, has only three, as its start site is situated just at the end of the first conserved region. LAP is the most commonly found active C/EBPβ isoform. A third, short form of C/EBPβ (20kDa), is called LIP (liver-enriched transcriptional-inhibitory protein), and it lacks all of the transactivation domain.

However, LIP has a bZIP domain, and therefore can act as a dominant negative inhibitor of C/EBP by forming non-functional heterodimers with other family members (Descombes and Schibler, 1991).

The N- and C-terminal regions of C/EBPβ are characteristic of the C/EBP family and have already been described. Upstream from the DNA-binding region of the protein is a regulatory region, which is subdivided in two regulatory domains, called RD1 and RD2. Posttranslational events take place in these domains, which appear to be important in modulating the transcription factor's function. These events are described in detail further on in this Introduction.

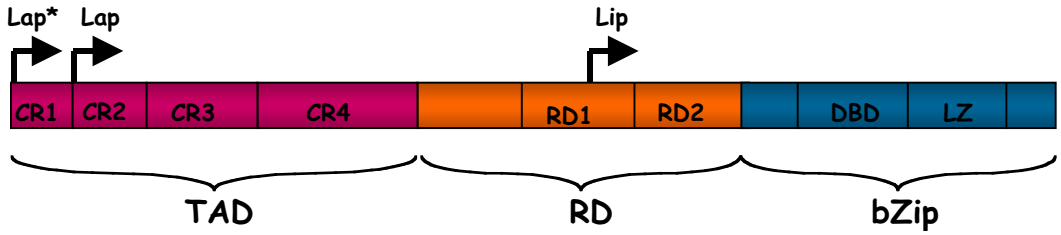



**Figure 1.5: Schematic representation of the C/EBPβ protein.** The arrows indicate the alternative start sites that give rise to the different C/EBPβ isoforms. The transactivation domain is at the N-terminus (TAD) with four conserved regions (CR1-4). RD is the regulatory domain, divided in two subregions (see below). The basic leucine zipper (bZIP) is composed of a DNA-binding domain (DBD) and the leucine zipper (LZ).

C/EBPβ expression is particularly high in the liver, adipose tissue, lung, spleen, intestine, kidney and myelomonocytic cells (Descombes *et al*., 1990; Poli *et al*., 1990; Akira *et al*., 1990). However, it has also been shown to be expressed in keratinocytes, where it plays a role in squamous differentiation (Zhu *et al*., 1999); in the mammary epithelium, where ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation take place only in the presence of C/EBPβ (Seagroves *et al*., 1998; Robinson *et al*., 1998); and in hippocampal neurons, where C/EBPβ is associated to long-term consolidation (Alberini *et al*., 1994).

Mice deficient for C/EBPβ are viable, although a small subset of pups die perinatally (Tanaka *et al*., 1995; Screpanti *et al*., 1995). The survivors display fasting hypoglycemia, decreased blood lipids, and impaired hepatic glucose output in response to hormonal stimulation (Liu *et al*., 1999). The lack of C/EBPβ causes an impairment in lipid droplet accumulation in interscapular brown adipose tissue (BAT) of newborn mice (Tanaka *et al*., 1997). The weight of epididymal white adipose tissue (WAT) of mice carrying a deletion of both C/EBPβ and C/EBPβδ is lower than that of wild type mice. Indeed, also mice lacking either C/EBPβ or C/EBPβδ tended to have lower epididymal fat pad weight, however in this case the difference was not significant.

Female C/EBPβ knockout mice are sterile due to a defect in ovarian follicle development (Sterneck *et al*., 1997). Recently, it has been shown that mice deficient for Id2, a negative regulator of basic helix–loop–helix (bHLH) transcription factors, exhibit a defect in lactation due to impaired lobuloalveolar development during pregnancy, very similar to the mice lacking C/EBPβ, and that Id2 is a target for C/EBPβ transcription (Karaya *et al*., 2005). Moreover, overexpression of the LIP inhibitory isoform of C/EBPβ correlates with breast tumors in humans (Zahnow *et al*., 2001), and gene expression profiling analyses in human tumors have shown that C/EBPβ is a principal effector of cyclin D1 activity in breast carcinomas (Lamb *et al*., 2003).

### 1.2.2 C/EBPβ in Macrophages

Several studies have proven C/EBPβ to play an important role in determining terminal differentiation and activation of macrophages. The expression of C/EBPα, -β and -δ is differentially regulated in the myelomonocytic lineage. C/EBPα is expressed in immature myeloblasts and at very low levels during the process of macrophage differentiation, a time during which C/EBPβ expression, on the contrary, is strongly induced (Natsuka *et al*., 1992). Upon activation of the macrophage, C/EBPα is shut down, whereas C/EBPβ appears to be important for terminal differentiation and mediating the immune response. C/EBPδ is also expressed at moderate levels alongside C/EBPβ in macrophages, however C/EBPδ-deficient mice do not seem to display immune defects.

One of the first studies on C/EBPβ(-/-) mice demonstrated that C/EBPβ is a critical transcription factor in bacteria killing and tumor cytotoxicity as well as in the G-CSF gene induction by macrophages (Tanaka *et al*., 1995). In particular, it appears that C/EBPβ deficient mice are more susceptible to *Listeria monocytogenes* infections than their wild type litter mates, and that this is due to the fact that the intracellular bacteria are able to escape from the phagosome to the cytoplasm.

In the same period, another work was published (Screpanti *et al*., 1995) that showed that C/EBPβ knockout mice are more susceptible to *Candida albicans* infection and that this correlates with an impaired production of IL-12 and a defective release of $NO_2$ anion by splenic macrophages. Moreover, these mice seem to display an imbalanced T-helper response attributed to a lymphoproliferative disorder. In particular, the ratio of B cells to T cells seems to be markedly shifted towards an excess of B cells, or a deficiency in T cells, which means that the antibody (B cell-mediated) response may be prevailing over the T lymphocyte-dependent cell-mediated immunity.

Several genes encoding for cytokines and other inflammatory mediators carry on their promoters C/EBP binding sites and have been reported to be targets for C/EBPβ transcription. Among these are the cytokines IL-6, TNFα, IL-1β, G-CSF, and IL-12 p40

(Matsusaka *et al*., 1993; Pope, 1994; Zhang *et al*., 1993; Dunn *et al*., 1994; Plevy *et al*., 1997), the chemokines IL-8, macrophage inflammatory protein (MIP) -1α, monocyte chemoattractant protein-1 (MCP1) (Matsumoto *et al*., 1998; Bretz *et al*., 1994), and the genes encoding lysozyme, cyclooxygenase (COX)-2, and inducible NO synthase (iNOS) (Goehte *et al*., 1994; Wadleigh *et al*., 2000; Lowenstein *et al*., 1993).

### 1.2.3 The C/EBPβ Promoter

Deletion studies on a region located upstream of the transcriptional start site of the C/EBPβ gene, performed by the use of luciferase reporter gene assays, demonstrated that C/EBPβ expression is directly linked to a small region in its promoter located at -60 to -120bp (Niehof *et al*., 1997). Within this region, two sites that are homologous to the classical CREB regulatory elements (CRE), or CREB consensus sequence, were identified. These two sites are incomplete CREB-binding DNA motifs, as only five out of eight nucleotides are conserved compared to the CREB consensus sequence. Nevertheless, CREB was shown to have binding affinity to CRE-1 and CRE-2 *in vitro* by gel shift experiments performed with oligonucleotides carrying the sequence of the C/EBPβ CREs (Niehof *et al*., 1997). *In vivo*, experiments in preadipocytes showed that increased C/EBPβ gene transcription correlated with the phosphorylation of CREB on Ser133, and the interaction of CREB with the C/EBPβ CRE elements was shown by chromatin immunoprecipitation (ChIP) (Zhang *et al*., 2003). CREB phosphorylation occurs in response to cAMP (Gonzales *et al*., 1989), increased intracellular Ca 2+ (Sheng *et al*., 1991), and growth factors like nerve growth factor (Ginty *et al*., 1994). Phosphorylation of CREB on Ser133 increases its binding to CREB binding protein (CBP), which mediates the link to the basal transcription machinery and therefore stimulates transcription of CREB-dependent genes (Kwok *et al*., 1994).

Moreover, a C/EBP binding site was found to be partially overlapping with CRE-1 (Niehof *et al*., 2001). This suggests the existence of an autoregulatory loop on the one hand, but also a synergistic regulation of C/EBPβ expression between C/EBPβ and CREB. Both transcription factors belong, in fact, to the bZIP class and are likely to be able to interact. The autoregulatory loop described by Niehof and colleagues is a positive one. This was shown by the decreased luciferase activity in cells cotransfected with a C/EBPβ expression vector and a luciferase reporter gene construct carrying the promoter of C/EBPβ with a mutation in the C/EBP binding site. Previous studies had identified two other sites, further upstream on the C/EBPβ promoter, to which C/EBPβ protein was capable of binding upon LPS stimulation of mouse liver cells (Chang *et al*., 1995). Interestingly, these C/EBP binding sites were not recognized by other C/EBP family members, indicating that the regulation loop is specific for C/EBPβ (Mink *et al*., 1999).

### 1.2.4 Posttranslational Modifications of the C/EBPβ Transcription Factor

C/EBPβ is a very rich substrate for posttranslational modifications. Phosphorylation takes place on numerous residues and is by far the most represented modification. However, recent studies have demonstrated C/EBPβ to be a substrate for acetylation and sumoylation. In contrast with the known positive regulatory effects of acetylation on other transcription factors, Xu and colleagues have shown that IL3-dependent *de*acetylation of a lysine residue in the basic region of C/EBPβ contributes to the induction of the Id1 gene expression in a hematopoietic cell line (Xu *et al*., 2003). In other words, acetylation of the basic region of C/EBPβ decreases its DNA binding affinity. The deacetylation of C/EBPβ seems to be mediated by STAT5, that recruits HDAC1 on the Id-1 promoter in the presence of C/EBPβ. Concerning sumoylation, the Williams laboratory has identified a five amino acid motif in the RD1 domain of C/EBPβ, which is a site for covalent attachment of the small ubiquitin-like modifier-1 (SUMO-1) protein. They show that attachment of SUMO-1 to the lysine within this motif decreases the transcriptional inhibitory function of the regulatory domain (Kim *et al*., 2002).

### 1.2.5 Modulation of C/EBPβ Activity by Phosphorylation

Phoshporylation plays a key role in the modulation of C/EBPβ function. It has been reported that C/EBPβ is normally a repressed factor in which negative regulatory regions mask its transactivation domain. Two regions shortly upstream from the DNA binding domain on the C/EBPβ protein were shown to repress transactivation, which was strongly enhanced if the two regions were deleted (Kowenz-Leutz *et al*., 1994). The two regions were defined regulatory domains, RD1 and RD2 respectively (Figure 2). Several putative phosphorylation sites occur in the serine/threonine-rich RD2 sequence and these are good candidates as mediators of a structural remodeling of the C/EBPβ protein, which could regulate its function (Williams *et al*., 1995). However this possibility has not been proven yet, and must be considered speculative.

What is certain is that phosphorylation occurs at least on two residues of the RD2 domain, and in particular Ser231 and Thr235 of the human isoform (corresponding to Ser 184 and Thr188 in the mouse) of C/EBPβ. This was demonstrated by transient expression of site-directed mutants on these residues and subsequent phosphopeptide mapping (Nakajima *et al*., 1993). In particular, Thr235 was found to be a substrate for MAP kinase phosphorylation and a target for *ras*-dependent activation of C/EBPβ. Since mutational activation of *Ras* plays an important role in skin tumorigenesis induced by a variety of carcinogens (Quintanilla *et al*., 1986), Zhu and coworkers (2002) studied the susceptibility of C/EBPβ null mice to carcinogen-induced skin tumors involving mutant *Ras*. They found that the mice were completely refractory to the tumors, and that v-Ha-

*ras* transgenic mice carrying the C/EBPβ-null mutation also showed a significant reduction in tumorigenesis. Moreover, they showed that Ha-*ras*-induced stimulation of C/EBPβ activity was abolished *in vitro* in primary keratinocytes derived from C/EBPβ-null mice and transfected with a mutant C/EBPβ where Thr188 (the mouse analog of Thr235 in human) was mutated to Ala.

More recently Thr188 was described as a target for Cdk2 phosphorylation alongside with a novel phosphoacceptor on Ser63 (Shuman *et al.,* 2004*)*. This does not exclude MAPK from phosphorylating Thr188, as one site can be targeted by multiple kinases, depending on the cellular context. Most coherently, phosphorylation of Ser63 and Thr188 by Cdks during specific stages of the cell cycle is reported to be essential for C/EBPβ to facilitate oncogenic transformation by H-Ras.

Controversial findings were recently published on the phosphorylation of the serine-rich region in the RD2 domain. Several researchers concur that some serines in this region are targets for GSK3β (glycogen synthase kinase 3β) phosphorylation (Piwien-Pilipuk *et al*., 2001; Tang *et al*., 2005; Zhao *et al*., 2005). However, if on one hand Tang claims the phosphorylation to occur on Ser184 and Thr179, and that the event of phosphorylation in adipocytes leads to acquisition of DNA-binding function, on the other Zhao and colleagues show that Thr188 and Ser184 (and possibly Ser 180 and Ser176) are the preferred targets for GSK3β phosphorylation in resting osteoblasts and that *de*phosphorylation will increase DNA-binding activity. It is hard to conceive that different cellular contexts can explain such opposing effects. Perhaps the *in vitro* procedures that have been used in these studies have produced some experimental artifacts, which could be clarified with appropriate *in vivo* approaches.

C/EBPβ is known to be phosphorylated on several other residues outside of RD2. Ser105 is a major phoshoacceptor for PKA *in vitro*, but this modification does not seem to affect C/EBPβ's DNA binding affinity, whereas phosphorylation of the same site by PKC enhances C/EBPβ transactivation activity (Trautwein *et al*., 1993). PKA and PKC are also able to phosphorylate Ser240 within the DNA binding-domain of C/EBPβ, this time markedly impairing DNA recognition (Trautwein *et al*., 1994). In pituitary cells, calcium-regulated phosphorylation of Ser276 within the leucine zipper domain of C/EBPβ stimulates transcription from a Ca-calmodulin-dependent protein kinase II-responsive element (Wegner *et al*., 1992). Activation of the p90 ribosomal S kinase (RSK) results in the phosphorylation of rat C/EBPβ on Ser105 and of mouse C/EBPβ on Thr217 and concomitantly stimulates proliferation in differentiated hepatocytes (Buck *et al*., 1999). Moreover, phosphorylation by RSK on Thr217 allows C/EBPβ to associate with procaspases 1 and 8, thereby inhibiting their processing and blocking the apoptotic cascade. This suggests that C/EBPβ plays an important antiapoptotic role in the liver.

Consistently, C/EBPβ-deficient mice display an enhanced programmed cell death in hepatic stellate cells.

### 1.3 Goal of the Project

The task of a transcription factor can be widely diverse according to the cellular context. C/EBPβ is expressed in a very broad range of tissues in the organism, and in each tissue it has been described to have a relatively unique function. The targets of gene expression for C/EBPβ can be entirely different from one cell type to another, or at different stages of the cell cycle, or even dependent on environmental conditions. As described above, the C/EBP transcription factors all share the same DNA-binding consensus sequence. However, despite a certain redundancy, they all maintain distinct functions. How can a molecule know what to do in each separate case? It could appear simpler if Nature had devised a separate and different transcription factor for each molecular requirement, highly specialized in one particular task. However, such a system would be energetically unfavorable to the organism if we consider the amount of additional genes, protein synthesis and metabolic organization that this would involve. Evolutionarily, it is by far more convenient to use the same tools for different purposes and to guide these tools through their tasks by an extremely accurate fine tuning. This is why it is important for scientists to understand the regulation of a transcription factor, both at the transcriptional and the posttranslational level.

In my thesis project I have endeavored to analyze the regulation of the C/EBPβ transcription factor in the most physiological conditions possible. Indeed, to avoid experimental artifacts due to protein overexpression, or the use of cell lines, or even to the absence of a cellular context, I have, whenever possible, used *in vivo* experimental models, such as the mouse. I have also addressed very specific questions about C/EBPβ regulation, both at the transcriptional and the posttranslational level, with the help of genetic engineering.

Shortly before and during the years of my PhD, several papers were published suggesting that CREB regulates C/EBPβ transcription (Niehof *et al*., 1997; Berrier *et al*., 1998; Bradley *et al*., 2003), and that it physically binds the C/EBPβ promoter during adipogenesis (Zhang *et al*., 2003). In order to dissect the effects of CREB regulation, and to understand when and where it is involved, I specifically deleted a short sequence on the C/EBPβ promoter that carries the two CRE elements, and made a mouse line carrying this deletion. The analysis of this mouse line is one of the main branches of my project.

The second part of my project focuses on some of the posttranslational modifications that modulate C/EBPβ activity. It is not difficult for a kinase to phosphorylate a protein where it finds a consensus sequence if both kinase and protein are the most highly represented molecules in the system, however this doesn't prove that the same event

takes place in physiological conditions. None of the numerous phosphorylations that have been described for C/EBPβ have been proven genetically, although most of them have been connected to a functional role. I have chosen to analyze the phosphorylation events that take place in the RD2 domain of C/EBPβ, and to understand their physiological role by mutating the phosphoacceptor sites to alanine. One site is the Thr188 phosphorylation site, already described to be involved in oncogenic *Ras* transformation in the skin (Zhu *et al*., 2002). In parallel, I also mutated three serines, shortly upstream from Thr188, some of which have recently been described as GSK3β phosphorylation sites (Tang *et al*., 2005; Zhao *et al*., 2005), but whose role still seems to be an open question. Once again mouse lines were made from these mutants and analyzed attentively.

It is hardly feasible to study a transcription factor in every tissue that it is likely to be expressed, particularly in the case of C/EBPβ for the reasons stated above. Furthermore, in many tissues when one C/EBP family member has been knocked down, another member, expressed at equally high levels, can take over the missing C/EBP and, at least partially, mask the effects of its absence. In the case of C/EBPβ, C/EBPα very often has given problems of redundancy. To ensure an accurate analysis, I chose to work on a tissue where C/EBPα is absent, and where C/EBPβ is by far the predominant isoform, that is the macrophages. By studying the transcriptional and posttranslational regulation of C/EBPβ in the macrophages, I aim at finding novel downstream targets of C/EBPβ transcription, and possibly learn more about the role of the transcription factor in the immune system.

# 2. MATERIALS AND METHODS

All the experiments in this thesis were carried out by myself, with the exception of the first part of the cloning of the R26(ARIAD) targeting construct, which was done by Elke Kurz. FACS analyses were done in collaboration with Peggy Kirstetter.

## 2.1 Molecular Biology

Standard protocols for molecular biology were taken from Molecular Cloning Laboratory Manual (2nd edition). For DNA preparation: MiniPrep, QIAquick PCR purification and Gel extraction kits (Qiagen) were used according to manufacturer instructions. Ligations were performed using T4 DNA ligase from New England Biolabs. Restriction enzymes were purchased from New England Biolabs. For cloning, *E. coli* strain XL1-blue was used, except for pcDNAI plasmids, for which the MC1061/P3 strain was used.

### 2.1.1 Plasmids

For testing the ARIAD system, the pL2N2-RHS3H/ZF3 plasmid (for simplicity it was referred to as pL2N2(ARIAD) in the Results) was purchased with the ARGENT™ Regulated Transcription Retrovirus Kit (Version 2.0; ARIAD), and the ZFHD-GFP plasmid, cloned by Elke Kurz, is a construct bearing the ZFHD motif followed by the IL2 promoter and then the GFP gene, on a pC4M-F2E expression plasmid backbone (from the ARGENT™ Regulated Heterodimerization Kit, ARIAD). The dimerizer (AP21967) used in this experiment is a chemically modified derivative of rapamycin and is also provided by the ARIAD kit.

