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# Gene regulation by Myc during B cell activation


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# List of abbreviations

| | |
|---|---|
| ATP | adenosine triphosphate |
| BCR | B-cell receptor |
| bHLHZ | basic helix-loop-helix-leucine zipper domain |
| BrDU | Bromodeoxyuridine |
| BSA | bovine serum albumin |
| CDK | cyclin-dependent kinase |
| cDNA | complementary DNA |
| ChIP | chromatin immunoprecipitation |
| CpG | C--phosphate--G |
| CTD | C terminal domain |
| DEGs | differentially expressed genes |
| DNA | deoxyribonucleic acid |
| E-box | enhancer box |
| eRPKM | exonic reads per kilobase per milion |
| FACS | fluorescence-activated cell sorting |
| FCS | fetal calf serum |
| FSC | forward scatter |
| GB | gene body |
| gDNA | genomic DNA |
| GO | gene ontology |
| GSEA | gene set enrichment analysis |
| HATs | histone acetyltransferases |
| HDACs | histone deacetylases |
| HSC | hematopoietic stem cells |
| INR | initiator element |
| IPA | ingenuity pathway analysis |

| | |
|---|---|
| LPS | lipopolysaccharide |
| Mad | Max dimerization protein 1 |
| MAX | myc-associated factor X |
| MBI | Myc box I |
| MBII | Myc box II |
| MBIII | Myc box III |
| MBIV | Myc box IV |
| MDRS | Myc-dependent serum response genes |
| miRNAs | micro ribonucleic acid |
| Miz1 | Myc-interacting zinc finger protein 1 |
| Mnt | MAX network transcriptional repressor |
| mRNA | messanger ribonucleic acid |
| mTOR | mammalian target for rapamycin |
| Myc | myelocytomatosis oncogene |
| NGS | next generation sequencing |
| p-TEFb | positive transcription elongation factor |
| PBS | phosphate buffered saline |
| PEST | peptide sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T) |
| PI | Propidium iodide |
| PTM | post- translational modifications |
| PWM | position weight matrix |
| qPCR | quantittive polimerase chain reaction |
| RAS | rat sarcoma oncogene |
| Rb | retinoblastoma protein |
| RNA | ribonucleic acid |
| RNAPolIII | RNA polimerase II |
| rRNA | ribosomal ribonucleic acid |
| SI | stalling index |

| | |
|---|---|
| SSC | side scatter |
| TAD | transcriptional activation domain |
| TAT-cre | TAT recombinase |
| TCR | T-cell receptor |
| TFII-I | general transcription factor II-I |
| TFs | transcription factors |
| TLR | toll-like receptors |
| TRAPP | transactivation-transformation domain-associated protein |
| tRNA | transfer ribonucleic acid |
| TSS | transcription start site |
| WDR5 | WD repeat-containing protein |

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# 1. Abstract

c-Myc is a transcriptional regulator required for the cellular response to proliferative stimuli. The gene expression programs regulated by Myc in physiological settings remain to be clarified. Here, we provide a complete characterization of Myc-dependent regulatory events in primary mouse B cells following activation by bacterial lipopolysaccharide (LPS). Taking advantage of cells homozygous for a conditional knockout allele of c-*myc*, we induced deletion before LPS stimulation, followed by genome wide profiling of mRNA levels and Myc-DNA interactions. In contrast with previous studies, in which Myc was proposed to directly drive transcriptional amplification at all active loci (Nie et al. 2012, Lin et al. 2012), our study revealed that Myc is required for the up- and down-regulation of distinct subsets of genes early after stimulation, occurring prior to the global increase in RNA production. These gene expression programs where partially overlapping with those regulated by Myc upon oncogenic activation, a distinction made not only in B-cells, but also in fibroblasts (Sabò et al., 2014, Perna et al. 2012). Our data also show that Myc dependent regulation can occur at the level of RNA Polymerase II loading, as well as elongation. Altogether these data provide an extensive picture of Myc' s action in response to a mitogenic stimulus, highlighting the importance of Myc-target genes in the remodeling of cellular physiology and metabolism. Systematic work will be needed to unravel which, among all the Myc-regulated genes, are critical in mediating this chain of events.

# 2.Introduction

Myc, the product of the *c-myc* proto-oncogene, is one of the most studied proteins in biomedical research. Myc normally integrates environmental signals in order to modulate different cellular processes including proliferation, apoptosis, energy metabolism and differentiation (Tansey, 2014) . Disruption of its tight regulation leads to aberrant cell cycle progression and carcinogenesis. Indeed Myc deregulation directly contributes to malignant transformation in multiple cell types and is a hallmark of many human cancers (Ciriello et al., 2013; Gabay et al., 2014; Meyer and Penn, 2008). Oncogenic activation of *c-myc* can occur directly or indirectly. Chromosomal abnormalities of the *myc* locus, such as gene translocation in Burkitt's B cell lymphomas (Küppers & Dalla-Favera, 2001), amplification in different carcinomas or insertional mutagenesis represent direct mechanisms of Myc overexpression. In these genetic rearrangements, the protein coding sequence of *c-myc* may remain intact, differently from other proto-oncogenes in which changes in protein sequences are needed for oncogenic activation. Most frequently, Myc is indirectly activated, since growth-regulatory pathways that induce or stabilize Myc in physiological conditions are themselves target of activating mutations (e.g. RAS, Wnt or Notch signaling) (He et al., 1998; Palomero et al., 2006; Sears et al., 1999). In addition, in many mouse models of Myc-driven tumors, the tumor cells become addicted to Myc overexpression, as inhibiting Myc can cause cell death, arrest and/or differentiation (Felsher and Bishop, 1999; Arvanitis & Felsher, 2006; Jain et al., 2002; ). Thus, Myc is usually required for tumour progression and maintenance also even if it's not mutated itself.