For stable expression of exogenous proteins, retroviral vectors were used. Wild type C/EBPβ, as well as the T188A and 3S/A mutants were cloned in the pBabePuro plasmid using the BamHI and EcoRI restriction sites. Infected cells were selected with 1μg/μl puromycin until complete death of uninfected controls. For transient transfections, wild type C/EBPβ and the T188A and 3S/A mutants were cloned into the pcDNAI expression plasmid using BamHI and EcoRI. For Cyclin D1-HA and C/EBPα transient expression, pCMV-cyclinD1-HA and pcDNA3-C/EBPα expression plasmids were kindly provided by Dr Tetsuhiro Fujimoto and Dr Oksana Bereshchenko respectively.

### 2.1.2 Targeting Constructs

The βΔCRE-neo targeting construct was obtained by cloning homology arms in the pSVKeo-X1 targeting vector, inserting the ZFHD sequence next to the *Neo* cassette, and knocking the resulting construct in the genomic clone of C/EBPβ. In detail, the 210bp DNA stretch (nt -370 to –161, left arm) immediately upstream from the first CRE element was cloned in the polycloning site of pSVKeo-X1 upstream of the *Neo* cassette using the AscI and SalI restriction sites. The 127bp DNA stretch (nt -108 to +19, right arm) immediately downstream of the second CRE element in the pSVKeo-X1 polycloning site was cloned downstream of the *Neo* cassette using the XhoI and NotI restriction sites. A 79bp linker DNA encoding the ZFHD recognition element was obtained by annealing complementary oligonucleotides carrying the ZFHD sequence, and cloned between the lox P site and the right arm using the PacI and XhoI restriction sites. This construct was inserted in the C/EBPβ genomic clone by ET recombination (Angrand *et al*., 1999). The C/EBPβ genomic clone was obtained by bringing together a 2kb XbaI-NotI fragment of the C/EBPβ promoter from the 9N1R plasmid (kindly provided by Dr. E. Sterneck), to the NotI-BamHI fragment, which includes the coding sequence and 6kb of the 3' utr, coming from the 3N2ΔB plasmid (also courtesy of Dr. E. Sterneck). The backbone of this construct is pBluescript-KS.

For the construction of the R26(ARIAD) targeting vector, the EcoRI-BamHI fragment, which includes transactivation domain (TAD), IRES and DNA binding domain (DBD) on the ARIAD pL$_2$N$_2$-R$_H$S3H/ZF3 vector, was cloned in a plasmid called pR26SALPA, which is a pBS plasmid in which the splice acceptor and polyA site from the original Rosa26 gene are cloned separated by a polylinker. Two homology arms from the ARIAD pL$_2$N$_2$-R$_H$S3H/ZF3 vector were cloned on either side of the floxed *Neo* cassette in pSVKeo-X1. The left homology arm was a 289 DNA stretch corresponding to the 3' end of the TAD of the ARIAD transcription factor, and it was cloned upstream of the pSVKeo-X1 *Neo* cassette using the KpnI and SalI restriction sites. The right homology arm was a 336bp DNA stretch corresponding to the first half of the IRES which immediately follows TAD on the ARIAD pL$_2$N$_2$-R$_H$S3H/ZF3 vector, and it was cloned downstream of the pSVKeo-X1 *Neo* cassette using the XhoI and SacII restriction sites. The resulting construct was used to knock in the floxed *Neo* cassette between TAD and the IRES in the pR26SALPA(ARIAD) vector by ET recombination. Finally, the 6kb long fragment carrying all the ARIAD construct from pR26SALPA(ARIAD) was cloned in the pR26-1 targeting construct (kindly provided by Dr. P. Soriano). This was achieved with an intermediate passage in the pUC19 vector in which a DNA polylinker was cloned between EcoRI and HindIII carrying the following restriction sites: NheI-AscI-PacI-SacI-NheI. An AscI-SacI 6kb fragment was cut out of pR26SALPA(ARIAD) by partial digestion and cloned in the polylinker. Then the fragment was cut out again with NheI

and cloned into pR26-1 linearized with XbaI, since the NheI and XbaI restriction sites have complementary overhangs.

The cloning strategy of the T188A and 3S/A mutants is extensively described in the Results. The first cloning step was done in pGL3 (Promega), which has the BspEI restriction site in its sequence. The first half of the C/EBPβ gene, generated as a BamHI-BspEI fragment by PCR, using an antisense oligo which created the silent BspEI restriction site in the C/EBPβ coding sequence, was cloned in pGL3. The fragment was excised by a BamHI-XmaI digestion and cloned in pBS. Next, an XmaI-EcoRI fragment, corresponding to the 3' portion (from just before Thr188 to the stop codon) of the C/EBPβ coding sequence was generated by PCR using a sense oligonucleotide that inserted the XmaI site as silent mutation and, in the case of T188A, also the mutation of the Thr188 to Ala. This fragment was inserted in the pBS construct with the BamHI-BspEI C/EBPβ fragment. The resulting plasmid was opened with BspEI and XmaI, and DNA linkers carrying the mentioned mutations were inserted. The mutations were inserted in the targeting construct by subcloning the 380bp BstBI-PstI fragment from the C/EBPβ coding sequence with the mutations into the NSRI plasmid, which carries a NotI-SalI fragment of the genomic clone of C/EBPβ with an ectopic EcoRI site just before the start site. The NotI-SalI fragment was then transferred into the pTV-flox-C/EBPβ targeting construct (NSRI and pTV-flox-C/EBPβ are a courtesy of Dr. A. Leutz).

To improve the targeting efficiency of the pTV-flox-C/EBPβ phosphorylation mutants, after linearizing the vector with a XbaI digestion, we annealed "DNA splinkers" to the overhangs (Kalisch *et al.*, 1986). The splinkers are oligonucleotides designed in a way that permits their self-annealing so as to form hair pin loops with overhangs complementary to those of the open targeting construct. This technique helps to avoid that the single stranded overhangs insert unspecifically in the genomic DNA of the ES cells.

## 2.2 ES Cells and Mouse Strains

### 2.2.1 ES Cell Transfection and Generation of Mouse Lines

E14.1 129/Ola ES cells were grown on mitomycin C-treated primary embryonic fibroblasts at 37°C, 5% $CO_2$ in Knockout DMEM with sodium pyruvate (Gibco), supplemented with 15% serum replacement, 100U/ml penicillin, 100μg/ml streptomycin, 2mM glutamine, 1% non essential amino acids, 0.1 mM β-mercaptoethanol and 1000U/ml leukemia inhibitor factor (LIF). Cells (15 million) were electroporated in 800μl of phosphate-buffered saline (PBS) with 30μg of linearized targeting vector DNA at 240V, 500μF for 6 msec in a Bio-Rad Gene Pulser. Cells were plated on gelatin-coated 10cm dishes and transferred 36 hr later to growth medium supplemented with G418

(150μg/ml). G418-resistant colonies were picked 9 days later. Homologous recombination was screened by genomic Southern blot hybridization.

For the generation of mutant mice, two ES cell clones derived from each construct were injected into C57BL/6 blastocysts and implanted in foster mothers. The resulting chimeras were bred for germline transmission. Agouti animals were genotyped, in order to distinguish the heterozygous from wild-type animals, using Southern blot analysis. Chimeras and the resulting mouse line were always crossed to C57BL/6 strains. Mice were kept in a clean (SPF) facility, and sacrificed by $CO_2$ asphyxiation.

### 2.2.2 Genotyping

For genotyping, genomic DNA was prepared from tails with the following protocol. Tails were incubated overnight at 55°C in 700μl lysis buffer (50mM Tris, pH8, 100mM EDTA, 100mM NaCl, 1% SDS, 0.5 mg/ml proteinase K). After incubation, 250μl of saturated NaCl was added and samples were centrifuged (10 min 14000 rpm). The supernatant was transferred to a new tube and 500μl isopropanol was added. The precipitated DNA was centrifuged (10 min 14000 rpm) and washed in 70% EtOH. DNA pellets were air dried and dissolved in 60μl TE buffer (10mM Tris pH8, 1mM EDTA).

βΔCRE mice were genotyped by PCR with primers designed to amplify the region around the deletion (and ZFHD insertion) on the C/EBPβ promoter:
left primer: 5'-CGTGTAGCTGGAGGAACGAT-3';
right primer: 5'-CGAGCGGGAGGTTTATAAGG-3'
producing a 363bp fragment for the wild type allele and a 420bp fragment for the mutant allele. PCR reaction: 94°C 2 min, 34x(94°C 1 min, 58°C 1 min, 72°C 1 min), 72°C 5 min. The reactions were carried out with Promega Taq Polimerase as described by the manufacturer, using 1μl DNA template and adding 5% DMSO.

R26(ARIAD) mice were genotyped by PCR as described by Soriano (1999) for R26 knock-in mice, except that the 3 primers were used 2 by 2 in two separate reactions.

3S/A and T188A mice were genotyped by Southern blot as described in the Results.

### 2.2.3 Southern Blotting

For screening of the ES cells, the confluent plates were washed with PBS. 50 μl of lysis buffer (10mM Tris, pH 7.5; 10mM EDTA pH 8, 10mM NaCl, 0,5% sarcosyl, 100μg/ml proteinase K) was added to each well and incubated ON at 55°C. The DNA was precipitated using 100μl EtOH/NaCl, washed three times with 70% ethanol, dried and digested with EcoRI ON at 37°C. 18μl of the ES DNA preparation was separated using 0.7% agarose gels, run at 30V for 15-18 hr. Before blotting, the gels were depurinated for 15' in 0.25M HCl, denaturated with 0.5M NaOH/1.5M NaCl, 2 times for 30 min, and finally neutralized with 0.5M Tris-HCl pH7.4/1.5M NaCl for 1 hr. Gels were

blotted by capillary blotting overnight onto a GeneScreen Plus membrane (Perkin Elmer) in presence of 20x SSC (3M NaCl, 0.3M $C_6H_5Na_3O_7$). The membrane was UV-crosslinked with a UV Stratalinker (Stratagene), and baked at 65°C for 1 hr. Prehybridization was performed in Quick-Hyb Buffer (Stratagene) for 2 hr at 65°C with rotation. The probes were prepared using Random Primed DNA Labeling Kit (Roche) and added to the membrane in Quick-Hyb buffer. For genotyping the βΔCRE, T188A and 3S/A ES cells and mouse lines, the 700bp external probe used was obtained by an EcoRI-XbaI digestion of the 9N1R plasmid. For counter-screening the T188A and 3S/A ES cell clones, a second internal 500bp probe was obtained by a SphI-XmaI digestion of the T188A construct.

Following hybridization, the blots were washed 3 times in 2x SSC/1% SDS and twice in 0.2x SSC/1%SDS at 65°C with rotation. Hybridized probes were visualized using a phosphoimager.

## 2.3 Cell culture

### 2.3.1 Cell Lines

J774 macrophages were grown at 37°C, 5% $CO_2$ in RPMI (Gibco), 10% Fetal Bovine Serum (FCS) (Gibco), 100U/ml penicillin, 100μg/ml streptomycin and 2mM Glutamine.

Q2bn quail fibroblasts, 293T cells and NIH3T3 murine fibroblasts were grown at 37°C, 5% $CO_2$ in Dulbecco's Modified Eagle's Medium (DMEM)(Gibco) supplemented with 10% FCS, 100U/ml penicillin, 100μg/ml streptomycin and 2mM Glutamine. Transfection in Q2bn cells was performed by calcium phosphate coprecipitation, whereas transfection in NIH3T3 cells was carried out with Lipofectamine Plus™ Reagent (Invitrogen) as described by the manufacturer. Cells were grown to subconfluence before splitting.

For inducing adipogenesis, NIH3T3 cells were infected in the presence of 20μg/ml polybrene (Sigma) with a virus encoding either wild type C/EBPβ or the indicated mutants. Cells were grown to confluence and then treated with 1μM dexamethasone (Sigma) and 2μg/ml insulin in growth medium for 48hr. The cells were then shifted back to plain growth medium until adipocytes appeared, around 3 days later. For Oil Red O staining, the cells were washed two times in PBS and fixed in 4% paraformaldehyde/PBS for 1 hr. Fixed cells were washed 3 times in PBS and stained with a filtered solution of 2.1g Oil Red O in 1.05 l of 57% isopropanol for 30 min. Cells were then washed in water and photographed. To measure the degree of staining, cells were air dried overnight and the dye was extracted with isopropanol (1ml per 6 cm dish) for 1 hr. Absorbance was read at 510nm.

### 2.3.2 Primary Macrophages

For the bone marrow-derived macrophages, femurs and tibias were collected from a mouse and crushed in a mortar in the presence of 1% FCS/PBS and filtered through a 45μm cell strainer (BD Biosciences). Cells were washed and resuspended in differentiation medium consisting of RPMI (Gibco), 20% FCS, 50μM β-mercaptoethanol, 100U/ml penicillin, 100μg/ml streptomycin, 2mM Glutamine and 20ng/ml macrophage colony stimulating factor (MCSF)(Sigma). The cells were cultured in differentiation medium for 6 days. Then MCSF was depleted and the cells were left in growth medium (as above but without MCSF). For macrophage activation, cells were treated (or not treated) with 100U/ml IFNγ (PeproTech) in growth medium, with 5% FCS, for 16 hr. Next, the cells were stimulated with 100U/ml IFNγ and 1μg/ml LPS from *E. coli* (Sigma) for 4 hr and then RNA or protein was extracted.

For the preparation of peritoneal macrophages, mice were killed 3 days after an intraperitoneal injection of 1ml 3% thioglycollate broth (Sigma). Exudate cells were harvested by washing the peritoneal cavity with 12ml PBS, washed and cultured in DMEM, 5% FCS, 100U/ml penicillin, 100μg/ml streptomycin and 2mM Glutamine.

## 2.4 Gene Expression

### 2.4.1 Affymetrix

Total macrophage RNA was prepared using Trizol (Sigma) as specified by the manufacturer. RNA was analyzed at the RH Microarray Center (Copenhagen University Hospital, Denmark) using Affymetrix Mouse Genome U74A GeneChip™. Data analysis was performed using Genespring software.

### 2.4.2 RT-PCR

First strand cDNA was synthesized from 1μg total RNA (prepared from tissues and macrophages with Trizol) using Ready-To-Go T-primed first strand kit (Amersham Bioscience). Relative mRNA levels were determined by real time PCR on a DNA Engine (MJ Research) using DyNAmo SYBR green qPCR kit (Finnzymes).

The same PCR program was used for all the RT-PCR analyses, except that the annealing temperature was adapted to each case (the annealing temperature is specified in table 2.1 next to each primer pair): 95°C 3 min, 40 cycles of (94°C 10 sec, $T_{anneal}$ 20 sec, 72°C 10 sec). The product content of the reactions was read after each PCR cycle. The identity of specific products was confirmed by a melting curve analysis.

RT-PCR primers were constructed using Primer 3 software (MIT, http://frodo.wi.mit.edu/primer3/primer3_code.html). cDNA sequence information was retrieved at the ENSEMBL data base (EBI, www.ensembl.org). A total reaction volume of 20μl was used: 5μl cDNA, 5μl of primer pair stock solution (1.2μM each) and 10μl

DyNAmo SYBR green qPCR kit (Finnzymes) according to the manufacturer. mRNA levels of each sample was evaluated in triplicates and dilutions of control cDNA were used to construct a standard curve. All cDNA levels were normalized to the level of ubiquitin cDNA. RT-PCR primers are listed in table 2.1.

**Table 2.1 RT-PCR primer list**

| Name | Left primer | Right primer | $T_{anneal}$ |
|---|---|---|---|
| C/EBPβ | GGAGACGCAGCACAAGGT | AGCTGCTTGAACAAGTTCCG | 60°C |
| Msr1 | CTGGACAAACTGGTCCACCT | TCCCCTTCTCTCCCTTTTGT | 58°C |
| Arginase 1 | AACACGGCAGTGGCTTTAAC | GAGGAGAAGGCGTTTGCTTA | 57°C |
| IL13α1 | CAGCTGGGATACAGGCATCT | TGGTTTCCACAGCATTTCAA | 58°C |
| Ccl8 | GGGTGCTGAAAAGCTACGAG | TTCCAGCTTTGGCTGTCTCT | 58°C |
| Ccl20 | AGAAAATCTGTGTGCGCTGA | AGGTCTGTGCAGTGATGTGC | 60°C |
| CD36 | TCCCTCACTGGAGGAAACTG | TGTGATATCTGGCCTTGCTG | 58°C |
| CD38 | CGAAGGAGCTTCCAGTAACG | AGCTTTCCACCAGTGTTGCT | 58°C |
| C-type lectin | TCTGACAGAGGCCGTTCTTT | CCACCTGGACCCTCTTACAA | 57°C |
| IL2β | GACTGAATGCAGCCTGTTGA | GGTCCCCAAAGCAACATAGA | 58°C |
| IL6 | TCCTTCCTACCCCAATTTCC | ACCACAGTGAGGAATGTCCA | 58°C |
| IL10 | CCAGGGAGATCCTTTGATGA | CATTCCCAGAGGAATTGCAT | 57°C |
| MARCO | GGAACATCTGGCTGGACAAT | GATTTCCATGTGGGTGGAAC | 58°C |
| metallothionein | ACCTCCTGCAAGAAGAGCTG | GCTGGGTTGGTCCGATACTA | 58°C |
| Mrc1 | CCAGGAACAAGTGACACGAA | AAGGACAAACCAATGCAACC | 57°C |
| Procollagen 1α1 | CAGTCGCTTCACCTACAGCA | GGTGGAGGGAGTTTACACGA | 58°C |
| Procollagen 1α2 | CCGTGCTTCTCAGAACATCA | GAGCAGCCATCGACTAGGAC | 57°C |
| Procollagen 3α1 | AAGGCTGAAGGAAACAGCAA | TGGGGTTTCAGAGAGTTTGG | 58°C |
| Procollagen 5α1 | GGCGGGACAGTATTTGAAGA | ATTTGGCCTCTGAGTGGATG | 58°C |
| Procollagen 5α2 | CAGCCAGTGTTCCACGTAAA | TCTGCGAGGCTTCTTTTGAT | 57°C |
| Procollagen 6α1 | GGAGACCCCTCCTCTTCATC | TTGAGGGAGAAAGCTCTGGA | 57°C |
| Procollagen 6α2 | ATCGTACACGCCATCAACAA | CTGTCACCCAGGCTGATCTT | 57°C |
| Procollagen C-pep | CAAAGCAGTACAAGCGGTCA | GCAGGGCACATACAACTTCA | 60°C |
| TIMP 1 | ATTCAAGGCTGTGGGAAATG | CTCAGAGTACGCCAGGGAAC | 57°C |
| TIMP 2 | GCTGTCCCAAGGACACAAAT | CTGAGGGGGAGTCCTTAACC | 57°C |
| TIMP 3 | AGCTGGCAAAGGCTTAAACA | CAAGCTTCCAGCCAAACTTC | 57°C |
| Ubiquitin | GATCCTCTTACCCCCTCGTC | CCTTTAGGCCACTCCTTCCT | 58°C |

## 2.5 Immunohistochemistry and Biochemistry

### 2.5.1 FACS Analysis

The bone marrow-derived macrophage population was stained in wells of a 96 well plate at 1.25x10(6) cells per sample. Cells were blocked with CD16/32 (an antibody against Fcγ receptors) (BD Pharmingen) in PBS/1%FCS for 5 min on ice. Then the cells were washed with PBS/1%FCS, centrifuged (2 min, 1200 rpm at 4°C), and stained with APC-conjugated CD45, Mac1, F4/80, Gr1, B220, CD4, CD8 and Ter119 antibodies (all from BD Pharmingen) in PBS/1%FCS for 15 min on ice in the dark. Finally, the cells were washed twice in PBS/1%FCS. Labeled cells were analyzed on a FACS Calibur (BD Biosciences). Data was analyzed using FlowJo software (Treestar).

### 2.5.2 Protein Extraction, SDS-PAGE, Anderson-PAGE and Western Blotting

For extracting proteins from mouse tissues, tissues were collected and snap frozen in liquid Nitrogen. Next, they were crushed in a mortar while still frozen and the powder was resuspended in lysis buffer (50mM Hepes, pH7.4, 10mM EDTA, 1% Triton X100, 10mM sodium orthovanadate, 50mM sodium pyrophosphate, 100mM sodium fluoride and 1 Complete Mini Protease Inhibitor tablet/50ml solution (Boerinhger). If lumps remained, the tissue was homogenized with an electric homogenizer. Whole cell protein extracts from cultured cells were obtained by resuspending the cells in RIPA buffer (150mM NaCl, 50mM Hepes, pH7.9, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate and 1 Complete Mini Protease Inhibitor tablet/50ml solution). Phosphatase inhibitors were added when necessary.

The extracts were cleared by centrifugation (10 min at 14000 rpm), and protein concentrations were determined using the Bio-Rad Dc protein assay (Biorad). 2x SDS loading buffer was added (100mM Tris pH6.8, 4%SDS, 20% glycerol, 0.2% bromophenol blue and 3% β-mercaptoethanol). The protein extracts were resolved on 0.75mm thick minigels (Bio-Rad apparatus) by SDS-PAGE. The resolving gel contained 15% acrylamide (Bio-Rad, stock solution 30% Acrylamide/bis-acrylamide 37.5:1 ratio), 375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% ammonium persulfate and 0.001% N,N,N',N'-tetramethylenediamine (TEMED)(BioRad). The stacking gel contained 4% acrylamide, 125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulphate and 0.001% TEMED. The gels were run in Laemmli running buffer (25 mM Tris base, 200 mM glycine, 0.1% SDS) at a 100V. Proteins were transferred onto Protran nitrocellulose (Schleicher & Schuell) using a Hoefer SemiPhor apparatus for 1 hr at a constant mA (1mA/cm$^2$ gel area) in transfer buffer (20 mM Tris base, 150 mM glycine, 0.1% SDS, 20% methanol).

Anderson-PAGE (Nebreda *et al.*, 1995) is an SDS-free PAGE with a different ratio of acrylamide to bis-acrylamide, which is able to separate proteins according to their charge

as well as their molecular weight, and therefore it is used to discriminate phosphorylated and unphosphorylated forms of the same protein. 15% resolving gels (30% acrylamide to 1% bis-acrylamide ratio = 174:1, 370mM Tris-HCl pH8.8) and 5% stacking gels (5% acrylamide, 0.13% bis-acrylamide, 125mM Tris-HCl pH6.8) were used. Large gels were poured (20cm long) and the protein extracts in SDS loading buffer were resolved at 100V for 20 hr in Laemmli buffer.

Western blots were blocked in 5% milk in TBS-T (0.1M Tris-HCl pH7.5, 150mM NaCl, 0.1% Tween-20 (BioRad)) for 1 hr at room temperature, and incubated with the indicated primary antibodies 1 hr at room temperature, or overnight at 4°C (in the case of the phospho-C/EBPβ(Thr235) antibody). Membranes were washed in TBS-T 3 times for 10 min before and after incubation with the secondary antibody against rabbit or mouse IgG (Jackson Immunolaboratories, 1:10000). Horseradish peroxidase conjugated secondary antibodies were used and ECL (Amersham Biosciences) revealed the signal.

For detecting C/EBPβ on Western blots α-C/EBPβ C19 antibody sc-150X (Santa Cruz Biotechnology, 1:1000) was used for most experiments. Where specified, the α-C/EBPβ Δ198 antibody sc-746 (Santa Cruz Biotechnology, 1:1000) was used instead. For detecting the phosphorylation on Thr188 of the C/EBPβ protein, a phospho-C/EBPβ(Thr235) Antibody (Cell Signaling Technology, 1:1000) was used. For detecting the HA-tagged Cyclin D1 we used an anti-HA antibody (Santa Cruz Biotechnology, 1:10000).

### 2.5.3 Chromatin Immunoprecipitation

J774 macrophages were grown to subconfluence in 10cm dishes. Cells were treated or not treated with 1μg/ml LPS for 15 min. Next, cells were crosslinked with 1% formaldehyde in the culture media for 10 min. Crosslinking was quenched by adding 0.125M glycin to the medium and incubating 10 min. Cells were then washed and scraped in cold PBS. Finally, the cells were pelleted and resuspended in ChIP lysis buffer (50mM Tris-HCl pH8, 10mM EDTA, 1% SDS) in a volume of 200μl/million cells. Cell lysates were incubated on ice for 10 min. Chromatin was sheared by sonication (4 bursts of 10 sec using a Sonoplus GM 200 sonicator (Bandelin Electronics), probe 72 at 20% output). After sonication, lysates were centrifuged 10 min at 4°C to eliminate the cellular debris. 200μl of the lysate was diluted 10 times in ChIP dilution buffer (16.7mM Tris-HCl pH8, 167mM NaCl, 1.2mM EDTA, 1.1% Triton X-100 and 0.01% SDS) and cleared by incubation with 30μl protein A beads (Amersham, 50% in ChIP dilution buffer) for 1 hr at 4°C with rotation. 4μg of anti-CREB antibody (Upstate) or unspecific rabbit IgG (Santa Cruz Biotechnology) were added and lysates were incubated 16 hr at 4°C on rotation. 30μl of a 50% Protein A bead solution was added, and the lysates were incubated 4 hr at 4°C on a rotating wheel. Beads were collected by centrifugation and

washed in ChIP dilution buffer, ChIP dilution buffer with 500mM NaCl, and twice in TE (10mM Tris-HCl pH8, 1mM EDTA). Finally, the beads were resuspended in 100µl TE and decrosslinked at 65°C in the presence of 1ng RNase for 4 hr. 50ng Proteinase K and SDS to 0.5% were added, and the samples were incubated 1 hr at 42°C. DNA was collected using Qiaquick gel extraction kit (Qiagen) according to the instructions of the manufacturer. Precipitated DNA was analyzed by PCR using the following conditions:
94°C 2 min, 30 x (94°C 1 min, $T_{anneal}$ 1 min, 72°C 1 min), 72°C 5 min. The annealing temperature was 58°C for C/EBPβ promoter primer pair, and 60°C for the control The reactions were carried out with Promega Taq Polimerase as described by the manufacturer, using 2µl DNA template and adding 5% DMSO. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

The ChIP primers were constructed using Primer 3 software (MIT, http://frodo.wi.mit.edu/primer3/primer3_code.html), based on sequence information retrieved from the ENSEMBL data base (EBI, www.ensembl.org). The primers used were specific for a 140bp region on the C/EBPβ promoter that includes the CRE elements. As an unspecific control, we used primers that amplified a 200bp DNA stretch on the C/EBPβ 3' utr, about 1.5kb downstream of the C/EBPβ coding sequence. The primers are the following:
β promoter left: 5'- CACTCCCCGCCGCGCCCTCTC -3'
β promoter right: 5'- CGAGCGGGAGGTTTATAAGG-3'
β 3' utr left: 5'- CGTTCTGCAAGCCCTGGG-3'
β 3' utr right: 5'- GAGTCGCTGGTCACCCCT-3'

### 2.5.4 Coimmunoprecipitation

For detection of protein-protein interactions in transiently transfected cells, 293T cells were transfected with 4µg DNA in total of pCMV-HA-tagged cyclin D1 and pcDNA1-C/EBPβ or the specified mutants, or pcDNA3-C/EBPα as negative control, using the calcium phosphate precipitation method. 40 hr after transfection, cells were washed and harvested in cold PBS. Whole cell extracts were prepared in CoIP lysis buffer (50mM Tris-HCl pH8, 150mM NaCl, 1mM EDTA, 0.5% NP40, 1mM DTT, and 1 Complete Mini Protease Inhibitor tablet/50ml solution (Boerinhger)). The lysates were precleared with 20µl Protein A beads (50% suspension in CoIP lysis buffer) at 4°C for 1 hr. Cleared lysates were incubated with 2µg C19 α-C/EBPβ (Santa Cruz Biotechnologies) for 3 hr at 4°C with rotation, and protein complexes were precipitated on 20µl 50% Protein A beads after a 1 hr incubation (4°C with rotation) of the samples with the beads. Immobilized complexes were washed 4 times with CoIP lysis buffer and finally decrosslinked by suspension in 2x SDS loading buffer (100mM Tris pH6.8, 4%SDS, 20% glycerol, 0.2%

bromophenol blue and 3% β-mercaptoethanol) and incubation at 98°C for 5 min. Samples were analyzed by Western blotting as described above.

## 2.6 *In Vitro* Assays

### 2.6.1 NO Assay

The NO assay was performed on peritoneal macrophages. Cells were plated in triplicates on a 96 well plate at 0.1 million cells/well. The cells were either left untreated, or treated with 10U/ml IFNγ and /or 10ng/ml LPS for 48 hr. $NO_2$ anion concentration in the medium was measured with the Greiss reagents (Ding *et al.*, 1988). To make the measurements more accurate and comparable from mouse to mouse, the NO concentrations were normalized to the degree of Thiazolyl blue (MTT) (Sigma) vital dye staining. Briefly, 20µl of a 1.25mg/ml MTT in culture medium solution was added to the well in which the cells were already in 100µl medium. Cells were incubated with the dye for 1 hr at 37°C. 60µl of medium was removed from each well, and then the cells were lysed with 140µl cold acidic isopropanol (isopropanol + 0.24% HCl). Absorbance was read at 570nm.

### 2.6.2 Reporter Gene Assays

Q2bn cells were plated at 3x10(5) cells/60mm dish and transfected by calcium phosphate coprecipitation with 200ng pcDNAI-C/EBPβ or the indicated mutants, 0.5µg pMim1Δ-Luc and 1µg pRSV-βgal. After 48 hr, cells were washed with PBS, and harvested by addition of 1ml TEN (40mM Tris-HCl pH7.5, 1mM EDTA pH8, 150mM NaCl) per dish. Cells were pelletted, washed in PBS and finally lysed with 100µl Reporter Gene Assay Lysis Buffer (100 mM potassium phosphate, pH7.8, 0.2% Triton X-100, 1 mM DTT). The cell debris was removed by centrifugation, and the lysate was used in either a luciferase or β-gal assay. Relative luciferase units were defined as β-gal normalized luciferase activity.

The luciferase activity was measured using a Lumat LB 9507 luminometer. The samples were measured using 50 µl lysate in 350 µl reaction solution (25 mM Gly-Gly pH 7.8, 2 mM ATP, 10 mM $MgSO_4$). 100 µl injection solution (25 mM Gly-Gly pH7.8, 0.2 mM Luciferin) was injected in each reaction.

The β-gal activity was measured as follows: 0.4ml ONPG (20mg/ml in ethanol) and 1.4µl MeSH was added to 10ml of the β-gal buffer (60mM $Na_2HPO_4$, 40mM $NaH_2PO_4$, 10mM KCl, 1mM $MgCl_2$). 50µl lysate was placed in a well on a 96 well plate and 150µl β-gal buffer was added and incubated at 37°C. The reaction was stopped with 50µl 1M $Na_2CO_3$ and the absorbance was measured at 414nm in an ELISA-reader.

For luciferase assays on NIH3T3 cells, the Dual Luciferase Assay Kit (Promega) was used. Subconfluent cells on 60mm dishes were transfected using the Lipofectamine

Plus™ Reagent (Invitrogen) with 200ng pcDNAI-C/EBPβ or the indicated mutants, 800ng pMim1-ΔLuc reporter and 2ng pRL-SV40 (the Renilla luciferase reporter). The assay was carried out according to the manufacturer's intructions, and read on a Lumat LB 9507 luminometer.

# 3. RESULTS

## 3.1 Generation of the βΔCRE Mouse Line

In order to understand the role of CREB in regulating C/EBPβ transcription, a mouse line was generated, in which the 52bp DNA segment carrying the two CRE elements (i.e. the TGACG motifs at –161 and –113 from the start site) on the C/EBPβ promoter were deleted. The deletion does not include the TATA box, which is situated at –86bp from the start site, however it slightly overlaps with a putative C/EBP recognition motif, because the T of the CRE element at –161 coincides with the T of the CAAT motif for C/EBP binding. In the site of deletion we inserted a DNA segment carrying a recognition motif for an artificial transcription factor commercialized by ARIAD. This was done in the future prospective of being able to artificially induce the expression of the C/EBPβ gene, and to reverse any phenotype obtained from the loss of CREB regulation. The *cis* recognition motif is called ZFHD, and its use will be described further on in this thesis. To enable selection of ES cells, a floxed *Neo* cassette was also inserted at the point of deletion, immediately upstream from the ZFHD. The engineered promoter was inserted into a plasmid carrying the genomic clone of C/EBPβ by ET recombination (figure 3.1A).

The resulting construct, which is the targeting vector, was checked by sequencing (not shown) and by transformation in the XL1-Cre bacterial strain, to ensure that a correct recombination and excision of the *Neo* cassette could be obtained (not shown). The targeting construct was linearized by cutting in the ScaI site inside the *AmpR* gene and transfected into ES cells. ES cell clones were selected with G418 and screened by Southern blot as shown on figure 3.1B. Positive clones (figure 3.1C) were injected in blastocysts and chimeric mice were obtained.

Once germline transmission of the deletion was achieved, the βΔCRE-neo mice were crossed to a deleter-Cre line, in order to eliminate the *Neo* cassette inside the C/EBPβ promoter by Cre recombination. The resulting line, called βΔCRE, is characterized by the substitution of the CRE elements in the C/EBPβ promoter with the ZFHD motif (figure 3.1D). The βΔCRE line is genotyped by PCR (see Materials and Methods). For simplicity, from here on the βΔCRE mutant allele will be referred to as DC.

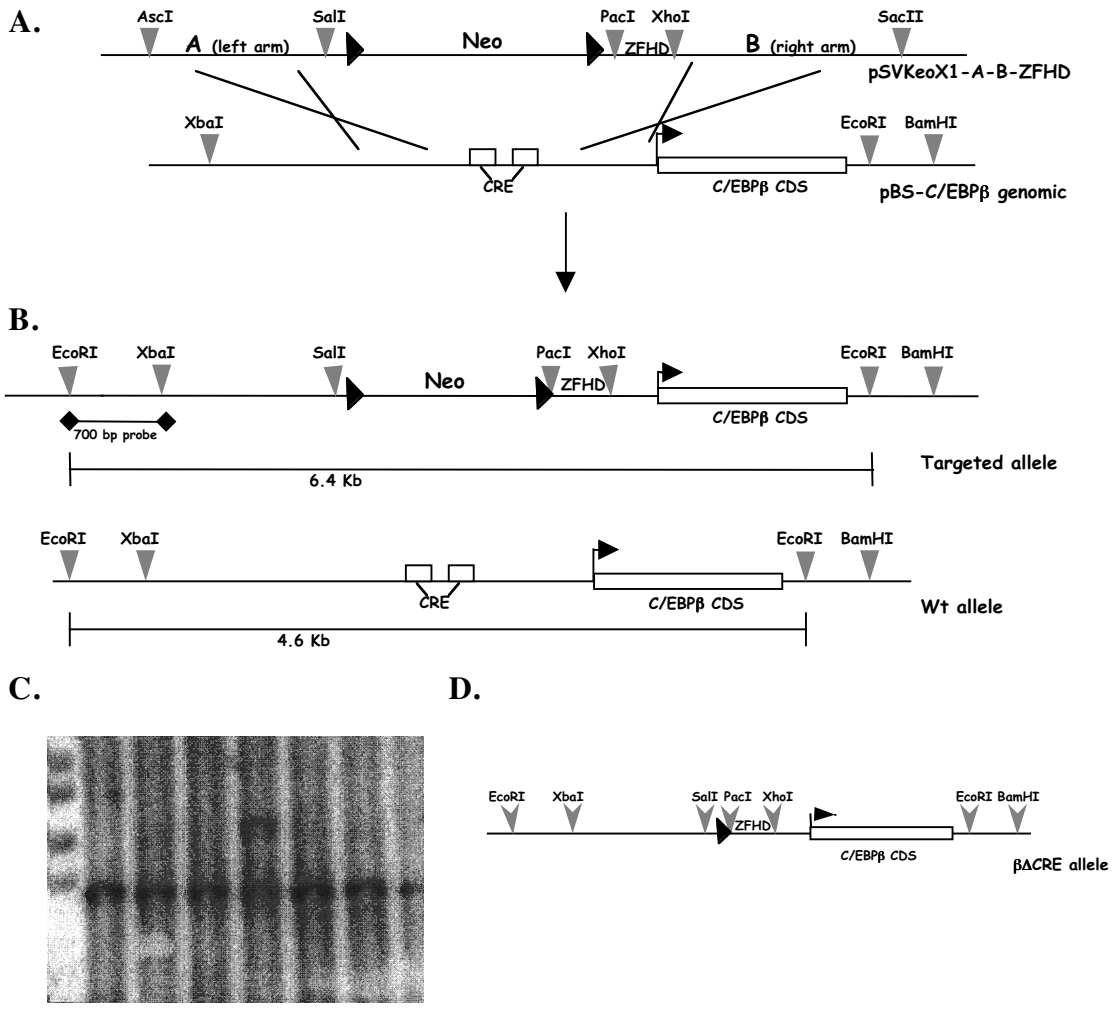



**Figure 3.1: Schematic representation of the targeting strategy.** A. Left and right homology arms flanking the CRE elements on the C/EBPβ promoter were cloned in the pSVKeo-X1 vector on the two sides of the *Neo* cassette followed by the ZFHD. After ET recombination in the genomic clone, the resulting vector was used to target the wild type allele (B.). ES cells were screened with the EcoRI-XbaI probe, external to the targeting construct, on EcoRI-digested genomic DNA. The lengths of the wild type and targeted allele digested fragments are indicated. C. The wt allele is 4.6 Kb long (lower band), whereas the mutant allele gives a band of 6.4 Kb (upper band on fifth lane). D. Schematic representation of the βΔCRE allele.

### 3.1.1 βΔCRE *DC/DC* Females Are Fertile

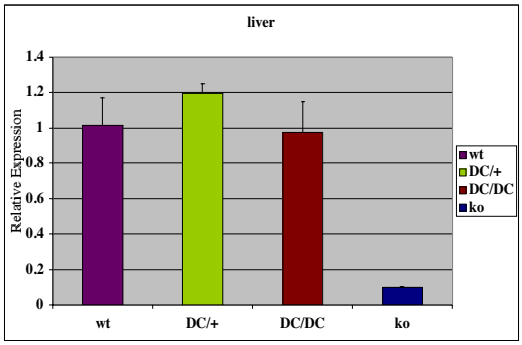
One of the most striking phenotypes of the C/EBPβ knock out mouse is the female sterility due to an impairment in the maturation of the granulosa cells in the ovaries (Sterneck *et al.*, 1997). In order to check the fertility of the βΔCRE homozygous (DC/DC) females, we set up matings between four DC/DC females and four DC/+ males.

Four DC/+ females were used as controls inside the same cage as the mutants. After 30 days of mating, 3 out of 4 controls were pregnant or had delivered, and two out of four DC/DC females were pregnant, and eventually delivered normal litters. Moreover, as was mentioned in the introduction, mice deficient for C/EBPβ, display impaired lobuloalveolar development of the mammary gland, which is associated with perturbed proliferation of mammary epithelial cells in the early phase of pregnancy (Seagroves *et al*., 1998). For this reason, we observed whether the DC/DC females had a problem in lactation. However, the DC/DC mothers were able to lactate, and the pups grew and were weaned at the same stage as litters born from control females. We can therefore conclude that βΔCRE homozygous mice do not display defects in female reproduction.