## 2.1 Myc

### 2.1.1 Myc's discovery

The discovery of Myc arose from studies on retroviruses associated with animal cancers. In 1911, the experiments of Peyton Rous showed that chicken leukemia and sarcomas are transmissible through cell-free filtrates. In the following 50 years it was established that many animal tumors could originate from viral infections.  During the 1960s and 1970s different retroviruses were isolated from avian neoplasms and in particular, the strain of virus called MC29 was shown to transform myeloid cells in myelocytomas, a solid tumour, or myelocytomatosis. The name gave to the genetic element responsible for transformation was v-*myc* from viral myelocytomatosis. The MC29 virus MYC protein was found to localize into the nucleolus of the target cell, suggesting a unique involvement in gene regulation and nuclear function respect to other retroviral oncoproteins. Then, a cellular homolog of this gene in uninfected vertebrate cells was found and called c-*myc* (Sheiness and Bishop, 1979). Finally in 1982, the c-*myc* gene was cloned and characterized opening an era of massive research efforts into understanding Myc's structure, biological functions and transcriptional regulation.

### 2.1.2 Biological functions of Myc in cellular physiology

Myc, in response to intracellular and extracellular stimuli such as cytokines, growth factors and mitogens regulates a plethora of different biological processes. It is able to induce cell cycle progression and growth, potentiate apoptosis, block differentiation, increase genome instability, stimulate angiogenesis, stromal remodelling, inflammation and thus drive transformation (Kelly et al., 1983; Eisenman, 2001; Eilers and Eisenman, 2008) (**Figure 1**). Myc may achieve these physiological and pathological outputs mainly through regulation of gene expression.

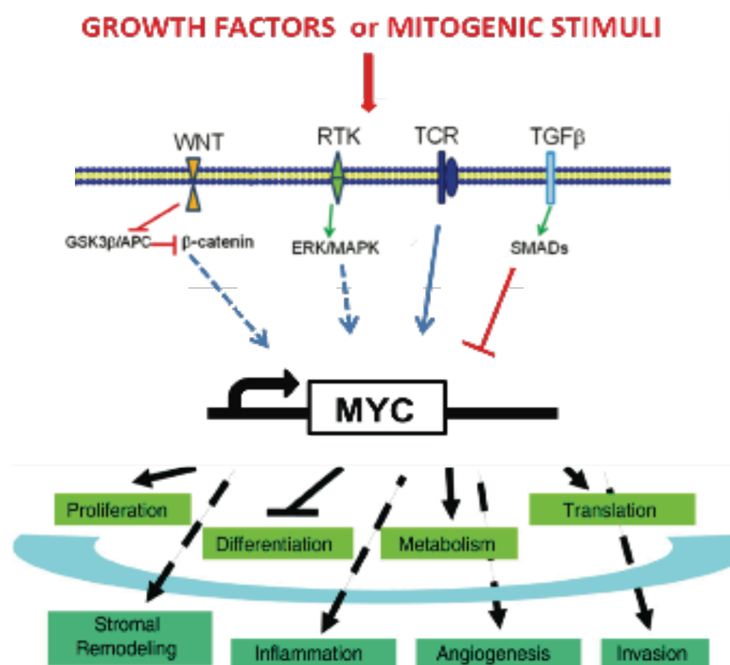



**Figure 1** ***Myc as an intracellular sensor of mitogenic stimuli.***
Schematic representation of the biological effects of Myc activation.
(Source: modified from Sodir and Evan *Journal of Biology* 2009 8:77 © 2009 BioMed Central Ltd)

In somatic cells, the main function of Myc is the tight regulation of proliferation. Germ line deletion of either N-*myc* or c-*myc* is lethal for the embryo beyond day 10 because of abnormalities in organs development and tissue growth (Davis A.C. et al 1993). For this reason, conditional myc knockout mice were generated to study Myc function in adult tissue. c-*myc* deletion in fibroblasts, keratinocytes and B cells compromises G0 to S phase progression upon stimulation with serum or cytokines (de Alboran et al., 2001; Rabbitts et al., 1985). On the other hand, Myc activation is sufficient to expedite cell cycle entry and reduce the requirement for mitogenic signals to maintain the cycling state. The main mechanism used by Myc to achieve this goal is the direct activation of cyclin/CDK expression such as cdk4, cyclin D, E and inhibition of cell cycle checkpoints (Hermeking et al., 2000; Yang et al., 2001). For example Myc induction of Cdk4-cyclinD and Cdk2-cyclinE promotes Rb hyperphosphorylation, the release of E2F from Rb and thus the S phase entry, which is also accelerated by a direct induction of E2F2 by Myc (Hermeking et al., 2000; Santoni-Rugiu eta l., 2000; Mateyak et al.,1999).

An important aspect of cell physiology is the ability to coordinate growth with cell division. Studies of Drosophila d*myc*, the orthologous of *myc*, showed that cells without d*myc* decreased cell mass accumulation and size, while cells overexpressing it are bigger than normal ones and more prone to progress in S phase to accelerate cell divisions (Johnston et al. 1999). Therefore, d*myc* influences growth with changes in cell doubling time suggesting a primary role in activation of the cell growth machinery. Studies in yeast on growth showed that cell cycle mutants had impaired cell growth (altered cell size and decreased in RNA content) independently of change in cell division (Fraser and Nurse, 1979). In primary B cells derived from Eμ-*myc* mice and in human B cell lines such as P493, it was demonstrated that ectopic c-*myc* expression induces cell growth, that is, an

increase in cell size and protein synthesis, independently from cell cycle phases (Iritani B & Eisenman 1999; Schuhmacher et al., 1999). Related to this, Myc promotes the increase in ribosome biogenesis and production of metabolic enzymes in order to satisfy the requirements of growing cells for ATP and metabolites (Johnston et al., 1999) (see paragraph 2.4.1).

Myc can also affect cellular differentiation. The presence or the absence of Myc can affect this process in opposite ways, depending on the cell context and stage of development. Down-regulation of *myc* expression can engage differentiation in B cell and in human promyelocytic leukemia cells (Schuhmacher et al., 1999; Bacon TA & Wickstrom E. 1991). In addition, Myc induction or overexpression blocks differentiation in various cell types such as keratinocytes in *vitro* (Dang CV., 2013) and *in vivo* (Lin K. et al., 2000). The physiological dowregulation of Myc is necessary, even if not sufficient, for B cell differentiation (Lin K. et al., 2000; Murn et al., 2009). Indeed, ectopic expression of Blimp1, a known inducers of plasma cell differentiation, induces also down-regulation of endogenous c-Myc, correlating well with the cessation of proliferation that occurs during terminal differentiation (Lin Y., et al. 1997). On the contrary, Myc can promote differentiation of the stem cell compartment: in particular, Myc can push the exit of hematopoietic stem cells (HSC) from the stem cell niche (Laurenti et al., 2008), and has a similar effect on human epidermal stem cells, favouring entry into the transit amplifying compartment and thereby initiating terminal differentiation (Gandarillas A. & Watt FM., 1997).