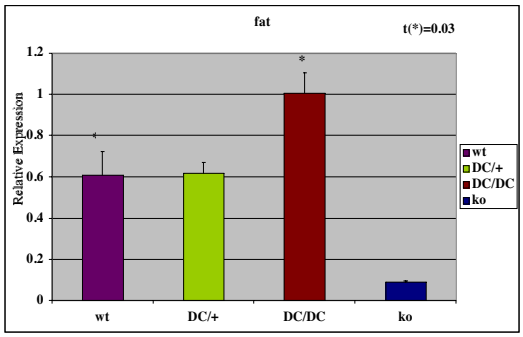
### 3.1.2 C/EBPβ Expression in βΔCRE Tissues

To have a general view of the level of expression of C/EBPβ in mice carrying a deletion in the promoter of this gene, we extracted RNA from several tissues where the transcription factor is known to be present and performed RT-PCR analysis for C/EBPβ. Tissue from C/EBPβ knockout mice was used as a negative control. In the liver, the levels of C/EBPβ mRNA are quite comparable between DC/DC and wild type mice (Figure 3.2A). In the fat there seems to be a stronger expression of C/EBPβ in the DC/DC (Figure 3.2B). In the brain, C/EBPβ was shown to be implicated in synaptic plasticity and memory consolidation (Alberini *et al*., 1994). Taubenfeld and coworkers have suggested that CREB is involved in regulating C/EBPβ expression in the pathway that leads to hippocampal learning (Taubenfeld *et al*., 2001). In detail, they observed a correlation between CREB activation and subsequent C/EBPβ upregulation following a spatial learning task. We therefore checked the levels of C/EBPβ mRNA in the hippocampus, and found them to be comparable to the wild type controls (Figure 3.2C). However, this is not too surprising considering the fact that the mice had not been made to perform a learning task before being sacrificed. The fact that C/EBPβ expression is somewhat higher in the DC/DC fat, could possibly be explained by the partial deletion of the C/EBP binding site on the C/EBPβ promoter due to its partial overlap with the CRE element. This would mean that C/EBPβ participates in an autoregulatory feedback loop, thereby restricting its own transcription when already present at high levels. We have further support of this theory from some data obtained with an affymetrix that will not be part of this thesis. Briefly, an affymetrix was carried out on NIH3T3 fibroblasts transduced either with C/EBPβ or with empty vector. A microarray is capable of detecting only the endogenous transcript, because the ESTs for the screening are chosen from the 3'utr of the gene, which is absent in an expression vector.

**A.**




**B.**




**C.**

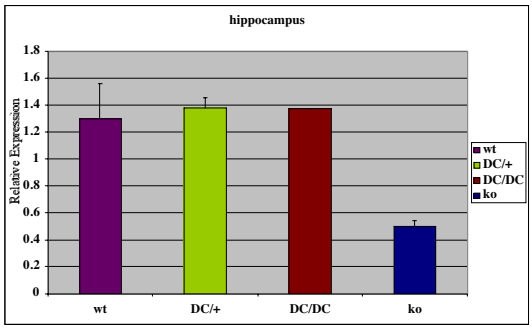



**Figure 3.2: Relative C/EBPβ mRNA levels in βΔCRE tissues.**
Expression in the liver (A), the fat pads (B), and the hippocampus (C). The signal obtained in the knockout sample (ko) should be considered background noise. n=2.

Therefore, we were able to monitor C/EBPβ endogenous expression in the presence of the exogenous protein. Indeed, we observed that the endogenous levels of C/EBPβ were consistently low compared to the empty vector-transduced cells (figure 3.3).

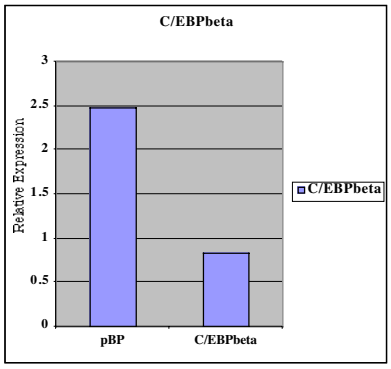



**Figure 3.3: Expression levels of endogenous C/EBPβ in cells overexpressing exogenous C/EBPβ.** Affymetrix on NIH3T3 fibroblasts transduced either with wild type C/EBPβ or with empty vector (pBP).

C/EBPβ is involved in adipogenesis, although the C/EBPβ knockout mouse doesn't present gross defects in the white adipose tissue (Tanaka *et al*., 1997). To check whether the higher expression of C/EBPβ in DC/DC fat could perturb its morphology, we weighed the epidydimal fat pads of βΔCRE mice and normalized the values to the total body weight of each mouse. Predictably, there was no difference between DC/DC mice

and their wild type littermates (not shown). Moreover, we made histological sections of the fat pads and stained the adipose tissue with eosin-hematoxilin. Once again there was no evident difference between mutant and wild type (data not shown).

### 3.1.3 CREB Physically Binds the C/EBPβ Promoter in Macrophages upon LPS Stimulation

Experiments conducted on the J774 macrophage cell line showed that the stimulation of the cells with LPS correlated with the phosphorylation of CREB, and therefore its activation, after 10 minutes of treatment, and the upregulation of C/EBPβ after four hours (Bradley *et al*., 2003).

To exclude the possibility that the upregulation of C/EBPβ following CREB phosphorylation could be a secondary, and not direct, effect in the signaling cascade, we checked whether CREB could be immunoprecipitated on chromatin bound to the C/EBPβ promoter. The ChIP experiment was performed on J774 macrophages in which protein-DNA interactions were crosslinked after a 15 minute LPS treatment. As shown in figure 3.4, CREB was immunoprecipitated on the C/EBPβ promoter only in the LPS-treated cells. The PCR that was used for this experiment is specific for a 140bp DNA fragment that covers the CRE elements on the C/EBPβ promoter.

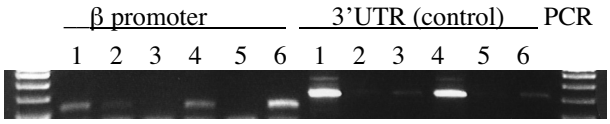



**Figure 3.4: ChIP on J774 macrophages.** J774 macrophages were treated (lanes 4-6) or not treated (lanes 1-3) with 1μg/ml LPS for 15 minutes. The PCR shows that CREB binds onto the C/EBPβ promoter only after LPS treatment (compare lanes 3 and 6 of β promoter PCR). The amplification of a DNA fragment ca 2kb downstream from the CRE elements (in the 3' utr of the C/EBPβ gene) was used as a control PCR.

These results suggested an interesting model to use for analyzing the βΔCRE mice. We decided to check whether the upregulation of C/EBPβ following LPS stimulation was dependent on CREB activation in primary macrophages as well.

### 3.1.4 IFNγ/LPS-Dependent Induction of C/EBPβ Expression Requires the CRE Elements on the C/EBPβ Promoter

Next, we tried to set up the best conditions for analyzing the βΔCRE primary macrophages. To find the time point with the strongest induction of C/EBPβ after LPS stimulation, we performed a time course in which primary macrophages of wild type mice were treated with LPS for 2-4-6-8 and 24 hours before RNA extraction. The RNA was reverse-transcribed and C/EBPβ expression levels were measured by real time PCR. Albeit a certain variability, the strongest induction took place between two and four hours (data not shown), with a smaller standard deviation at four hours. We found that an overnight pre-treatment with IFNγ, with the aim of priming the macrophages, reduced the variability significantly. For future experiments, we chose to prime the macrophages with IFNγ and to stimulate with IFNγ and LPS for four hours.

βΔCRE primary macrophages were obtained from bone marrow cultures in the presence of M-CSF (see Materials and Methods). To make sure that our macrophage population was relatively pure, we stained the wild type cells with conjugated antibodies against specific markers for the major components of the hematopoietic system and analyzed them by FACS. The primary cells were very high in Mac-1 and F4/80 (figure 3.5A and B respectively), typical macrophage markers, and showed a significant level (43%) of Gr-1 (figure 3.5C). Gr-1 is considered a granulocyte specific marker, however a certain class of macrophages, termed natural suppressor (NS) cells, originates from granulocyte-monocyte progenitors, and express Gr-1, Mac-1 and F4/80 as surface markers (Atochina *et al*., 2001). B lymphocytes, detected with B220 and CD-19 (figure 3.4D), and T lymphocytes, detected with CD-4 and CD-8 (figure 3.5E), were practically absent, as were the erythrocytes, marked by Ter-119. We therefore considered our population to be sufficiently pure to proceed with our studies.

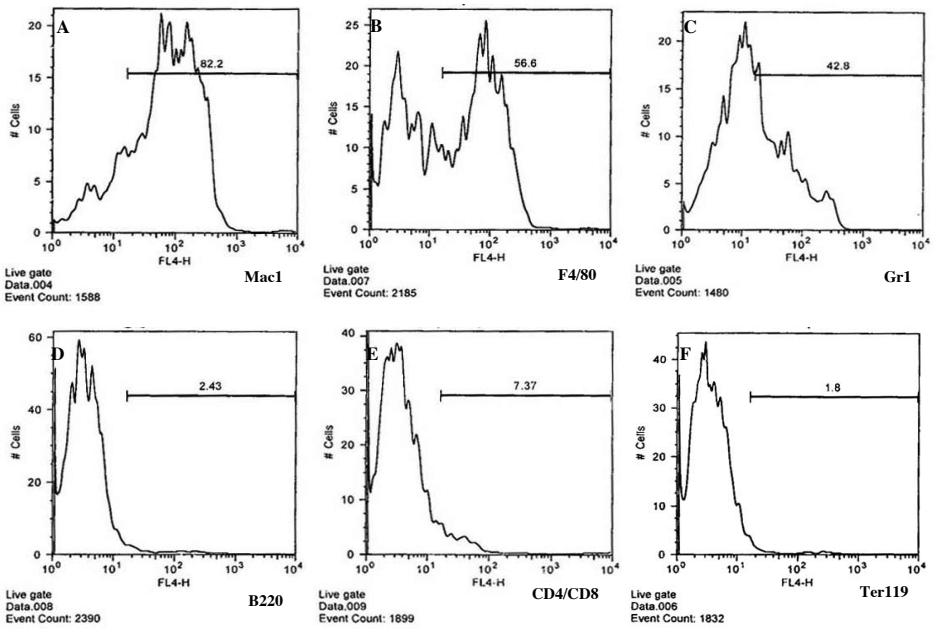



**Figure 3.5: FACS analysis of cultivated bone marrow-derived macrophages.** Bone marrow cells from wt mice were cultured for 6 days in the presence of M-CSF and stained with antibodies specific for macrophages (A and B), granulocytes (C), B-lymphocytes (D), T-lymphocytes (E) and erythrocytes (F). The gate was set on CD45 (a marker for all bone marrow-derived cells) positive cells.

DC/DC and wild type primary macrophages were treated overnight with 100ng/ml IFNγ and the following morning 1μg/ml LPS was added to the culture and incubated for 4 hours. RNA was extracted from the cells and C/EBPβ expression levels were determined by RT-PCR. As shown in figure 3.6, IFNγ/LPS stimulation induced a 3 fold upregulation of C/EBPβ, whereas this was not the case for the DC/DC macrophages.

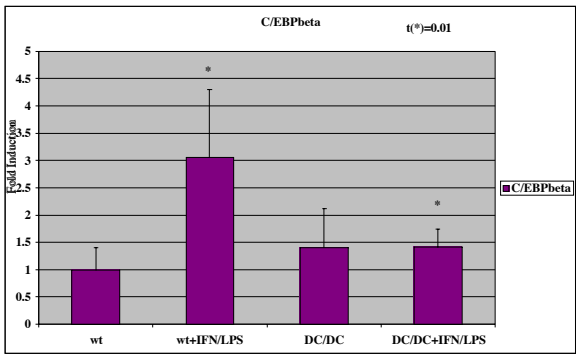



**Figure 3.6: C/EBPβ expression following IFNγ/LPS stimulation.** The experiment was repeated several times with similar results. n=6. The difference between wt+IFN/LPS and DC/DC+IFN/LPS is significant as indicated by Student's t test [t(*)=0.01].

This experiment clearly indicates that the CRE elements on the C/EBPβ promoter are necessary for IFNγ/LPS-dependent induction of C/EBPβ in primary macrophages. This information is new, as the CREB-dependence of C/EBPβ activation is not only a correlation, but a proof *in vivo* in macrophages.

### 3.1.5 Affymetrix Analysis on IFNγ/LPS-Stimulated βΔCRE Macrophages

The experiment described above turned out to be a very useful tool to find novel downstream targets for C/EBPβ following IFNγ/LPS-stimulation. We performed an Affymetrix DNA micro-array analysis on total macrophage RNA of DC/DC+IFNγ/LPS samples, from two different mice, and two wt+IFNγ/LPS controls. In Table 3.1 are reported the genes resulted from the data analysis that we considered most pertinent to this study and that presented a fold regulation of at least 2.

| Affy ID | Gene description | Fold regulation |
|---|---|---|
| | **Downregulated in DC/DC** | |
| 1452250_a_at | procollagen, type VI, alpha 2 | 46.54 |
| 1419549_at | arginase 1, liver | 31.32 |
| 1422437_at | procollagen, type V, alpha 2 | 25.10 |
| 1427883_a_at | procollagen, type III, alpha 1 | 23.93 |
| 1423669_at | procollagen, type I, alpha 1 | 19.14 |
| 1450857_a_at | procollagen, type I, alpha 2 | 17.46 |
| 1437165_a_at | procollagen C-proteinase enhancer protein | 15.90 |
| 1416740_at | procollagen, type V, alpha 1 | 14.52 |
| 1448590_at | procollagen, type VI, alpha 1 | 9.70 |
| 1449334_at | tissue inhibitor of metalloproteinase 3 | 7.81 |
| 1460227_at | tissue inhibitor of metalloproteinase 1 | 7.64 |
| 1457871_at | macrophage bacteria-binding receptor MARCO | 7.11 |
| 1454677_at | tissue inhibitor of metalloproteinase 2 | 4.72 |
| 1428942_at | metallothionein 2 | 3.65 |
| 1450430_at | mannose receptor, C type 1 | 3.15 |
| 1425435_at | Macrophage scavenger receptor type 1 | 3.11 |
| 1425951_a_at | C-type lectin, superfamily member 10 | 3.06 |
| 1421239_at | Interleukin 6 signal transducer | 2.94 |
| 1450136_at | CD38 antigen | 2.78 |
| 1419684_at | small inducible cytokine A8 (Ccl8) | 2.69 |
| 1450883_a_at | CD36 antigen | 2.07 |
| 1422029_at | Ccl20 | 2.06 |
| 1454783_at | Interleukin 13 receptor, alpha 1 | 2.04 |
| 1448759_at | Interleukin 2 receptor, beta chain | 2.02 |
| | **Upregulated in DC/DC** | |
| 1450330_at | Interleukin 10 | 3.24 |

**Table 3.1: Affymetrix data analysis on wt+IFNγ/LPS versus DC/DC+IFNγ/LPS macrophage total RNA.** n=2. The normalized values of samples with the same genotype were averaged and used to calculate the fold regulation.

All the genes listed in table 2 were counterchecked by RT-PCR using primary macrophages from new DC/DC and wild type mice. Three of the genes were reconfirmed and these are arginase 1, macrophage scavenger receptor 1 (Msr1), and interleukin 13 receptor α1 (IL-13α1)(figure 3.7A, B, C). All the other genes were not confirmed by RT-PCR.

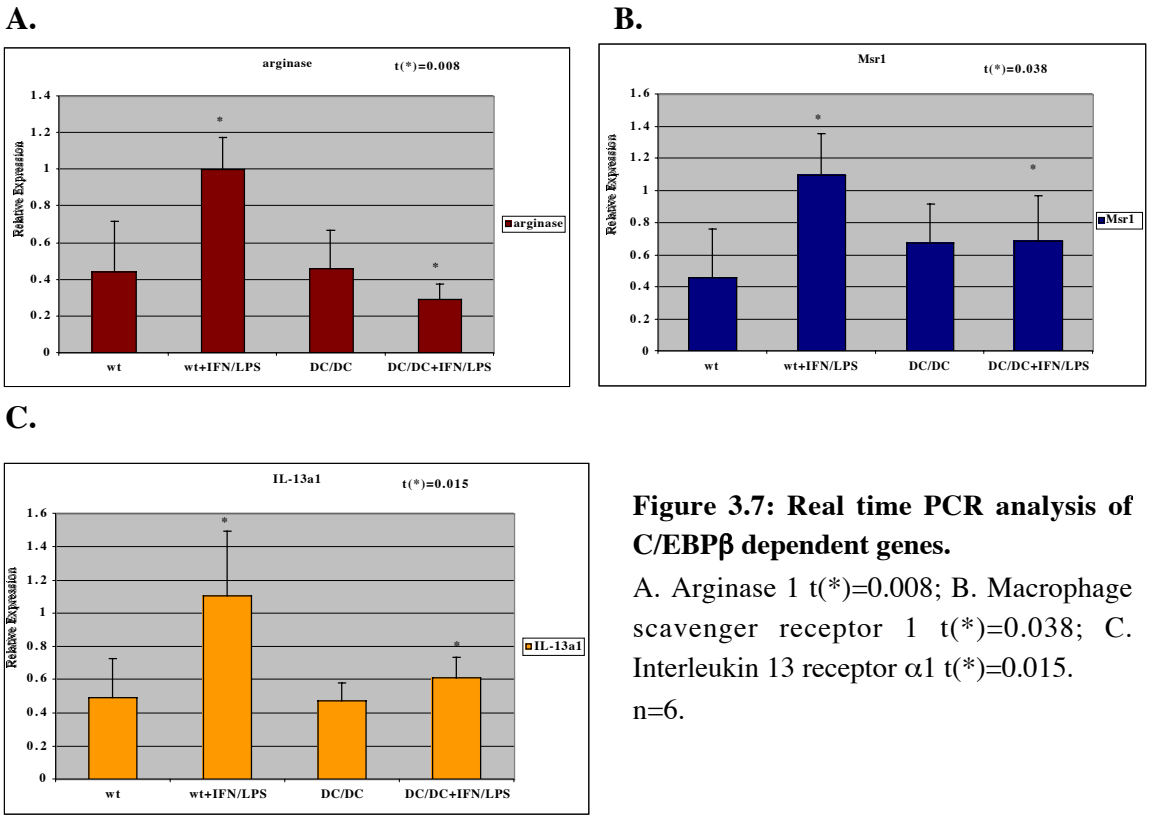



**Figure 3.7: Real time PCR analysis of C/EBPβ dependent genes.**
A. Arginase 1 t(*)=0.008; B. Macrophage scavenger receptor 1 t(*)=0.038; C. Interleukin 13 receptor α1 t(*)=0.015. n=6.

### 3.1.6 βΔCRE Mice Display an Enhanced NO Production in Response to LPS Treatment

As a preliminary characterization of the functionality of the βΔCRE macrophages, we decided to check the release of nitric oxide (NO) of the cells in response to LPS and/or IFNγ. Thioglycollate-elicited peritoneal macrophages were plated at low density in a 96-well dish and treated for 48h with LPS and/or IFNγ. NO release in the culture medium was determined with the Greiss reagents (see Materials and Methods). When the macrophages are treated with LPS and IFNγ together, DC/DC and wild type cells display a very similar NO release. However, when the macrophages are treated with the reagents individually, the DC/DC cells react by releasing more NO than the wild type. The result is not significant in the IFNγ treated samples, due to a high standard deviation, but it is significant in the LPS-treated samples (t=0.004) (figure 3.8).

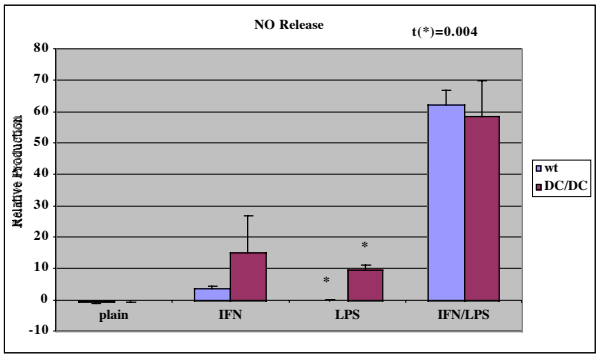



**Figure 3.8: Production of NO by macrophages.** Thioglycollate-elicited peritoneal macrophages were cultured for 48h in the absence or presence of IFNγ and/or LPS. NO release was determined. n=3. t(*)=0.004. Values were normalized to the cell number, quantified by MTT staining (see Materials and Methods).

### 3.2 Preliminary Studies on the ARIAD Transcription Factor

As mentioned in the first paragraph of this section, we would like to use an artificial transcription factor to switch on C/EBPβ transcription in the βΔCRE mouse in a tightly regulated conditional manner. We chose to use the ARGENT™ Regulated Transcription Retrovirus Kit commercialized by ARIAD. The kit provides retroviral vectors encoding a transcription factor that is expressed as two individual peptides: a transactivation domain (TAD) and a DNA binding domain (DBD). The two peptides can reconstitute a sequence-specific transcriptional activator if they are brought together via a non-covalent interaction. This interaction is achieved by a dimerizer, a cell-permeant organic molecule with two separate motifs that each bind with high affinity to a specific protein module. The dimerizer, which is provided with the kit, is a chemically modified derivative of rapamycin, and it is non-toxic to cells or mice if administered below a certain dosage limit. The DNA binding domain of the ARIAD transcription factor recognizes a unique composite DNA sequence called ZFHD, which we inserted in the C/EBPβ promoter in the place of the CRE elements (figure 3.9).

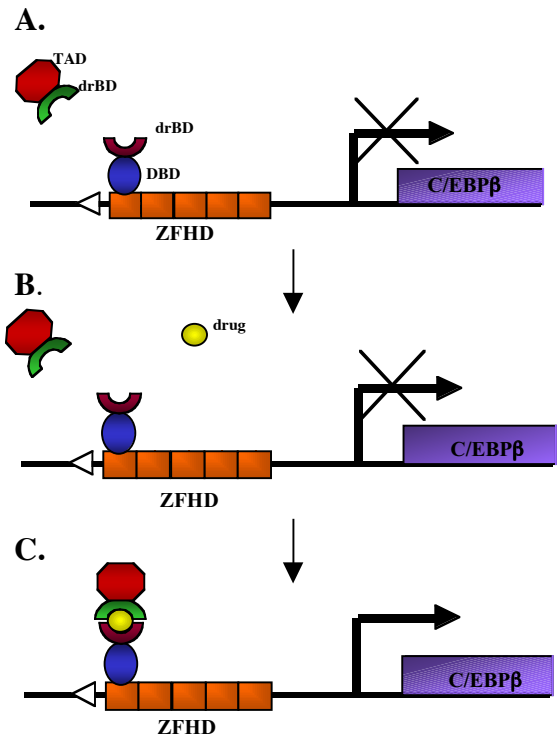



**Figure 3.9: Schematic representation of the ARIAD transcriptional activity.**
A. The ARIAD transcription factor comprises 2 separate peptides. One is a transactivation domain (TAD) and the other a DNA binding domain (DBD). Both peptides also have a drug binding domain (drBD). The DBD recognizes a specific sequence (ZFHD) which has been cloned in the C/EBPβ promoter. B. The two subunits of the ARIAD transcription factor cannot interact unless a drug (or dimerizer) is supplied to the system. C. The drug will bridge the two peptides and the TAD can now activate C/EBPβ transcription.

Our aim was to create a mouse expressing the ARIAD transcription factor in a time and tissue conditional manner. Before proceeding with the cloning of the mouse, we checked whether the system could work *in vitro*, on cells transfected with our βΔCRE targeting vector and an expression vector for the ARIAD transcription factor.

Q2bn cells were transfected with the retroviral vector encoding the ARIAD transcription factor (pL2N2ARIAD), a reporter plasmid with the GFP gene directly downstream of the ZFHD binding site, and the βΔCRE-neo targeting vector (figure 3.1B) or the βΔCRE vector with the *Neo* cassette floxed out (figure 3.1D). The cells were treated with the dimerizer (20nM) for 24h, and then on one hand they were analyzed by FACS to detect GFP expression (figure 3.10A), and on the other protein extracts were analyzed by western blot to detect the expression of C/EBPβ (figure 3.10B).

The green fluorescence, detected only in the sample where the dimerizer had been added, tells us that the system doesn't leak, since the functional activity of the ARIAD transcription factor is entirely dependent on the presence of the dimerizer. The western blot takes us to a similar conclusion, since expression of C/EBPβ is obtained only when the C/EBPβ with the ZFHD in the promoter is transfected together with the ARIAD vector and the dimerizer is present (lanes 9 and 10). Moreover, the western shows that we can induce C/EBPβ protein expression with this system. The *Neo* cassette in the βΔCRE-neo construct does not interfere with C/EBPβ expression because the ZFHD motif is downstream from it. Therefore we can conclude that the *cis* ZFHD motif is necessary and

sufficient for the activation of a gene in a system where the ARIAD transcription factor is expressed and the dimerizer is supplied.

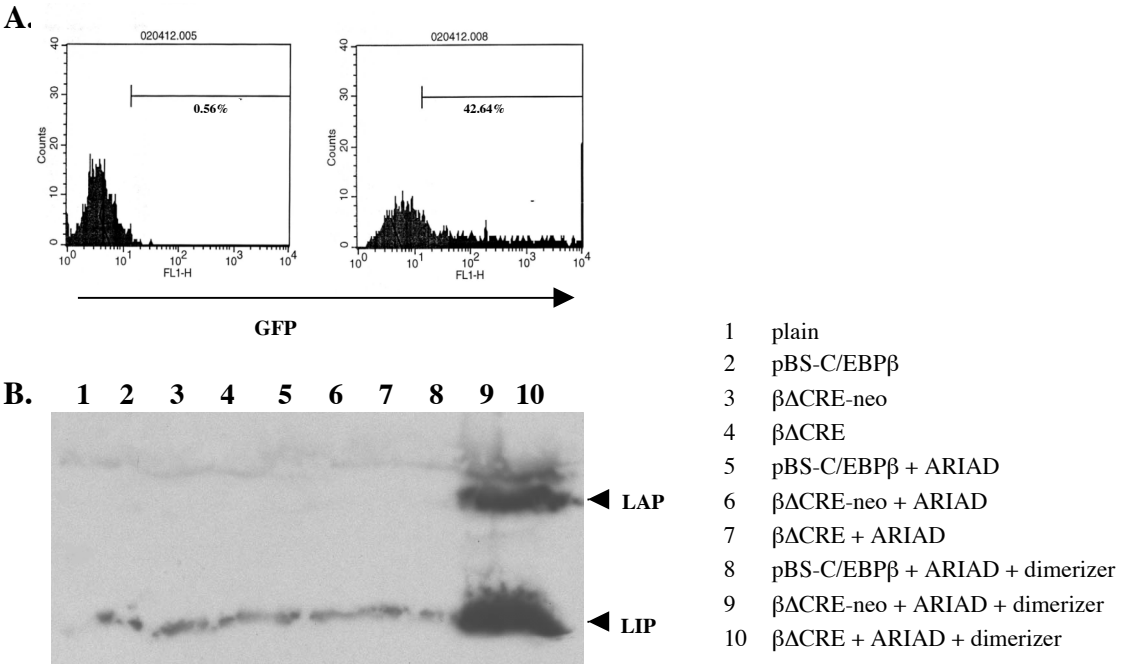



**Figure 3.10:** ***In vitro*** **assay of the ARIAD system.** A. Q2bn cells transfected with pL2N2(ARIAD) and a ZFHD-GFP plasmid not treated (left panel) or treated (right panel) with dimerizer were analyzed by FACS to detect green fluorescence. B. Western blot on transfected Q2bn cells. C/EBPβ (LAP higher band, and LIP lower band) was detected with the Δ198 α-C/EBPβ antibody.

### 3.2.1 Generation of the R26(ARIAD) Knockin Mouse Line

In order to use the ARIAD system in the βΔCRE mouse, we decided to make a mouse line where the ARIAD transcription factor is cloned in the first intron of the Rosa26 (R26) gene. Rosa26 is ubiquitous, and the disruption of its gene does not affect the tissues in any known way (Soriano *et al.*, 1999). The construct provided by ARIAD ensures that the transcription factor cassette is expressed from the retroviral LTR on a bicistronic transcript. The first cistron carries the gene for the transactivating peptide, and the second, separated from the first by an IRES derived from the encephalomyocarditis virus, encodes the gene for the DNA binding domain (pL2N2(ARIAD) in figure 3.11). To have an inducible tissue specific expression of C/EBPβ using the ARIAD system in the mouse, we inserted a stop cassette, in the form of the *Neo* gene followed by its poly(A) tail, between the genes encoding the two individual ARIAD peptides. This way, if Cre recombinase is not expressed in a particular tissue, then only the TAD peptide will be expressed, and alone it will be unable to transactivate the C/EBPβ gene. If, on the other

hand Cre recombinase is expressed in a given tissue, the *Neo* cassette with its stop cassette will be floxed out, and we will obtain equal transcription of both the TAD and DBD peptides. However, transactivation will only be obtained in the presence of the dimerizer.

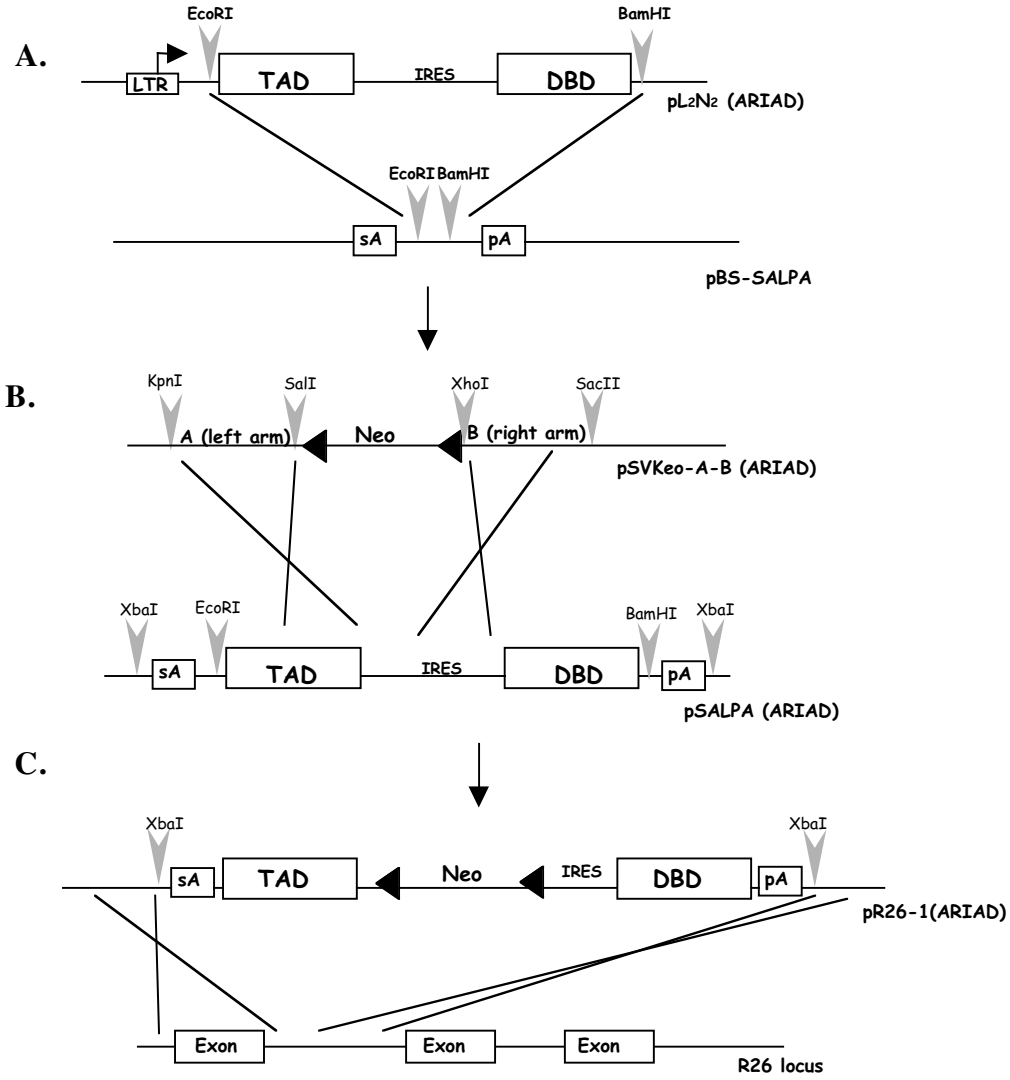



**Figure 3.11: Cloning procedure for the R26(ARIAD) mouse.** A. The ARIAD construct was transferred in pBS-SALPA, which carries R26 splice acceptor (sA) and polyadenylation site (pA). B. ARIAD homology arms were cloned in pSVkeo-X1 flanking the *Neo* cassette. The *Neo* cassette was inserted in the pSALPA(ARIAD) vector between the TAD gene and the IRES sequence by ET recombination. C. The XbaI fragment containing the complete ARIAD cassette was inserted in the R26-1 targeting vector, and used to target the R26 locus.

The pL2N2(ARIAD) construct was transferred into the pBS-SALPA vector, a plasmid which carries a splice acceptor and a polyadenylation site from the original R26 gene separated by a polycloning site (figure 3.11A). The splice acceptor is necessary for the

ARIAD cassette to be correctly spliced from the first intron of the R26 gene. Next, The insertion of the *Neo* cassette between the TAD and DBD genes was performed by ET recombination between the pSVKeo-X1 plasmid, in which homology arms (A and B) were cloned on either side of the *Neo* cassette, and the pSALPA(ARIAD) construct (figure 3.11B). Finally, the whole ARIAD cassette was transferred into the targeting vector for the R26 gene. This vector, called R26-1, has homology arms that enable the insertion of the exogenous cassette in the first intron of the R26 locus (figure 3.11C).

The R26-1(ARIAD) construct was sequenced to make sure that it had been correctly cloned, and it was transformed in the XL-1 Cre bacterial strain, to check that the *Neo* cassette would be floxed out as expected (data not shown). The R26-1(ARIAD) targeting vector was transfected in ES cells, which were put in selection with G418. The clones were screened by Southern blot analysis (see Materials and Methods). Seven heterozygous clones were obtained (figure 3.12), and one was injected in mouse blastocysts.

We obtained germline transmission of the R26(ARIAD) knockin. The mouse line is now in the process of being crossed to several tissue specific Cre lines, and screened, to determine whether the ARIAD system works *in vivo*, as well as it does *in vitro*.

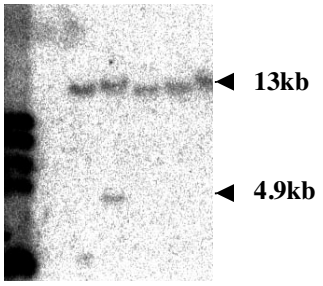



**Figure 3.12: Example of R26(ARIAD) positive clone.** The southern is performed with an EcoRI digestion and the probe is external to the targeting construct. The wild type band is about 13kb in size, whereas the knockin band is around 4.9kb (in the third lane is an example of a heterozygous clone).

### 3.3 Study of C/EBPβ Phosphorylation Mutants

If the sequences of the C/EBPβ genes from different species are aligned, four regions on the sequence stand out as highly conserved. The first two conserved boxes are the conserved regions in the transactivation domain, and at the C-terminus the bZIP domain is notoriously conserved among all the C/EBP family members. The fourth region is placed in the RD2 domain (see Introduction). This serine-rich region carries several phosphorylation sites, some of which are still debated upon (e.g. the serines upstream of Thr188), and others that are by now fully acknowledged (e.g. the Thr188) (figure 3.13). We chose to develop these studies further, with the advantage of using genetically engineered animal models.

```
rLAP      AYLGYQATPSGSSGSLSTSSSSSPPGTPSPADAK
          |||||||||||||||||||||||||||||||||||
mLAP      AYLGYQATPSGSSGSLSTSSSSSPPGTPSPADAK
          ||||||| |||||||||||||||||||||||||||
hLAP      AYLGYQAVPSGSSGSLSTSSSSSPPGTPSPADAK
           ||||| ||||||| |||||||||||| |
cLAP      SYLGYQSVPSGSSGNLSTSSSSSPPGTPNPSESSK

T188A                                  *
3S/A                     *    *    *
```

**Figure 3.13: Sequence alignment of the C/EBPβ RD2 region.** RD2 domains of rat, mouse, human and chicken were aligned. Thr188 (in bold) was mutated to alanine to generate the T188A mutant. The three underlined serines were all mutated to alanine to create the 3S/A mutant.

Thr188 of the mouse C/EBPβ has been shown to be a target for MAPK phosphorylation (Nakajima *et al.*, 1993) and to be involved in skin tumorigenesis (Zhu *et al.*, 2002). However, all the studies on this phosphorylation site were done *in vitro*. Therefore we proposed to gain further insight on the physiological role of Thr188 by making a mutant mouse line, where the threonine was substituted by an alanine. In parallel, we chose to make a mouse model in which serines 176, 180 and 184, that have recently been shown to correspond to GSK3β phosphorylation sites (Zhao *et al.*, 2005; Tang *et al.*, 2005) were mutated to alanine.

#### 3.3.1 Generation of the T188A and 3S/A Mutants

To make point mutations in the RD2 region, we chose the strategy of cloning a DNA linker, with the mutations, inside the C/EBPβ gene. In this region, there are no suitable restriction sites for inserting the linker, so we created two unique restriction sites by

making silent mutations. In particular, nucleotides 511-516, which are normally agc ggc (a serine and a glycine), were changed into tcc gga (always a serine and a glycine), which creates a BspEI restriction site. This mutation was made by amplifying by PCR a fragment of the C/EBPβ gene spanning from the start site to the mutated nucleotides, using an antisense oligonucleotide that carried the mutation. In a similar fashion, nucleotides ccc ggc (557-562)(a proline and a glycine) were changed into ccc ggg, which creates a XmaI restriction site without altering the amino acidic sequence. In this case a mutated sense oligo was used to amplify a fragment from the point of mutation to the C/EBPβ stop codon. To make the 3S/A mutant, a DNA linker (created by annealing sense and antisense oligos) with the 3S/A mutations was ligated between the BspEI and XmaI restriction sites. In the case of the T188A mutant, the point mutation was made directly on the same oligo that was used to create the XmaI site, and a wild type linker was cloned between the BspEI and XmaI restriction sites (figure 3.14).