Another aspect of Myc biology is its ability to sensitize cells to different apoptotic stimuli rather than directly inducing apoptosis by itself. In B-lymphocytes, for example, anti-CD40 stimulation with consequent Myc induction promotes the surface expression of CD95 and

makes them susceptible to cell death. In addition, it was demonstrated in fibroblasts and myeloid cell lines that, upon serum deprivation, overexpression of Myc can promote apoptosis (Evan G.et al 1992; Askew DS et al 1991). In addition c-Myc deficient B cells were shown to be resistant to CD95 induced cell death or staurisporine, another apoptotic stimulus (de Alborán et al., 2003). On the contrary, it was shown that down-modulation of c-*myc* expression correlates with induction of apoptosis in B cells (Wu et al., 1996). Among the main mechanisms adopted by Myc to induce apoptosis are the disruption of the equilibrium between pro-apoptotic BH3-only protein and anti-apoptotic proteins Bcl-2 and Bcl-X (Hemann et al., 2005; Eischen CM et al., 2001) or the activation of ARF-MDM2-p53 axis during tumorigenesis (Eischen et al., 2001; Oster et al., 2002). The ability of Myc to drive both programmed cell death and proliferation in a well-balanced way is a safeguard mechanism to prevent uncontrolled growth and tumour onset.

### 2.1.3 Structure of Myc proteins

In mammalian cells, there are three different gene family members of Myc proteins, *c-Myc*, *N-myc*, *L-myc,* which share the same general topology with almost 40% of sequence homology and show very similar functions, but different patterns of expression (Bull et al., 2001; Xu et al., 1991). The presence of multiple Myc family members may reflect the different spatial and temporal requirements of Myc activity in development and in adult tissues with a consequent overexpression of each protein in specific cancer types. In particular, c-Myc is usually overexpressed in most blood borne and solid tumors whereas N-myc is more selectively amplified in neuroblastoma and L-myc in small cell lung carcinomas (Nau et al., 1985; Weiss et al., 1997). The structural organization of Myc

proteins is conserved through evolution and resembles the conformation of a typical sequence-specific DNA binding transcriptional regulator (**Figure 2**). Among the three paralogs (c, N, L-myc) the main similarities lie in the conserved regions (**Figure 2**).

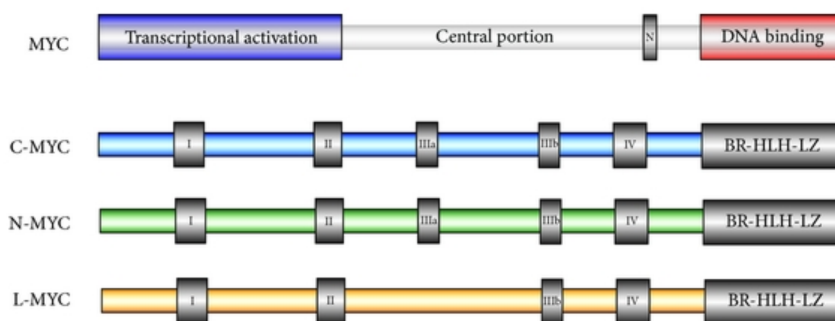



**Figure 2** ***General architecture of MYC proteins.***
The main structural domains of MYC are shown schematically at the top of the figure. Transcriptional activation, central portion, canonical nuclear localization sequence 'N' and region of DNA binding via interaction with MAX are reported. A representation of the different family members (C, N and L-MYC) is shown underlining the conservation of the sequences and the localization of Myc boxes even if the proteins are different in length. (Source: Tansey, W. (2014). Hindawi Publishing Corporation New Journal of Science, Volume 2014, Article ID 757534, 27 pages; Copyright © 2014 William P. Ğansey.).

We can schematically subdivide Myc structure in three main parts, as described in details in (Conacci-Sorrell et al., 2014; Tansey, 2014) (**Figure 3**):

1- An amino-terminal region constituting the transcriptional activation domain (TAD), a region sufficient for trascriptional activation when fused with a DNA binding domain (DBD), that contains the conserved Myc boxes (MBI, MBII) (Kato et al., 1990).
2- A central portion rich in glutamic acid, threonine and proline residues (PEST) with two conserved Myc boxes (MBIII and MBIV) and a nuclear localization sequences.

3- A carboxy-terminus domain of ≈ 100 amino acids comprising the basic helix-loop-helix-leucine zipper (bHLH-LZ) domain, which mediate protein-protein interactions and DNA-binding (Blackwell T.K., et al., 1990; Prendergast G.C. & E.B. Ziff, 1991).

Unlike the central region, which is still poorly understood, the other regions are very well characterized. The N-terminus TAD is required for Myc's transforming activity *in vitro* (Stone et al., 1987). Myc box I (MBI) is required for gene activation and ubiquitination/proteasomal degradation of Myc. Point mutations in this domain (T58, S62 or S71) affect its half life (around 20-30 min in many normal cells) and increase its stability in many tumors (Salghetti et al., 1999). Myc box II (MBII) is necessary for Myc transforming activity *in vitro* and *in vivo*, gene activation (Oster et al., 2002) and is a key domain for binding of coactivators such as components of histone acetiltransferases complex (TRAPP, GCN5, TIP60, TIP48) (McMahon et al., 1997). The MBI-MBII-TAD region has an important role in trascriptional regulation because it is also bound by other co-factors of Myc such as p-TEFb complex or the bromodomain protein BRD4 (see below) (Eberhardy SR & Farnham PJ., 2001; Rahl PB and Young R., 2014).