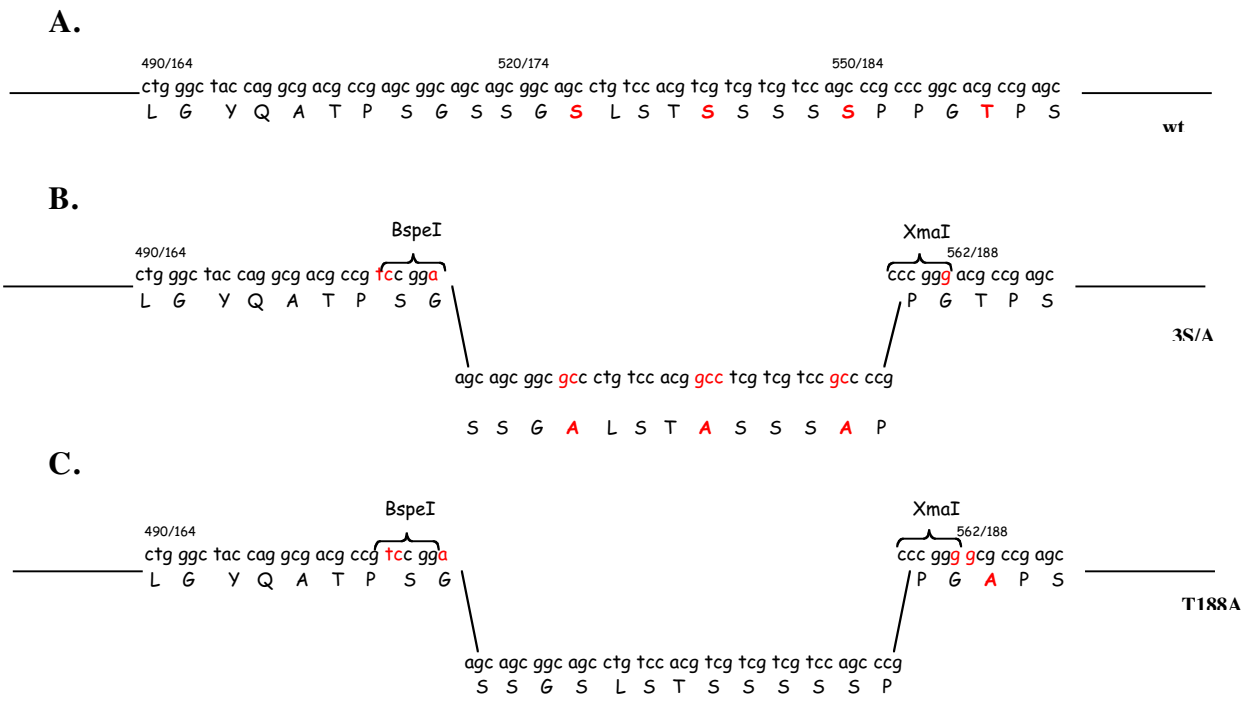



**Figure 3.14: Schematic representation of the point mutations made on the C/EBPβ gene.** A. Close-up of the RD2 domain on the wt C/EBPβ. The amino acids we want to mutate are highlighted in red. B. Insertion of the 3S/A linker between the BspEI and XmaI artificially created sites. C. Insertion of a wt linker between BspEI and XmaI. The T188A point mutation was created together with the XmaI restriction site.

The mutated clones were checked by sequencing and by restriction mapping (data not shown).

### 3.3.2 Anderson on the 3S/A and T188A Phosphorylation Mutants

Before proceeding to make a mouse, we analyzed the phosphorylation mutants *in vitro*, in the attempt to find a model system that could help us answer some basic questions on the nature of these sites.

In 1999, Ross *et al*. demonstrated that C/EBPα is a target for GSK3β phosphorylation. This was done by treating 293T cells with lithium, a natural inhibitor of GSK3β. The region in which C/EBPα is phosphorylated, just upstream from the bZIP domain, corresponds to where our three serines are located in the C/EBPβ gene. Moreover, GSK3β is known to phosphorylate serines or threonines that are positioned four amino acids upstream from another serine or threonine, and this is the pattern in which C/EBPβ serines 176, 180 and 184 fall into.

The 3S/A and T188A C/EBPβ mutants were cloned into the pBabePuro retroviral vector, which was used to infect NIH3T3 mouse fibroblasts. The cells were treated with LiCl following the protocol in Ross *et al*., and the protein extracts were separated on an Anderson protein gel, a SDS-free acrylamide gel in which proteins migrate according to their charge rather than their molecular weight (figure 3.15).

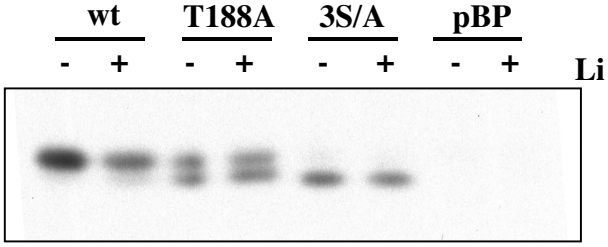



**Figure 3.15: Anderson on C/EBPβ phosphorylation mutants.** NIH3T3 cells infected with wt C/EBPβ, T188A, 3S/A or empty vector (pBP), were treated (+) or not treated (-) with 25μM LiCl for 48h. Cell lysates were run on an Anderson gel.

There is a clear shift in C/EBPβ migration between wt and 3S/A, confirming our hypothesis of there being one or more phosphorylations on the three mutated serines. The T188A mutant migrates as a duplet. The protein is seemingly present in a double, phosphorylated and unphosphorylated, state. What we expected from this experiment was to see a shift in the migration of the wild type C/EBPβ treated with lithium, similar to the shift of the 3S/A mutant. This does not seem to be the case, although there is a very faint band in the wt + Li sample at the right level. Considering the recent publications that showed, although somewhat controversially, that some of these serines are GSK3β targets (Zhao *et al*., 2005; Tang *et al*., 2005), it is probable that our experiment wasn't stringent enough. Possibly, NIH3T3 fibroblasts are less sensitive to lithium than the 293T cells used in the work by Ross and collaborators. However, this Anderson gel gave us interesting insights on the possible mechanism of phosphorylation, which will be discussed further on in this thesis (see Discussion).

### 3.3.3 *In Vitro* Functional Assays on the C/EBPβ Phosphorylation Mutants

In the attempt to find a functional role for the 3S and T188 phosphorylation sites, we screened our mutants for their capability to carry out some of the known functions of the C/EBPβ transcription factor.

In the first place we asked whether our mutants were capable of transactivating a reporter gene. Past findings, in fact, suggested that the RD2 domain is an important region, involved in regulating C/EBPβ transactivational activity (Kowenz-Leutz *et al*., 1994). We performed luciferase reporter assays using *mim-1*, a known target of C/EBPβ transcription, as reporter gene. Q2bn quail fibroblasts were transfected with wild type C/EBPβ, 3S/A or T188A expression vectors along with the luciferase reporter, normalizing with β-gal (figure 3.16A). The experiment was repeated in a similar way in NIH3T3 mouse fibroblasts using the Dual Luciferase Assay kit (Invitrogen) (figure 3.16B)).

**A.** **B.**

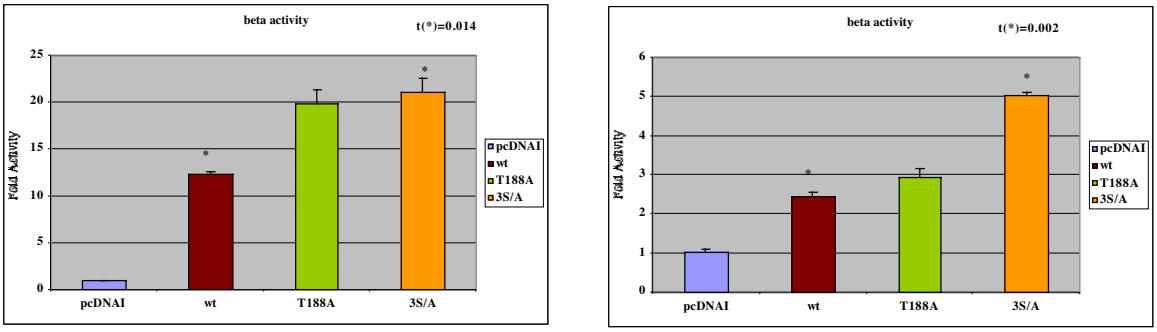



**Figure 3.16: Luciferase reporter assays on C/EBPβ and the phosphorylation mutants.** The *mim1* promoter was used for the reporter gene. A. Q2bn cells transfected with empty vector, wild type C/EBPβ or the mutants were tested for luciferase and normalized with β-gal. B. NIH3T3 cells transfected with empty vector, wild type C/EBPβ or the mutants were tested with the Dual Luciferase Assay Kit. n=2.

It is evident that the mutations in the phosphorylation sites do not impair C/EBPβ's transactivational activity. On the contrary, it appears that the activity is enhanced. This result is not consistent for the T188A mutant, but it is significant in both assays for the 3S/A mutant.

Next, we asked whether the phosphorylation mutants were capable of inducing NIH3T3 differentiation into adipocytes when treated with an appropriate hormonal cocktail. In fact, it is known that C/EBPβ overexpression in NIH3T3 cells triggers adipogenesis when a cocktail composed of insulin, IBMX (isobuthylmethylxanthine), and

dexamethasone is added to the culture medium (Wu *et al*., 1995). NIH3T3 fibroblasts infected with pBP, wild type C/EBPβ, T188A and 3S/A retroviral vectors, were treated with the above described differentiation medium for two days, and then shifted to normal growth medium. After three additional days in culture, all the cells expressing C/EBPβ, whether wild type or mutated, had differentiated into adipocytes, whereas the pBP control sample did not (figure 3.17). Adipocytes were stained with Oil Red O, a lipid-specific dye, to visualize fully differentiated cells. The dye was then extracted from the cells with isopropanol and absorbance was measured to quantify adipogenesis. As expected, there was no significant difference between the mutants and the wild type control (not shown). While trying to make the experimental conditions more stringent, we observed that differentiation could be induced with equal success if the cells were treated with dexamethasone alone, as long as C/EBPβ was overexpressed. However, also in this case there was no difference in the differentiation capacity between the wild type C/EBPβ and the phosphorylation mutants (data not shown).

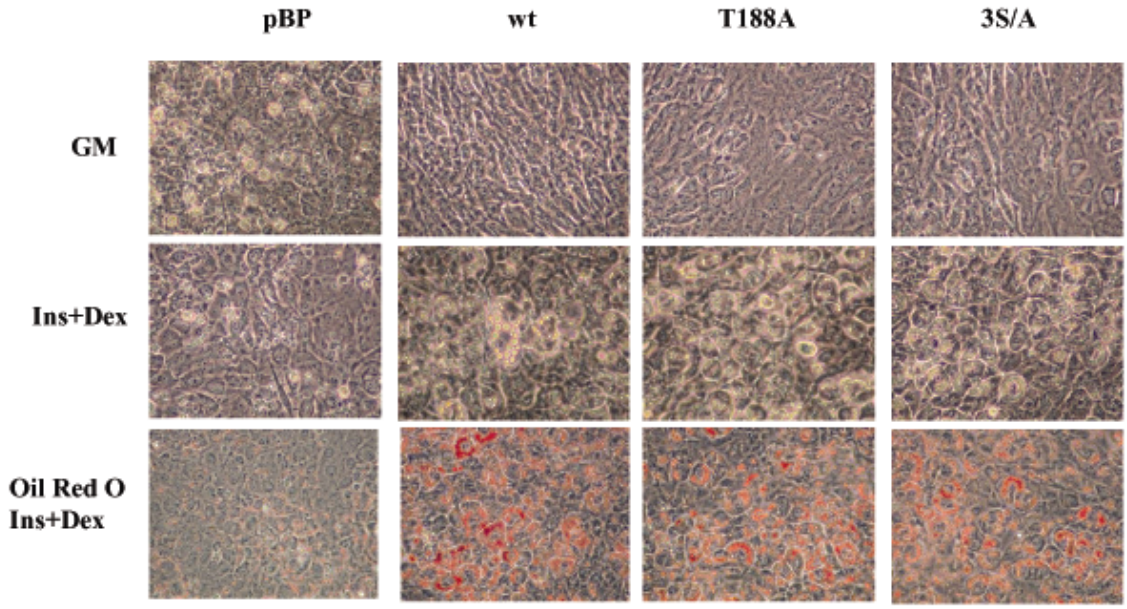



**Figure 3.17: Differentiation assay on NIH3T3 fibroblasts.** Cells were infected with empty vector (pBP), wild type C/EBPβ (wt) and mutant clones (T188A and 3S/A). Cells were grown in normal growth medium (GM, first row), or in adipocyte differentiation medium (Ins+Dex, second row). In the bottom row, differentiated cells from row 2 were stained with Oil Red O.

Finally, we asked whether the phosphorylation sites on the RD2 domain could be important for mediating protein-protein interaction between C/EBPβ and coactivators of C/EBPβ transcription. As a working model, we took the interaction between C/EBPβ and Cyclin D1. Indeed, C/EBPβ was shown to be involved in the regulation of genes affected

by oncogenic cyclin D1 overexpression (Lamb *et al*., 2003). In their work, Lamb and coworkers show that cyclin D1 and C/EBPβ physically interact, so we tried to repeat the experiment with our phosphorylation mutants (figure 3.18).

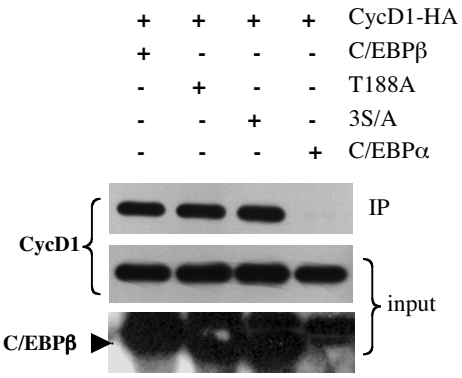



**Figure 3.18: Physical interaction between cyclin D1 and the C/EBPβ phosphorylation mutants.** HA-tagged cyclin D1 was transfected in 293T cells in combination with C/EBPβ, T188A, 3S/A and C/EBPα (as a control) expression vectors. Whole cell lysates were immunoprecipitated with an antibody against C/EBPβ and the Western blot was probed with anti-HA and anti-C/EBPβ antibody. A non-specific band is recognized just above C/EBPβ.

Once again, the phosphorylation mutants seem to be perfectly functional *in vitro*.

In conclusion, we can say that the T188 and the 3S phosphorylation sites are involved in modulating the transactivational activity, although this doesn't seem to influence C/EBPβ's role in inducing adipogenesis. Moreover, the phosphorylation sites do not seem to be involved in mediating protein-protein interaction (at least not in the specific interaction with cyclin D1). These findings seem to exclude a role of the RD2 domain in enabling configurational changes that can either permit or block the transcription factor's functional activity.

As often happens for subtle mutations, the best way to find the functional role of the 3S and T188 phosphorylation sites is to study them in physiological conditions, and therefore to make a mouse model out of the 3S/A and T188A mutants.

### 3.3.4 Generation of the 3S/A and T188A Mouse Lines

The C/EBPβ phosphorylation mutants were cloned in the NS-RI vector (courtesy of A. Leutz), which is a plasmid that carries all of the coding sequence of C/EBPβ plus part of the promoter and the 3' utr. About ten nucleotides upstream of the start codon, this plasmid has an ectopic EcoRI restriction site, which is particularly useful for genotyping. The point mutations were cloned into NS-RI by substituting the BstBI-PstI fragment of the wild type C/EBPβ with the same fragment from the phosphorylation mutants (figure 3.19A). Next, the whole C/EBPβ cassette from NS-RI was transferred into the pTV-flox targeting vector (also courtesy of A. Leutz) with NotI and SalI (figure 3.19B). The pTV-flox plasmid encodes all of the C/EBPβ genomic clone, with a floxed *Neo* cassette inserted in the 3' utr of the C/EBPβ gene. Moreover, pTV-flox has a thimidine kinase (*TK*) cassette, which enables an additional negative selection strategy on the ES cells (by

administration of gancyclovir) if the selection by neomycin resistance isn't stringent enough.

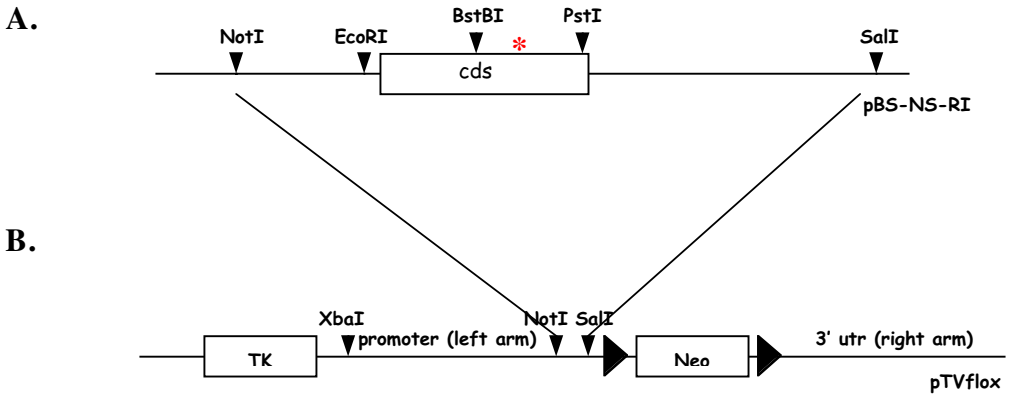



**Figure 3.19: Schematic representation of 3S/A and T188A cloning procedure.** A. Point mutations were inserted in the NS-RI plasmid by exchanging the BstBI-PstI fragment from this plasmid with the one from the previously described mutants (figure 3.14). The red asterisk indicates point mutations, whether 3S/A or T188A. B. The NotI-SalI fragment from NS-RI was then cloned into pTV-flox.

The pTV-flox(3S/A) and pTVflox(T188A) targeting constructs were transfected into ES cells and clones were selected by resistance to G418. About 200 clones were picked for each construct and screened by Southern using the same EcoRI-XbaI probe used for the βΔCRE ES cells, which is external to the targeting construct, on an EcoRI digestion. The mutant gives a band of 2.9kb, thanks to the presence of the ectopic EcoRI site just before the C/EBPβ start site, whereas the wild type is around 4.9kb long. We obtained one positive clone for the 3S/A mutant, and four for the T188A. These clones were double checked with a different Southern strategy. The ES cell DNA was digested with XbaI and BspEI, and screened with a SphI-XmaI probe, internal to the C/EBPβ gene. The mutants are characterized by a 2.8kb band, due to the ectopic BspEI restriction site, which was put in the C/EBPβ coding sequence by silent mutation to make the point mutations in the first place (figure 3.14). The wild types, instead, give a 3.5kb band. In figure 3.20 are examples of the two Southern strategies.

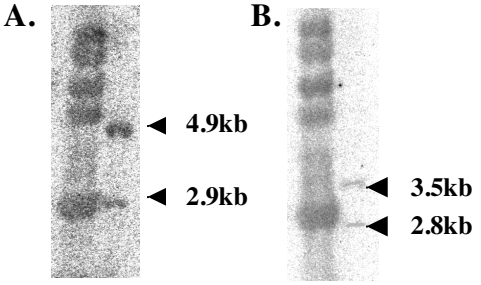



**Figure 3.20: Southern analysis on 3S/A and T188A clones.** A. Example of a positive clone screened by an EcoRI digestion and the EcoRI-XbaI external probe. B. Example of a positive clone counterscreened by a BspEI-XbaI digestion and the SphI-XmaI internal probe.

A positive ES cell clone for each construct was injected in mouse blastocysts. Germline transmission was obtained for both mutants, which are now established mouse lines. The *Neo* cassette was excised from the 3'utr of the C/EBPβ gene by crossing the lines with a deleter-Cre mouse.

Both mouse lines were analyzed for female fertility. Fertility tests were set up in a similar way to the one described for the βΔCRE line. Homozygous females were fertile in both mutants (data not shown).