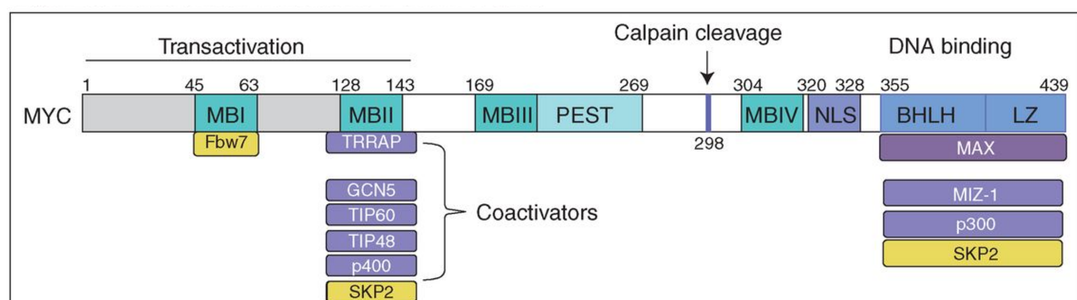



**Figure 3** ***Organization of Myc structure and its interacting partners.***
Different portions of Myc structure are very well described. Transcriptional binding partners of Myc are indicated in violet and major ligases involved in Myc turnover are coloured in yellow. (Source: Modified from Conacci-Sorrell, et al. (2014). Cold Spring Harb Perspect Med *4*, a014357; Copyright @2014 Cold Spring Harbor Laboratory Press)

In physiological conditions, Myc does not homodimerize (Prendergast G.C. & Ziff E.B. 1991) but, through the bHLH-LZ domain at the C-terminal, heterodimerizes with the same domain of another bHLH-LZ protein, Max (Blackwood E.M & Eisenman R.N. 1991). They form a complex, which bind major groove of the DNA by forming a fork-like structure (Ferre D'Amare et al., 1993; Ferre D'Amare et al., 1994). Helices 1 and 2, part of bHLH-LZ of each monomer, create a hydrophobic core that stabilizes the dimeric structure, while the leucine zipper regions form a coiled coil and lastly the zipper interactions determine the dimerization specificity (Soucek et al., 1998). DNA binding occurs through recognition of a consensus sequence "CACGTG", called " Enhancer box" (E-box) or variants thereof (Lüscher B. and Larsson L.G, 1999; Eilers M. & Eisenman R.N. 2008) **(Figure 3; Figure 4)**.

Interaction of Myc with Max is required for the correct Myc folding and its biological activities such as regulation of gene expression, proliferation, transformation and apoptosis (Amati et al. 1992; Amati et al.1993a; Amati et al. 1993b). The complex formation itself is an important point of Myc regulation, since Max is expressed also in the absence of Myc and can dimerize with additional bHLH-LZ such as Mnt or some Mad members (O'Shea JM. & Ayer DE., 2013). These members of the Max network behave much like Myc and therefore they can compete for available Max and for Myc/Max binding sites.

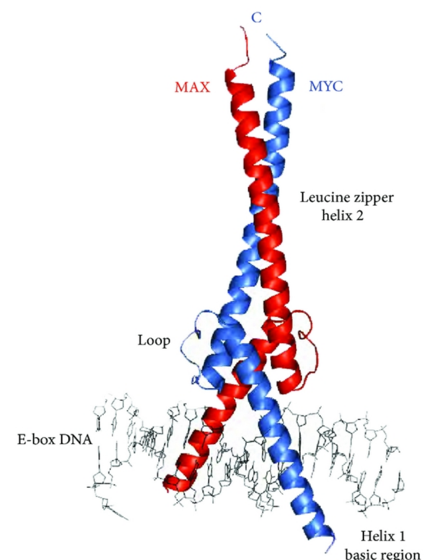



**Figure 4** ***X-ray structure of MYC_MAX.*** bHLHZ dimers bound to E-box DNA sequences (Source: Tansey,W.New Journal of Science 2014; Copyright © 2014 William P . €ansey).

The physiological status of the cell may dictate the outcome of this competition (Ayer et al. 1993; Hurlin et al 1997). In addition to the heterodimerization with Max, each steps of

the life of Myc protein is tightly regulated. Myc activity is controlled at the level of transcription, post-transcription, which involved mRNA stability, translation and by post-translational modification (PTMs) such as phosphorylation, acetylation, glycosylation and ubiquitination (Oster et al., 2002; Hann SR., 2006; Farrell & Sears, 2014; Salghetti et al., 1999).

## 2.2 Mechanisms of transcriptional regulation by Myc

### 2.2.1 Myc binding to the genome: sequence recognition and different modes of Myc-chromatin interactions

Interaction with Max is necessary for the basic domain of Myc to bind DNA at the so-called E-box sequences and drive gene expression (Kretzner et al., 1992; Amati et al.1992; Amati et al., 1993a). In this regard, chromatin immunoprecipitations (ChIP) on CpG Island arrays firstly confirmed that Myc and Max bound to identical promoters (Mao et al., 2003). With the development of high throughput ChIP technologies, many labs have identified *in vivo* the Myc DNA-binding sites. The first features found to be enriched among Myc-binding sites was the CpG island (Fernandez et al., 2003; Lüscher and Vervoorts, 2012), which is associated with active chromatin (Deaton A.M. & Bird A., 2011). Indeed, Myc-bound promoters are always associated with an active chromatin context delineated by precise histone marks (H3K4me3, H3K4me2, H3K27ac and so on), presence of the basal transcriptional machinery and hypersensitivity to DNAase I digestion (Fernandez et al., 2003; Zeller et al., 2006; Deaton and Bird, 2011; Guccione et al. 2006; Sabò et al., 2014; Lin C. et al, 2012). There are no cases in which Myc results to bind heterochromatin even if E-boxes are present (Lin C. et al., 2012; Sabò et al., 2014).

Hence, sequence recognition by Myc is not possible before recognition or access to open chromatin context. Thus, ChIP-seq experiments performed in many systems, showed that Myc binds open and active promoters and distal sites (Sabò et al.2014; Lin C. et al 2012). As an example, in serum stimulated fibroblasts, at low and physiological level, Myc preferentially binds promoters already marked by H3K4me3, pre-loaded RNAPolII and contained E-boxes sequences rather than sites that lack the E-boxes (Perna et al 2012; Sabò et al 2014). With increasing Myc levels, the number of bound promoters increases and Myc starts also to bind distal sites (Lin C. et al 2012; Sabò et al. 2014). All of these distal peaks are identified as active enhancers based on the presence of chromatin features such as H3K4me1, me3, H3K27ac marks and high H3K4me1/H3K4me3. These sites are already active before Myc overexpression and binding to them (Nie et al.,2012; Lin C. et al., 2012; Sabò et al., 2014). In this context, Myc can also bind lower affinity E-boxes (variants or non canonical) and also other sequences indicating that Myc/Max binding to the DNA could be less sequence specific (Guccione et al., 2006; Fernandez et al., 2003). At the highest Myc levels, as in tumors or cell lines, Myc can be cross-linked at virtually all active promoters and enhancers of the genome, a phenomenon called 'invasion' (Lin C. et al. 2012). This widespread binding to the genome is therefore less selective and includes E-boxes variants that have lower affinity for Myc-Max *in vitro* or other sequences (Lin C. et al., 2012).

Our lab recently proposed a general model of Myc binding to DNA, possibly valid also for other transcription factors (Sabò A. & Amati B., 2014; Kress et al 2015). Myc/Max recruitment to chromatin can be explained through successive steps (**Figure 5**). As already mentioned, the packing of DNA into an inaccessible heterochromatic state prevents the Myc/Max binding (**Figure 5a**). When positive determinants for recruitment such as

histone marks and cofactors are present, the heterodimer starts to interact with chromatin facilitated by contacts with chromatin-associated protein or the basal transcriptional machinery (**Figure 5b**). The Myc/Max dimer is then engaged on DNA in a sequence independent manner, allowing local scanning (**Figure 5c**). After DNA scanning, Myc may stabilize on high affinity binding sites (**Figure 5d**). This kind of binding mode is also supported by the structural studies of bHLH-LZ proteins such as Myc-Max with DNA backbone (Sauvé et al., 2007). In particular, after formation of an initial complex partially folded, basic region of bHLH-LZ dimer screens DNA in search of favouring interactions (DNA-assisted folding). Afterwards, through the binding to an E-Box, the basic region is stabilized in its α-helical conformation; whereas if it doesn't happen, the complex lacks its conformational stability (Sauvé et al., 2007).

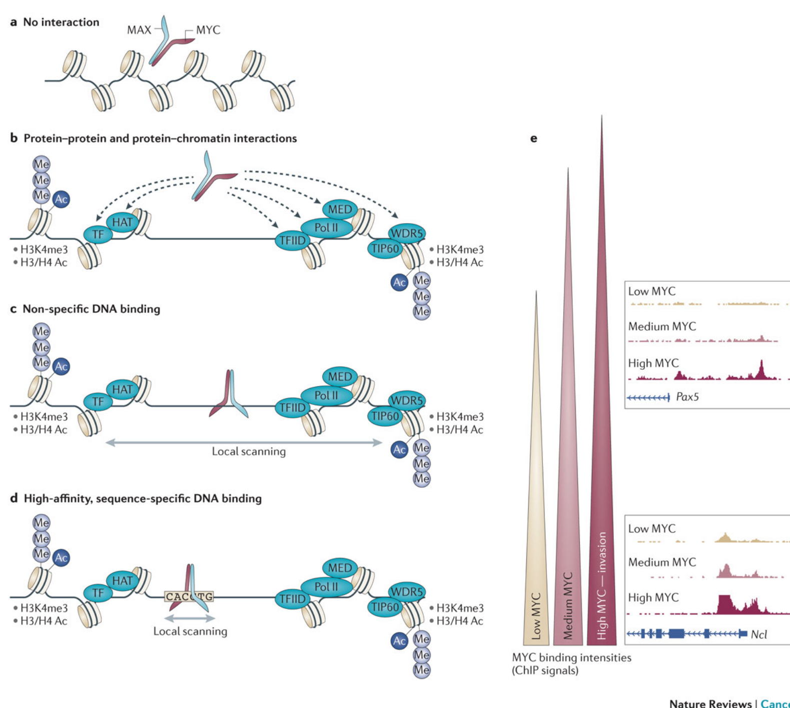



**Figure 5** ***Schematic model for Myc/Max interaction with chromatin.***
Different modes of Myc-chromatin interactions are represented in details on the left of the picture. Two examples of Myc ChIP-seq tracks for high affinity Myc binding site (*Ncl*, nucleolin) and low affinity binding site (*Pax5*, paired box 5) in mouse B cells are reported on the right. (Source: Kress et al 2015, *Nature Reviews Cancer* (2015); Copyright © 2015, Rights Managed by Nature Publishing Group:)

In summary we proposed that that Myc/Max heterodimer is firstly recruited to open chromatin domain, such as active promoters and enhancers via interactions with cofactors and then, almost simultaneously, the complex scan DNA and stabilize on high affinity binding sites. These binding modes lead to a continuum of DNA-binding intensities and they can be maintained at low medium and high Myc expression levels (**Figure 5e**) (see paragraph 2.2.3).

### 2.2.2 Cooperation between Myc and others transcription factor

Analysis of ENCODE (Encyclopaedia of DNA Elements) ChIP-seq datasets demonstrated that only a subset of Myc/Max sites actually binds to canonical and non-canonical E-boxes (Soufi et al., 2015; Neph et al., 2012). Around 40% of Myc-binding sites, especially when outside promoters, lack E-boxes and are localized in a more relaxed sequence environment leading to the hypothesis that on those sites Myc could have been recruited through indirect DNA binding (Soufi et al., 2015). In general, two TFs can bind to neighbouring sites (co-binding) or one TF can bind to another that, in turn, binds directly to DNA (tethered binding). The analysis of the binding motifs different from E-boxes in Myc ChIP-seq experiments shown significant enrichment for other TFs binding sites that can indirectly tether Myc to chromatin such as CTCF, GA-binding protein- α chain (GABPA) and activating protein 1 (AP1) (Wang J. et al., 2012), but the relevance of these observations remains to be addressed. In terms of co-binding, Myc was shown also to interact with many TFs. These Myc partners found with motif analysis were AP-2, HIF1, Sp1, YY1, NF-Kb. (Lüscher and Vervoorts, 2012; Oster et al., 2002; Wang J. et al 2012). AP-2 can antagonize Myc-mediated transcriptional activation by competing with Myc/Max for DNA binding to target promoters of genes mainly involved in apoptosis or, in absence