### 3.3.5 T188A and 3S/A Protein Expression and Migration in Animal Tissues

Protein extracts were made from several different tissues from the T188A and 3S/A mice. In the first place, we checked how the endogenous mutant protein would migrate on an Anderson gel (figure 3.21).

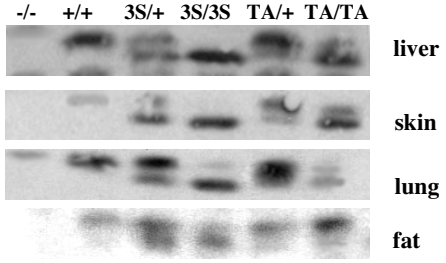



**Figure 3.21: Anderson Western blot on phosphorylation mutant tissues.** Whole cell extracts were made out of the indicated tissues, and run on an Anderson gel. The blot was probed with an anti-C/EBPβ antibody.

The pattern of migration of the phosphorylation mutants is similar to what we obtained *in vitro* (see figure 3.15). In addition, we now have heterozygous samples, which are useful to compare the relative quantities of wild type and mutant protein. For example, in the 3S/+ sample of the lung, and possibly the fat, the wild type allele of C/EBPβ seems to be more than the mutant allele. This could be explained by a relative instability of the 3S/A protein in these tissues. All the TA/+ samples show a stronger band corresponding to the wild type allele. This is not surprising, since we know that T188A migrates as a duplet, and that one band in the duplet migrates at the same height as the wild type C/EBPβ, thereby enriching that particular band. However, it is interesting to note that in all the

TA/TA tissues except the fat, the band corresponding to the unphosphorylated form of the protein is more abundant than the phosphorylated. It is possible that in the fat C/EBPβ phosphorylation is regulated in a different way in comparison to other tissues.

To analyze the T188A tissues, we had the additional advantage of the existence of an antibody specific for the phosphorylated Thr188. Liver tissue extracts were run on a traditional SDS-PAGE, and the western was probed with the anti-phosphoT188-C/EBPβ antibody (figure 3.22).

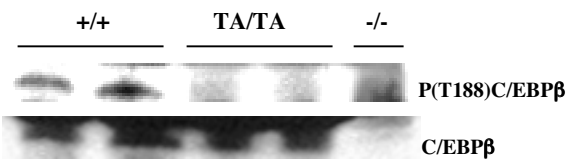



**Figure 3.22: Detection of T188-phosphorylated C/EBPβ on liver extracts.** Western blot was done using α-P(T188) and α-C/EBPβ antibody.

C/EBPβ phosphorylated on Thr 188 is clearly missing in the TA/TA samples, whereas the anti-C/EBPβ antibody shows that the protein is there.

### 3.3.6 Study of the Phosphorylation Mutants in Macrophages

Since the βΔCRE mice gave interesting results on the role of C/EBPβ in the macrophages, we were curious to know whether phosphorylation on the RD2 domain was important in regulating the transcription factor's activity in these cells.

Primary bone marrow-derived macrophages were treated overnight with IFNγ and then with IFNγ/LPS for four hours. cDNA was made from total RNA extracts, and the samples were then analyzed by RT-PCR to see whether there was an anomaly in the expression of the C/EBPβ IFNγ/LPS-dependent target genes (i.e. arginase 1, Msr1, IL13α1). In the T188A macrophages the expression of the C/EBPβ target genes, as well as that of C/EBPβ itself was similar in wild types and TA/TA samples. All these genes were regularly induced in response to IFNγ/LPS treatment (data not shown). Surprisingly, in the 3S/A macrophages there was a misregulation of C/EBPβ as well as some of its target genes, but not an inhibition of induction in the LPS-treated samples, as was the case for the DC/DC mice, but rather an excessive upregulation (figure 3.23). This was the case for the expression of C/EBPβ, arginase 1 and IL13α1, but not for Msr1 (not shown). These results suggest an autoregulatory role for the three serines in the RD2 domain. In particular, the phosphorylation on these residues should enhance the inhibitory effect of C/EBPβ on its own transcription. The absence of these phosphorylation sites is sufficient to abrogate the autoregulational capability of the transcription factor. The overexpression of some target genes of C/EBPβ obtained with the IFNγ/LPS-treated 3S/3S sample can either be a direct consequence of the

overexpression of the mutated C/EBPβ, or it can be explained by a higher transactivational potential of this mutant.

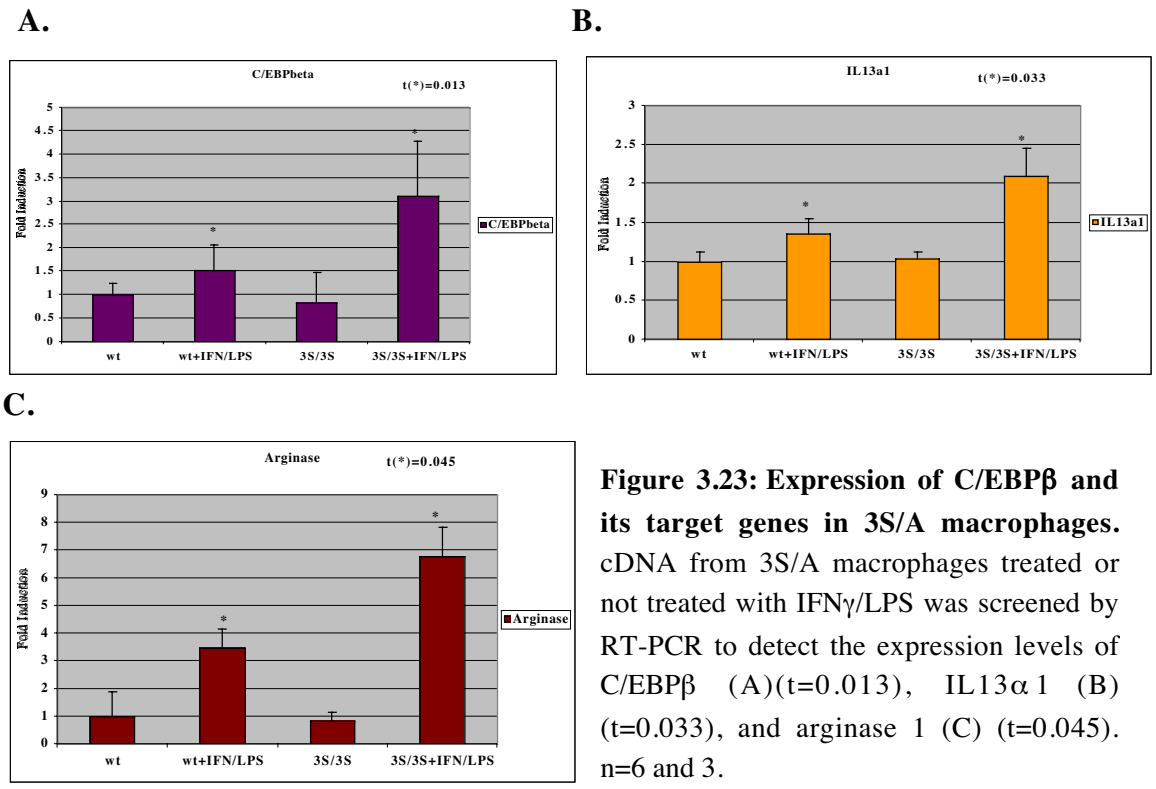



**Figure 3.23: Expression of C/EBPβ and its target genes in 3S/A macrophages.** cDNA from 3S/A macrophages treated or not treated with IFNγ/LPS was screened by RT-PCR to detect the expression levels of C/EBPβ (A)(t=0.013), IL13α1 (B) (t=0.033), and arginase 1 (C) (t=0.045). n=6 and 3.

Since we have shown that there is a strong over-induction of C/EBPβ in the LPS-treated 3S/3S macrophages at the transcriptional level, we were interested to see what happened at the protein expression level. 3S/A macrophages were obtained from bone marrow as usual, and were treated with IFNγ/LPS for four hours. Whole cell extracts were separated on an Anderson gel and the blot was probed for C/EBPβ (figure 3.24). In this experiment, we can see that IFNγ/LPS treatment does not change the phosphorylation of the wild type C/EBPβ, and the upregulation is probably not strong enough to be detected by Western. However, it is clear that the expression level of the IFNγ/LPS-treated 3S/3S and 3S/+ protein is higher in comparison to the unstimulated counterpart. These results further support the theory that serines 176, 180 and 184 are involved in the C/EBPβ autoregulatory loop.

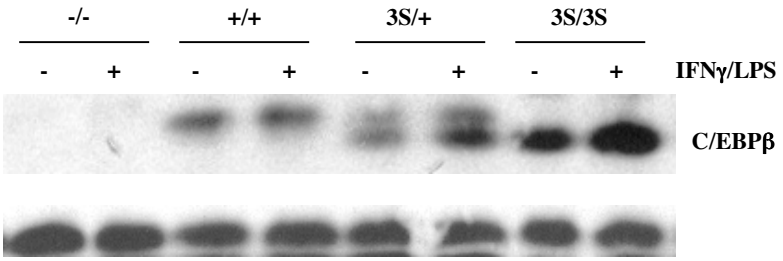



**Figure 3.24: Anderson on IFNγ/LPS-treated 3S/A macrophages.** 3S/A IFNγ/LPS-treated or not treated macrophage whole cell extracts were run on an Anderson gel. The blot was probed with an anti-C/EBPβ antibody. In the bottom panel is a non-specific protein recognized by the same antibody, which can be used for normalization.

# 4. DISCUSSION

### 4.1.1 CREB is a Direct Activator of C/EBPβ Gene Transcription in Macrophages

In the first tissues where we measured C/EBPβ expression levels (liver, brain and fat; figure 3.2), we never obtained an inhibition of C/EBPβ expression due to the absence of the CRE elements. This does not necessarily mean that CREB is not required at all for C/EBPβ transcription in these tissues, but simply that it is not necessary for basal transcription, whereas modified environments, such as activated states should be further investigated. Although CREB has been shown to regulate C/EBPβ transcription in the fat (Zhang *et al*., 2003), βΔCRE mice do not show an aberrant phenotype in the adipose tissue, as neither do C/EBPβ knock out mice unless C/EBPβ is depleted in combination with C/EBPδ (Tanaka *et al*., 1997). Indeed, C/EBP family members can often be redundant with one another, taking over the missing member's function in its absence. In Nature this ability can have a protective role, but in science it can make it particularly difficult to dissect the function of one specific C/EBP family member in a given tissue.

In macrophages, C/EBPβ is the predominant C/EBP isoform (Natsuka *et al*., 1992). Very low levels of C/EBPδ are expressed, and are probably insufficient to make up for a deficiency in C/EBPβ. For this reason we chose to work on primary macrophages to investigate on the role of CREB in regulating C/EBPβ gene transcription.

The first proof that CREB can physically interact with the C/EBPβ promoter comes from the work of Zhang *et al*. (2003). In this work a chromatin immunoprecipitation (ChIP) was performed on 3T3-L1 preadipocytes and MEFs (mouse embryo fibroblasts) showing that CREB is found on the C/EBPβ promoter at all times, but phosphorylated (and thus active) CREB is found only after adipogenesis is stimulated in these cells. In macrophages, past publications have shown a correlation between CREB phosphorylation and C/EBPβ activation dependent on LPS stimulation (Bradley *et al*., 2003), but these observations were never counter-proved by demonstrating the interaction between transcription factor and promoter *in vivo*. We did this by performing a ChIP on the J774 macrophage cell line, to detect the presence of CREB on the C/EBPβ promoter in conditions of macrophage activation by LPS (figure 3.4). Our findings show that CREB is not on the promoter prior to LPS-mediated activation, as opposed to what is observed in preadipocytes or MEFs, supporting the notion that the system of a gene's regulation is

very specific to the cell type. Moreover, the physical presence of CREB on the C/EBPβ promoter has been circumscribed to a 140bp DNA stretch that includes the CRE elements, giving us sufficient confidence that the βΔCRE mutant will abrogate CREB-mediated regulation of the C/EBPβ promoter.

Primary macrophages are a slightly more complex system than a macrophage cell line. A strong variability from mouse to mouse can be a drawback to devising reproducible experiments. For this reason we spent time on finding the best conditions for activating the macrophages. In our experimental model LPS could upregulate C/EBPβ expression, but the fold regulation was random from one mouse to the other. We found that priming the macrophages with IFNγ enhanced the phenotype and reduced variability significantly. Consequently, except where specified, the results reported are obtained by the combined effect of LPS and IFNγ activation.

RT-PCR analyses showed that C/EBPβ cannot be upregulated in response to IFNγ/LPS-stimulation in βΔCRE DC/DC macrophages (figure 3.6). This means that C/EBPβ induction during macrophage activation is dependent on CREB and its ability to drive C/EBPβ transcription. However, since we partially deleted the C/EBP binding site that overlaps with one of the CRE elements, we cannot exclude that the upregulation of the C/EBPβ gene in response to IFNγ/LPS is also C/EBPβ-dependent.

### 4.1.2 Novel Targets for C/EBPβ Transcription in Macrophages: Msr1

We took advantage of our experimental model with the βΔCRE macrophages to find new downstream targets of C/EBPβ transcription in activated macrophages. The results of the affymetrix were vast and must be analyzed further, however we tried to select the genes that appeared most interesting and inherent to the macrophage (table 3.1). Through RT-PCR analysis, we identified three genes that are C/EBPβ-dependent, and these are Msr1, arginase-1, and IL13α1 (figure 3.7).

Msr1 is the murine homologue of SR-A (the class A human scavenger receptor). Scavenger receptors are trimeric transmembrane glycoproteins subdivided in six domains: the N-terminal cytoplasmic, spacer, α-helical coiled-coil, collagenous and C-terminal domains (Ashkenas *et al.*, 1993). The ligand binding region is in the positively charged collagenous domain of Msr1. Scavenger receptors were originally defined by their ability to recognize modified (i.e. oxidized or acetylated) forms of low density lipoprotein (LDL), and therefore their implication in atherogenesis (see Introduction) (Brown and Goldstein, 1983). Msr1 deficient mice crossed to ApoE knockouts (which have high plasma cholesterol and develop atherosclerotic lesions) have higher levels of plasma cholesterol than controls and still develop atherosclerotic lesions, although smaller than those of single ApoE knockouts, consisting mainly of foamy macrophages

(Suzuki *et al*., 1997). The presence of atherosclerotic lesions and foamy macrophages in the double knockout mice suggests that other scavenger receptors may also participate.

Liver sinusoidal Kupffer cells express Msr1, and they are believed to protect the body by scavenging cholesterol and atherogenic particles from the blood compartment, thereby reducing the accumulation of modified lipoproteins in the interstitial space of the vessel wall (Van Berkel *et al*., 1991).

However, scavenger receptors are also able to bind a broad range of polyanionic ligands, including LPS and lipotechoic acid (LTA), and they have been implicated in host defense against bacterial infections (Krieger, 1997). Msr1(-/-) mice are more susceptible to *Listeria Monocytogenes* infection than wild type controls (Suzuki *et al*., 1997). In general, Msr1 can mediate binding and ingestion of a range of microorganisms (Peiser *et al*., 2000).

To further support the role of C/EBPβ in regulating Msr1, we would like to assay the foam cell formation *in vitro*, on βΔCRE macrophages. This can be done by culturing the primary macrophages in the presence of modified LDL, and measuring the lipid intake of the macrophages by Oil Red-O staining. Moreover, we are in the process of screening the βΔCRE mice for susceptibility to *Listeria Monocytogenes* infection, which could be, at least partly, attributed to the incapacity of upregulating Msr1.

### 4.1.3 Novel Targets for C/EBPβ Transcription in Macrophages: Arginase 1 and IL13α1

As mentioned in the Introduction, arginase is an enzyme that converts L-arginine to L-ornithine and urea. More commonly, in macrophages L-arginine is used by inducible nitric oxide synthase (iNOS) to synthesize NO, a crucial host-protective, antimicrobial effector molecule, as well as a potential host-destructive mediator in diverse settings of immunopathology (Kröncke *et al*., 1995). Amphibians and mammals express two isoforms of arginase, called 1 and 2. Both isoforms catalyze the same reaction, but they are encoded by different genes and differ with respect to cellular distribution and mode of regulation (Jenkinson *et al*., 1996). Arginase 1 is a cytosolic enzyme, expressed almost exclusively in the liver, and arginase 2 is a mitochondrial enzyme with widespread tissue distribution. In murine macrophages, the arginase 1 isoform is upregulated in the context of a Th2 immune response, whereas arginase 2 is constitutively expressed (Munder *et al*., 1999). On one hand arginase participates in the regulation of NO synthesis by competing for the common substrate L-arginine (Modolell *et al*., 1995). On the other, it is involved in fibrogenic and reparative processes, via collagen synthesis, or antiinflammatory actions, via production of polyamines, a byproduct of ornithine (Jenkinson *et al*., 1996).

That C/EBPβ can regulate arginase 1 transcription had already been demonstrated *in vitro* (Pauleau *et al*., 2004; Chowdhury et al., 1996) and suggested *in vivo* (Sonoki *et al*.,

1997). ChIP experiments, performed on the RAW macrophage cell line, detected C/EBPβ, STAT6 and the coactivator CBP (CREB binding protein) on the arginase enhancer following IL4 treatment (Pauleau *et al.*, 2004).

In our experiments, we find an impairment of arginase 1 upregulation in the DC/DC primary macrophages in response to IFNγ/LPS treatment, which correlates with the impaired induction of C/EBPβ in the same samples. This not only shows that arginase 1 gene expression is C/EBPβ-dependent *in vivo*, but we also demonstrate that the activation initiates through CREB signaling. Since arginase competes with iNOS for the same substrate, it can be expected that cells in which arginase is downregulated display an enhanced NO production. This seems to be the case regarding the NO release of βΔCRE macrophages. When DC/DC macrophages are stimulated with LPS alone, they produce significantly more NO than the wild type cells (figure 3.8). This is not the case when we treat the cells with LPS and IFNγ, possibly because the signal to produce NO is much stronger in these conditions, and the substrate has not yet been totally sequestered by arginase.

When NO production studies were performed on macrophages from C/EBPβ knockout mice, peritoneal macrophages were comparable to the wild type controls in producing NO upon IFNγ/LPS stimulation (Tanaka *et al.*, 1995), but splenic macrophages from C/EBPβ (-/-) mice previously infected with *Candida albicans* displayed a severe impairment in NO production (Screpanti *et al.*, 1995). In neither case were the macrophages stimulated with LPS alone, but the NO release of the peritoneal macrophages treated with IFNγ and LPS is comparable to our own results. The experimental conditions used by Screpanti *et al.*, instead, are not comparable with ours.

Arginase 1 was also shown to be responsive to IL13 signaling (Munder *et al.*, 1999; Pauleau *et al.*, 2004), and the receptor for IL13 is another gene that we found to be regulated by C/EBPβ.

Interleukin 13 (IL13) is an immunoregulatory cytokine secreted predominantly by activated Th2 cells (Minty *et al.*, 1993). IL4 and IL13 are related cytokines that belong to the same α-helix superfamily, and they share many functional properties. IL13 mediates its effects via a complex receptor system that includes IL4Rα (IL4 receptor α chain) and at least two other cell surface proteins, IL13Rα1 and IL13Rα2. Both IL13Rα1 and IL13Rα2 are members of the hematopoietin receptor superfamily (Miloux *et al.*, 1997). IL13Rα1 binds IL13 with low affinity by itself, but, when paired with IL4Rα, it binds IL13 with high affinity and forms a functional IL13 receptor capable of signaling. Consistent with the fact that IL4 and IL13 share common subunits, they also share common signaling pathways. Studies in STAT6-deficient mice have revealed that IL13 signaling utilizes the JAK/STAT pathway and specifically STAT6 (Takeda *et al.*, 1996)). Signaling through IL4Rα/IL13Rα1 is thought to occur via IL4Rα, because both IL4 and

IL13 stimulation of the complex results in activation of signaling intermediates characteristic of IL4 responses, including phosphorylation of IL4Rα, insulin-receptor substrate-2, JAK1, and Tyk2 (Welham *et al.*, 1995). Although IL4 and IL13 have many overlapping functions, they also have distinct roles. In parasitic infection models, IL13 has a critical role in the Th2-dependent expulsion of *Nippostrongyloides brasiliensis* (Urban *et al.*, 1998). Furthermore, IL13 has recently been shown to be a key mediator of allergic inflammation independent of IL4 in mouse models whereby IL13 blockade prevented allergen-induced airway inflammation (Grunig *et al.*, 1998).