of overlapping DNA binding sites, it can specifically interact with Myc preventing Myc/Max complex from DNA binding and so inhibit Myc functional activation (Gaubatz et al., 1995). Under low oxigen conditions which usually characterized a tumor environment, the transcriptor factor HIF-1 can cooperate with Myc to control the expression of metabolic genes including those encoding for glycolytic enzimes (Dang et al., 2008). In order to promote uncontrolled cell cycle progression (Zeller et al., 2006), overexpressed Myc may cooperate with E2F1. In addition, it was reported that Myc, STAT and E2F1 may interact with different portion of p300/CBP suggesting that these TFs can at least in part cooperate by co-recruitment of p300/CBP (Bedford et al., 2010).

Finally, Myc can also co-bind DNA with TFs and negatively affects their activities, such as in the case of Myc interaction with Sp1 and Miz1 (see also paragraph 1.2.6 for Miz1). As an example a study of $p21^{Cip1}$ promoter which is a repressed Myc target, outlined the presence of several Sp1 binding sites. Myc binds the zinc finger domain of Sp1 and may form a complex with Sp1/Sp3. Since Sp1 is required for p21 transcription, it is possible that Myc may down-regulate p21 transcription by sequestering Sp1 out of promoters (Gartel et al., 2001).

### 2.2.3 Global versus selective transcriptional control by Myc

Even if there is a vast amount of literature describing RNA profiling studies in response to modulation of Myc levels, the identification of a definitive Myc signature is still missing. One reason is that Myc *per se* is a mild transcription factor, inducing only 2-3-fold change in mRNA expression levels. Next, since Myc is able to interact with several other transcription factors as described above, a very complex network is orchestrated.

Paradoxically with the advent of next generation sequencing for genome-wide studies, different views of Myc gene regulation have emerged. Two recent papers (Lie C. et al., 2012; Nie et al., 2012) describe a new way of Myc action in transcription and they introduce the 'transcriptional amplifier model'. According to that model, Myc does not work as a sequence specific transcriptional activator or repressor of specific gene programs (as classically thought) but it functions as a direct activator or amplifier of all the genes already 'on' in a given cell type either at physiological or pathological levels. Indeed, in LPS-activated B cells, embryonic stem cells (Nie et al., 2012) and tumour cells (Lie C. et al., 2012), Myc is highly expressed and able to invade all open regulatory elements of the genome both promoters and enhancers. This phenomenon of invasion was directly associated to an higher level of RNA per cells in these Myc overexpressing cells compared to cells with low Myc (co-called "RNA amplification"). However, these observations are also compatible with an alternative model by which Myc can activate and repress selected target genes, with RNA amplification occurring as a secondary consequence. Indeed, in our work (Sabò et al., 2014), we demonstrated that Myc either in tumours or normal cells, with or without invasion on all active elements of the genome, can selective up or down-regulate genes. These genes mainly control growth, metabolism, cell size, protein translation, ATP content and mitochondrial mass increase. All of these physiological changes can lead to a global transcriptional rate increase, which is deciphered in higher amount of RNA (das Neves et al 2010; Marguerat and Bähler, 2012). In relation with this, we reported that Myc chromatin invasion and its specific gene regulation could or not imply into a cell metabolic switch which in turn feedback on RNA amplification (Sabò et al., 2014). As an example, upon serum stimulation of fibroblasts, Myc does not invade all active chromatin but is essential for cell cycle entry and activation of a set of Myc-dependent serum response (MDSR) genes, which precedes the increase in

RNA levels and cell size (**Figure 6**). Upon supra-physiological expression of Myc in already proliferating fibroblasts, invasion of active chromatin with selective gene regulation was observed without further RNA amplification (**Figure 6**). On the other hand, in Myc overexpressing tumors such as in the Eμ-*myc* model, Myc regulates transcription of selective genes but its widespread binding to active chromatin and total RNA increase are concomitant processes making difficult to unravel their cause-to-effect relationships. Returning to the idea that Myc can regulate specific genes, Walz et al., 2014 showed in cancer human cell lines that, concomitant with the enhanced degree of Myc-regulatory elements binding due to Myc abnormal expression, there is a direct Myc gene activation and repression in this last case through the suppression of Miz1-target genes. Thus, this second model, more close to a classical view of Myc action, re-established the importance of specific Myc target genes independently of Myc chromatin invasion and RNA amplification.

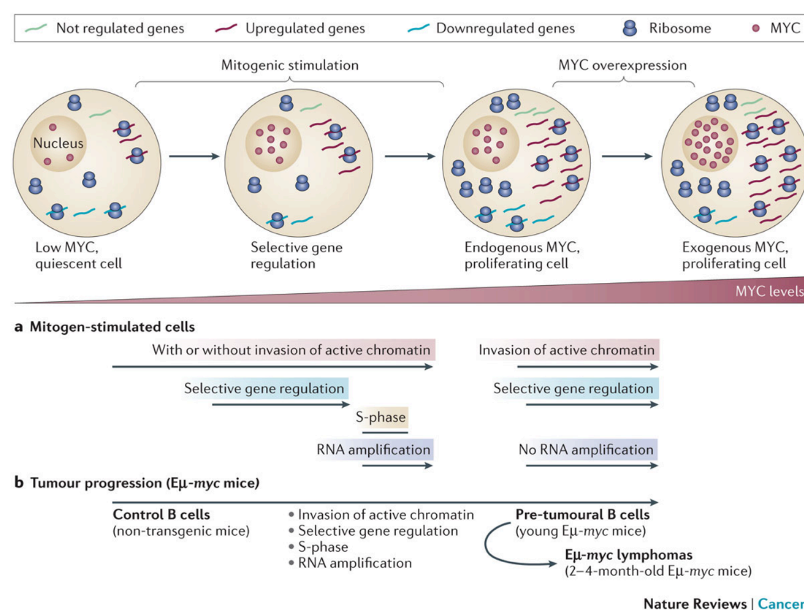



**Figure 6** ***Summary of events occurring after mitogenic stimulation and/or Myc overexpression.*** Comparative studies on mitogen-stimulated cells such as serum stimulated fibroblasts and LPS activated B cells or Myc overexpressing cells such as 3T9 MycER and Eμ-myc tumour B cells shown that RNA amplification and chromatin invasion are separable phenomena**.** (Source: Kress et al 2015; *Nature Reviews Cancer* (2015) doi: 10.1038/nrc3984; Copyright © 2015, Rights Managed by Nature Publishing Group:)

### 2.2.4 Myc-dependent activation: Interplay between Myc and RNAPolII

The importance of Myc/Max binding to the DNA through interactions with other effector proteins is very well documented. Since ChIP-seq experiments shown Myc binding mainly on pre-engaged RNAPol2 promoters, it’s possible that Myc could be recruited on DNA through components of the general transcriptional machinery such as TFII-I (Roy et al., 1993) and in addition through other transcriptional molecules or complexes. For example, Myc is known to interact with many histone-acetyltransferases (HATs) and HAT-containing complexes such as GCN5/PCAF, Tip60, p300/CBP, as well as with the adaptor protein TRAPP (transformation- transactivation domain-associated protein), itself a subunit of the GCN5/PCAF and Tip60 complexes (McMahon et al., 1997; Lance RT. & Tansey WP, 2015). All of these interactions are thought to result mainly in core histone acetylation (Frank SR. et al., 2001), which is likely to contribute to Myc-dependent transcriptional activation. However, It ‘s not really clear how all these cofactors work in order to promote a functional transcriptional regulation. Furthermore, Myc also recruits the core Mediator through the SAGA complex containing the acetyltransferases GCN5 (Liu X et al 2008) and interacts with WD repeat-containing protein (WDR5) an H3K4me3 associated protein for the binding to its target sites in the genome (Thomas LR et al. 2015). Myc has also been reported to recruit different components of the SWI/SNF complex, a multiprotein apparatus that activates transcription by remodelling chromatin in an ATP-dependent manner. In particular, the bHLHZip domain of Myc directly interacts with hSNF5, key component of SWI/SNF complex and this interaction permits the Myc-mediated expression of reporter genes (Cheng et al 1999). In conclusion, the transcription activation function of Myc involves at least in part the recruitment of histone acetiltransferase and chromatin remodelling complexes.

Another regulatory step of the activation of target genes by Myc is the promoter recruitment and clearance of RNA polymerase II (RNAPolII) (Cole M and Cowling V. 2008; Oster et al., 2002). Even though Myc regulates the transcription of rRNA and tRNA genes by RNA polymerase I and III respectively, the vast majority of Myc target genes are mRNA-encoding genes transcribed by RNAPolII. Transcription factors can regulate their target genes by affecting transcriptional initiation (RNAPolII recruitment) or transcriptional elongation (pause release and RNAPolII elongation). In particular, the C-terminal domain (CTD) of RNAPolII undergoes a cycle of phosphorylation and dephosphorylation during the various steps of transcription. RNAPolII is recruited to promoters in a hypophosphorylated form (transcriptional initiation) and then is phosphorylated on Ser5 by the transcription factor TFIIH. Subsequently, RNAPolII produces a short transcript and a pause factor induces a pausing usually 20-50 bp downstream of the transcriptional start site. Specific signals and cofactors then stimulate transcriptional elongation via RNAPolII phosphorylation on Ser2 by P-TEFb complex. Transcriptional termination is finally stimulated by recognition of polyadenylation site sequence by factors associated with RNAPolII during elongation. A CTD dephosphorylation occurs at the termination step to also promote a new cycle of transcription (Cole M and Cowling V. 2008). All genome-wide studies so far (Lie et al.2012; Nie et al 2012;Sabò et al.2014; Walz et al.2014) agree with the fact that TSS-associated Myc binding sites are pre-marked not only by open chromatin but also by the presence of RNAPolII. Even if Myc has been reported to enhance the loading of the polymerase on target promoters (Martinato et al. 2008), other data suggest that it mainly modulates transcriptional elongation by recruiting the P-TEFb complex (cyclin T1 and Cdk9) which phosphorylates the carboxy-terminal domain of RNAPolII on Ser2 favouring transcriptional elongation (Rahl et al. 2010; Lin C. et al.2012; Bouchard et al., 2004) (**Figure 7)**. The role of Myc on RNAPolll elongation is at the basis of the general

amplifier model according to which increased Myc binding leads to increased rate of RNAPolII elongation and consequently higher levels of transcribed mRNAs (Rahl et al., 2010; Wolf et al., 2014; Rahl PB and R.Young 2013). However, the general increase in the transcriptional RNAPolII activity of a cell undergoing metabolic activation and cell size expansion is a highly conserved process, which pre-dates Myc in evolution (Marguerat and Bähler, 2012; das Neves et al., 2010; Lloyd A., 2013). The growth in cell size correlates with RNA amplification but the cause-to effect relationship between these changes is still unclear and how changes in transcriptional elongation of Myc's target genes take part to these processes need to be elucidated.