As was the case for arginase 1, we found a correlation between the impaired C/EBPβ upregulation in the DC/DC+LPS/IFNγ macrophages and the impaired IL13α1 upregulation in the same conditions. This shows that the upregulation of IL13α1 is C/EBPβ-dependent. An interpretation of this result is that C/EBPβ has a role in making the macrophage responsive to IL13 signaling, and that DC/DC macrophages are consequently unresponsive to such stimulation. This possibility is further discussed below.

### 4.1.4 C/EBPβ: a Molecular Switch from M1 to M2 Macrophages?

As was described in the Introduction, macrophages can elicit two opposing immune responses, depending on the stimuli that they receive. Macrophages induced in Th1-dominated immune responses, secrete multiple inflammatory mediators (e.g. IL1, IL6 and TNFα) and are termed inflammatory macrophages or M1 macrophages. M1 macrophages possess cytotoxic and antimicrobial effector functions based on their ability to produce NO (MacMicking *et al.*, 1997). On the contrary, M2 macrophages are elicited by the Th2 immune response and display antiinflammatory properties. M2 macrophages produce arginase 1, which, as mentioned above, will generate urea and polyamines, which nourish cell growth and promote tissue repair (Jenkinson *et al.*, 1996).

Macrophages are capable of bridging the innate to the acquired immune response through antigen presentation and the release of cytokines (Unanue and Allen 1987). Several studies have described an innate response that leads to expansion of suppressor macrophage populations (Angulo *et al.*, 1995; Atochina *et al.*, 2001). These immunoregulatory cells, termed natural suppressor (NS) cells, originate from granulocyte-monocyte progenitors, express Gr1, Mac1 and F4/80 surface markers, and are capable of inhibiting proliferative responses of naïve or activated T cells. There are two different subpopulations of NS macrophages. Classically activated macrophages are IFNγ-dependent, and are found in the bone marrow and peripheral lymphoid organs in cancer patients and during viral infections (Young *et al.*, 1996; Cauley *et al.*, 2000). Alternatively activated macrophages are IL4-dependent, and are found in the peritoneal

cavity of mice in response to the filarial nematode *Brugia malayi* (Goerdt and Orfanos, 1999).

In our experiments, FACS analyses have shown that our macrophage population expresses Mac1 and F4/80, which are the traditional macrophage markers, but also, to a significant extent, Gr1 (figure 3.5). The expression of Gr1 either accounts for the presence of granulocytes in our culture (an unlikely event considering the culture conditions), or suggests the presence of NS macrophages. Moreover, the percentage of Gr1 expression (43%) and that of the two macrophage-specific markers (82% and 57%), suggests an overlap of at least one of the macrophage markers with Gr1 in these cells.

The fact that C/EBPβ was found in our experiments to regulate the expression of arginase 1 and IL13α1 receptor subunit, leads us to the tempting supposition that the transcription factor might activate a molecular mechanism that enables the macrophages to switch from the M1 to the M2 type. Indeed, arginase 1 is a typical agonist of the Th2-driven response, and IL13α1 would allow the cell to be responsive to IL13, which is notoriously a cytokine produced by Th2 cells to drive an M2 antiinflammatory response. In addition, Atochina and coworkers (2001) have shown that the antiproliferative effect of NS cells on CD4(+) T cells was dependent on the production of IFNγ and NO. In our experiments, C/EBPβ-driven arginase and IL13α1 expression is dependent on IFNγ/LPS stimulation, and we have also shown that in these conditions the cells are able to produce NO (figure 3.8).

Studies on C/EBPβ knockout mice have shown that with age these mice develop skin lesions, swelling in the mucosal regions and splenomegaly (Screpanti *et al.*, 1995). These lesions are more dramatic in mice exposed to pathogens than in mice raised in SPF conditions. Moreover, these same mice display high levels of IL6, a typical inflammatory cytokine, in the blood serum compared to their littermate controls. This scenario suggests a pronounced inflammatory immune response typical of M1 macrophages. In the light of our experiments, this feature could be interpreted as an underrepresentation of antiinflammatory M2 macrophages due to the absence of C/EBPβ.

### 4.1.5 A Broader View and Future Perspectives

One can't ignore the fact that C/EBPβ has been shown in many cases to regulate genes involved in the Th1 response, such as COX2, which catalyzes the production of prostaglandins (Inoue *et al.*, 1995), iNOS (Lowenstein *et al.*, 1993), IL6 (Matsusaka *et al.*, 1993) and TNFα (Pope, 1994), just to name a few. Our own finding of Msr1 would probably fall into the same category. However, a transcription factor is not expected to have a univocal role, particularly because its activity is often modulated by the presence of other coactivators. Some of the genes stated above were screened by RT-PCR in our experimental model (e.g. COX2, TNFα and IL6), and found to be regularly induced in

response to LPS/IFNγ stimulation (data not shown). Most of these genes have binding sites for the proinflammatory transcription factor NFκB on their promoters, or also PU.1 in the case of COX2. In this case, the C/EBPβ deficiency in the DC/DC macrophages apparently doesn't perturb the system, since other potent activators are still present. Accordingly, also in M2 cells C/EBPβ will act in concert with other coactivators. A good candidate is STAT6, which was shown to regulate arginase 1 transcription together with C/EBPβ (Pauleau *et al.*, 2004), and which is essential for IL13-mediated functions in macrophages (Takeda *et al.*, 1996). Moreover, found in inflammatory zone 1 (FIZZ1) gene induction in allergically challenged lungs was found to be an IL4- or IL13-driven process in which STAT6 and C/EBPβ are critical mediators (Stutz *et al.*, 2003).

It is possible that in our experimental model we have a mixture of natural suppressor M2 cells and M1 macrophages, which respond differently to IFNγ/LPS treatment. It was shown that activation of NS cells can induce a rapid expansion of these cells in a T cell–independent manner (Atochina *et al.*, 2001). To understand what sort of populations we are dealing with it would be interesting to test our macrophages some time after activation, and see whether the population displays an overall increased level of Gr1, and perhaps is impaired in NO production. Theoretically, DC/DC macrophages, compared to the wild types, would be expected to have lower levels of Gr1 and higher NO production in these conditions. Moreover, additional M2 markers such as IL10 or IL4Rα should be checked.

A test on βΔCRE mice for susceptibility to parasites, which normally exploit M2 macrophages for a successful infection, could produce interesting insights on the role of C/EBPβ in facilitating, perhaps, the infection. Since NS cells have been found in lymphoid organs during viral infections (Cauley *et al.*, 2000), and the replication of HIV-1 in macrophages seems to be specifically inhibited by the LIP isoform of C/EBPβ (Tanaka *et al.*, 2005), we are also investigating on the viral susceptibility of the βΔCRE mice. However, this is work in progress, and nothing can be anticipated yet.

In addition, tumor growth increases NS cell activity (Young *et al.*, 1996). In recent publications, C/EBPβ deficient mice were found to be resistant to carcinogen induced skin tumors (Zhu *et al.*, 2002), and C/EBPβ was also found to be a principal effector of cyclin D1 activity in mammary gland human cancer (Lamb *et al.*, 2003). In these publications, the prooncogenic role of C/EBPβ was attributed to a molecular process taking place in the tumoral cells. However, it would be interesting to investigate whether C/EBPβ in resident tissue macrophages could have a role in permitting the expansion of the tumor cells.

### 4.2 The Potential and Future Perspectives for the R26(ARIAD) Knockin Mouse

The functionality of the ARIAD transcription factor has been tested in cell lines and shown to work in a very stringent manner (Pollock *et al.*, 2000; 2002; and our own data, figure 3.10). The system was also tested *in vivo* via an intramuscular injection of adeno-associated viral vectors encoding the ARIAD transcription factor and the target gene (Rivera *et al.*, 1999). The R26(ARIAD) mouse would be the first example of a transgenic mouse line expressing the artificial transcription factor. The presence of the *Neo* cassette in the middle of the bicistronic transcript allows a tissue specific expression of both components of the transcription factor dependent on the tissue specificity of the Cre line the mouse is crossed to. The possibility of feeding the dimerizer to the mouse, or injecting it intraperitoneally, allows the activation of the transcription factor in a time-specific manner. Ideally, should the system work, after crossing the R26(ARIAD) mouse to the βΔCRE line, we would be able to artificially induce C/EBPβ gene expression in any tissue depending on the availability of a specific Cre mouse line. Furthermore, should the system prove to be functional, it would be possible to clone the ZFHD motif in the promoter of any given gene to artificially regulate its transcription.

At the moment we are trying to assay the system in primary cells extracted from the R26(ARIAD)+/-βΔCRE DC/+ mice. We have crossed the R26(ARIAD) mouse either to a CD4-Cre (T cell specific) or to a Lys-Cre (macrophage specific) mouse line. The idea is to purify the T cells and macrophages from each mouse, put them in a culture dish, and add the dimerizer in the culture medium. After a 24h treatment we harvest the cells and purify RNA and protein to detect the expression of C/EBPβ. The expression should be specific to the tissue in which Cre is expressed and to the presence of the dimerizer. If the system works, the mice are then ready for *in vivo* approaches, in which we could control the dependence on C/EBPβ expression in our macrophage experiments. However, more importantly, the system could be a precious tool to investigate the role of C/EBPβ in tissue determination during development, for example by an ectopic induction of the gene in specific tissues.

#### 4.3.1 Migration Pattern of the Phosphorylation Mutants: Can There Be Cooperativity?

Our phosphorylation mutants target specific phosphoacceptors in the RD2 domain just upstream from the bZIP domain of the C/EBPβ protein. By running protein extracts from NIH3T3 cells transduced with the T188A or 3S/A mutants on an Anderson gel, we were able to confirm the involvement of the mutated sites in phosphorylation events. For the 3S/A mutant we cannot say whether all three of the mutated serines are normally phosphorylated. Of particular interest is the migration pattern of the T188A mutant. The fact that T188A migrates as a duplet, both from *in vitro* and *in vivo* whole cell extracts

(figures 3.15 and 3.21), cannot be attributed only to the T188 point mutation. If the entire population of the protein is mutated, then it should all migrate in the same way, unless some labile event, which can modify protein migration, is taking place in this particular mutant. One band in the duplet migrates like the wild type C/EBPβ, and the other migrates at the same height of the 3S/A mutant. The labile event, which apparently takes place in about half of the protein population, could be another phosphorylation, which requires phosphorylated Thr188 for stability. The most obvious candidates for this additional phosphorylation are the three serines that are mutated in 3S/A, not only because of their proximity to Thr188, but also because the lower T188A band migrates exactly like 3S/A. Additional support to this theory is given by the observation that in the TA/TA fat tissue extracts, the upper T188A band is more abundant than the lower, whereas in all the other tissues it is the contrary (figure 3.21). Since it has been shown that GSK3β phosphorylates C/EBPβ in the fat (Tang *et al.*, 2005), and it is possible that there is more active GSK3β in the fat than in the other tissues analyzed, this could lead to a higher amount of phosphorylation on the serines even in the suboptimal condition of the absence of phosphorylation on residue 188. Analyses done on the phosphorylation of synthetic peptides carrying the T188A mutation, showed that GSK3β phosphorylation was impaired, again suggesting a sequential phosphorylation by MAPK and GSK3β performed on T188 and the preceding serines (Tang *et al.*, 2005). Moreover, the requirement of a docking site for phosphorylation is characteristic of GSK3β. This kinase is known to phosphorylate serines or threonines that are located four amino acids upstream of another threonine, and that are spaced four amino acids one from the other (see figure 3.13). The fact that the higher band of the T188A C/EBPβ migrates as the wild type suggests that the wild type C/EBPβ is phosphorylated only transiently on Thr188, just the time required to enhance GSK3β phosphorylation. This model, although in many ways supported by Tang *et al.*, still remains to be elucidated by functional assays.

### 4.3.2 Controversy Between Published and Personal Data on the Roles of the T188 and 3S Phosphorylation Sites

According to Tang *et al.* (2005), the mutation of T188 and the GSK3-targeted serines abolishes DNA-binding and transactivation activities of C/EBPβ. However, our luciferase assays clearly show that the mutants are not impaired in their transactivational capacities. On the contrary, our mutants, particularly the 3S/A, have an enhanced transcriptional activity *in vitro* (figure 3.16). This finding is in agreement with the work of Zhao *et al.* (2005), which shows that mutation of the GSK3β target sites enhances activity. Moreover, Tang *et al.* show that by treating 3T3-L1 preadipocytes with inhibitors of MAPK and GSK3β, adipogenesis is completely blocked and C/EBPβ is

incapable of binding the aP2 gene (an adipogenic target of C/EBPβ in the fat) promoter. However, our experiments definitely show that the T188A and 3S/A mutants are perfectly capable of driving adipogenesis under hormonal stimulation (figure 3.17). The MAPK and GSK3β inhibitors used by Tang obviously blocked a signaling pathway that is upstream of C/EBPβ. The conserved and enhanced activity of T188A and 3S/A discredits the possibility that these phosphorylations in the RD2 domain could somehow change the three-dimensional configuration of the protein to influence its transactivational capacities, as suggested by some (Kowenz-Leutz *et al*., 1994; Williams *et al*., 1995). A similar scenario would likely have the effect of on or off, and not modulate the degree of activity.

### 4.3.3 Phosphorylation for Autoregulation: Is It a Positive or a Negative Loop?

Our experiments on gene expression in the 3S/A primary macrophages produced very interesting results on the mechanism of autoregulation of the C/EBPβ gene. The overinduction of C/EBPβ in the 3S/3S macrophages treated with IFNγ/LPS suggests that an impairment in autoregulation is taking place. Most likely, 3S/A has lost the capability of inhibiting (or limiting) its own transcription. This is shown both by the higher expression of the gene screened at the mRNA level (figure 3.23A), and by the stronger signal for the 3S/3S samples, particularly the activated ones, on the macrophage Anderson western blot (figure 3.24). That C/EBPβ is subjected to autoregulation was already shown by Niehof *et al*. (2001). The motif on the C/EBPβ promoter responsible for autoregulation is, as mentioned before, adjacent and partly overlapping with the most distal CRE element from the start site. In the βΔCRE mice we partly disrupted this C/EBP binding site by deleting the CRE elements. In all the RT-PCR analyses that we performed for C/EBPβ expression in the βΔCRE mice, we always observed a slightly higher basal level of C/EBPβ expression in the untreated DC/DC sample, although statistically not significant (figure 3.6). This observation supports the idea that a negative autoregulatory feedback loop can take place on that particular location of the C/EBPβ promoter in determined conditions, such as quiescence. In addition, the affymetrix in figure 3.3 shows that the overexpression of exogenous wild type C/EBPβ causes the downregulation of the endogenous, again proving the existence of a negative feedback loop.

The higher induction of the C/EBPβ target genes in the 3S/3S macrophages stimulated with IFNγ/LPS (figure 3.23B and C) could be explained as being the consequence of the higher expression of C/EBPβ. However, if we combine this result with the luciferase assays, it is also possible that the 3S/A mutant simply has a stronger transactivational capacity than the wild type transcription factor. On the other hand, this would probably mean that the autoregulatory feedback loop is positive instead of

negative. In other words, instead of wild type C/EBPβ sitting on the promoter to prevent further transcription by other transcription factors, it could directly activate its own transcription, and the 3S/A mutant would be all the more efficient in doing so. The autoregulatory loop was described as being positive also by Niehof *et al*. (2001). This explanation, however, doesn't take into account the slightly higher basal transcription of the βΔCRE DC/DC mutant, and the downregulation of the endogenous C/EBPβ in cells overexpressing an exogenous analog. It is tempting to believe that the 3S/A protein has stronger transactivational abilities on one hand, but weaker autoinhibitory properties on the other. Practically, the two mechanisms should be considered distinct, and modulated perhaps by the interaction with different cooperating proteins.

The vicinity and partial overlap of the C/EBP binding site to the CRE element on the C/EBPβ promoter may suggest an interaction between C/EBPβ and CREB, which is not impossible, both being bZIP proteins. If the feedback loop is positive, then the interaction with CREB would most certainly enhance activation. If it is negative, then maybe C/EBPβ could mask CREB's activation site, or rather, the presence of C/EBPβ on the C/EBP site could be of steric hindrance to the binding of CREB on the CRE element, without there being any interaction. With the elements that we have, it is difficult to determine which interpretation is the correct one, and most of what we have said is reduced to speculation. It will take further studies to have an answer.

### 4.3.4 The Importance of a Mouse Model

The activity of the T188A mutant doesn't seem to be reproducibly enhanced. T188A was comparable to the wild type in one of the two luciferase assays (figure 3.16B), and also in the ability to autoregulate C/EBPβ gene expression and induce C/EBPβ target gene expression in the macrophages (not shown). If the theory on the cooperativity between the T188 and 3S phosphorylation sites is true, then the higher migrating protein could be sufficient to modulate C/EBPβ activity in the cell, and the phenotype would be intermediate to wild type-like. However, this would mean that the T188A phosphorylated on the three serines is dominant on the unphosphorylated, and this takes us back to the theory of the negative feedback loop. Again, this is pure speculation.

The anti-phospho-T188 antibody was very useful to confirm that the T188A mice actually carried the intended mutation (figure 3.22). The migration pattern of this protein remains an intriguing puzzle and we have done our best to interpret it. Cooperativity or no, we know with certainty that our point mutations have hit distinct phosphorylation sites. Regarding the 3S/A mutant, we cannot be sure that all three of the mutated serines are involved in the phosphorylation. However, due to the considerable shift in protein migration, it is reasonable to believe that at least two of the serines are involved, and

these would probably be Ser180 and Ser184. Once again, this cannot be asserted for certain.

The cloning of mice carrying the point mutations has been helpful in clarifying controversial issues on these phosphorylation sites, and making us confident that we are not dealing with experimental artifacts. We believe that further studies of the T188A mouse *in vivo* will help us understand the role of this MAPK phosphoacceptor in each cell type and in physiological conditions. This mouse would also be useful to confirm the findings of Zhu *et al.* (2002) that the T188 phosphorylation site is involved in the signaling of oncogenic *ras* causing C/EBPβ to have an important role in carcinogen-induced skin tumors. Their studies regarding Thr188, in fact, were based on *in vitro* experiments. Moreover, the. tumor model on the T188A mice would be a very useful tool to uncover the role of this phosphorylation site in respect to C/EBPβ function. For this reason, we are starting a collaboration with the lab of Robert Smart, which is where Zhu and coworkers performed their studies.

Taken together, the two projects described in this thesis have produced new insights on the transcriptional and posttranslational regulation of the C/EBPβ transcription factor. During these studies we have run into numerous controversies on the published data regarding the regulation of C/EBPβ's activity, many of which have been discussed here. We believe that our studies have in many ways clarified some of these points, thanks to the use of an *in vivo* model, which is the nearest we can get to true physiological conditions. Moreover, we were also able to find novel targets for C/EBPβ transcription in the macrophage, which were fundamental in suggesting a broader view of the role of C/EBPβ in this cell type. It will take more investigation to prove this potential immunosuppressive role in the macrophages, but most certainly it will have to be done *in vivo*.

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