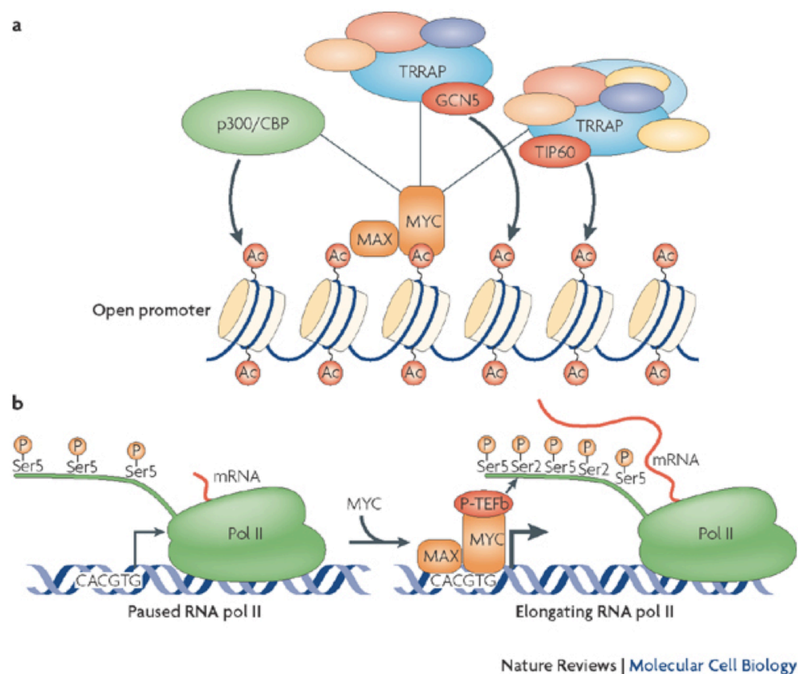



**Figure** ***7 Chromatin remodelling and promoter clearance of RNAPolII for Myc induced transcriptional activation.***
Myc-Max dimer recruits acetyltransferases, which modify chromatin in an open and active state. Then, Myc can induced pause release of RNAPolII on the mRNA targets by recruiting the P-TEFb, which phosphorylates RNAPolII on Ser2 and so, promotes transcriptional elongation. (Source: Transcription-independent functions of MYC: regulation of translation and DNA replication Michael D. Cole and Victoria H. Cowling (2008) *Nature Reviews Molecular Cell Biology*; Copyright © 2008, Rights Managed by Nature Publishing Group).

### 2.2.5 Myc-dependent repression

Even if the mechanisms of Myc-mediated transcriptional repression are less understood compared to the ones of activation, many studies indicate that Myc may repress genes with anti-proliferative properties such as cell cycle inhibitors, cell adhesion molecules and tumour suppressive miRNAs (Tansey, 2014). For example Myc can induce EZH2 expression, a member of the polycomb complex, directly or through the repression of miRNAs such as miR-26a and miR-26b which are negative regulators of Ezh2 (Koh et al., 2011; Sander et al., 2008). The existence of direct Myc mediated repression has been recently questioned and gene repression by Myc ascribed to technical artefacts of the normalization of gene expression data (Lovén et al., 2012). Indeed, when considering cells with different level for RNA (such as cells with High Myc versus cells with Low Myc), the normalization of RNA profiles to the average expression or to the reference housekeeper genes, as is commonly done in gene expression studies, would be inappropriate because genes that are simply less induced compared to the average population are classified as 'repressed'. In light of this, the authors of the amplification models propose the measurements of RNA levels for cell equivalent in order to compare and analyze gene expression datasets (Lin et al. 2012; Nie et al. 2012; Lovén et al., 2012; Wolf et al.2014).

Since the transcriptional output scale proportionally with cell size (Marguerat and Bähler, 2012), we demonstrated that the concept of repression is still valid in the context of bigger cells overexpressing Myc (Sabò et al 2014). A known mechanism of repression driven by Myc is via its association with Miz1 (**Figure 8**). Miz1 is a zinc–finger-containing protein that binds 'initiator' elements (INR) close to the transcription start site (TSS) of selected genes and induces their expression. When complexed with Myc through interaction with its C-terminal helix–loop–helix domain, Miz1, loss the interactions with

its co-activators, the histone acetiltransferase p300 and nucleophosmin Npm1 (Staller et al. 2001; Wanzel et al 2001). Nucleophosmin can also be displaced from Miz1 by the ribosomal protein RPL23, transcriptionally induced by Myc (Wanzel et al. 2008). Myc can also recruit the DNA methyltransferase Dnmt3a that methylates the promoters of Miz1-target genes (Herkert and Eilers, 2010) (**Figure 8**). Therefore, both MYC and MIZ1 are transcriptional activators that form a repressive complex upon binding to each other. Indeed, it has been proposed that the ratio of MYC and MIZ1 bound to each promoters correlates with the direction of transcriptional response (Walz et al.2014). This 'anti-activation' mode through which Myc acts on Miz1 is a mechanism of Myc–mediated repression reported also for Myc and Sp1, C/EBPα and other transactivators described in the previous paragraph (Gartel et al 2001). Finally, Myc can also repress transcription via recruitment of histone deacetylases (HDACs) to chromatin, inducing deacetilation and thus nucleosome compaction, a chromatin environment refractory to transcription (Kurland & Tansey 2008).

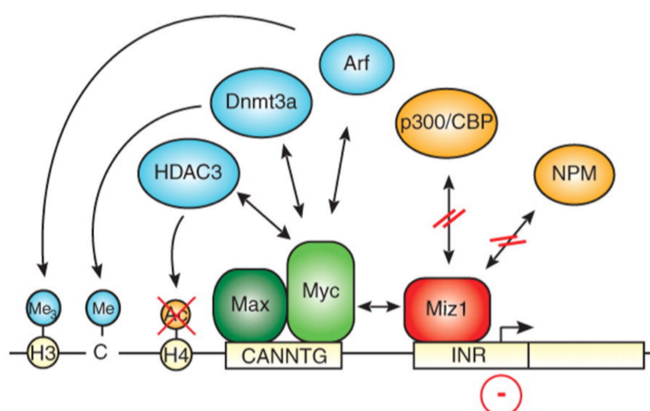



**Figure 8** ***Myc transcriptional repression mediated by Miz1 and HDACs/Dnmt3a recruitment to chromatin.***

Schematic representation of Myc transcriptional repression by Miz1 and histone deacethilases HDACs/Dnmt3a. (Source: Modified from Barbara Herkert and Martin Eilers Genes Cancer. 2010 Jun; 1(6): 580-6; © The Author(s).

## 2.3 Myc role in lymphocytes

The humoral immunological response depends on plasma cells, the sole producers of antibodies. Thus, activation of mature B cells, secretion of antibody and survival of plasma cells needs to be tightly regulated. Cellular development and commitment in the B cell lineage takes place in the bone marrow, originating from hematopoietic stem cells (HSCs) (**Figure 9**). Rearrangement of immunoglobulin heavy-chain (IgH) gene segments in pro-B cells leads to precursor (pre)-B cells. After a phase of active proliferation, pre-B cells rearrange their antigen receptors and migrate to the periphery (spleen and other lymph nodes) as quiescent G0 lymphocytes.

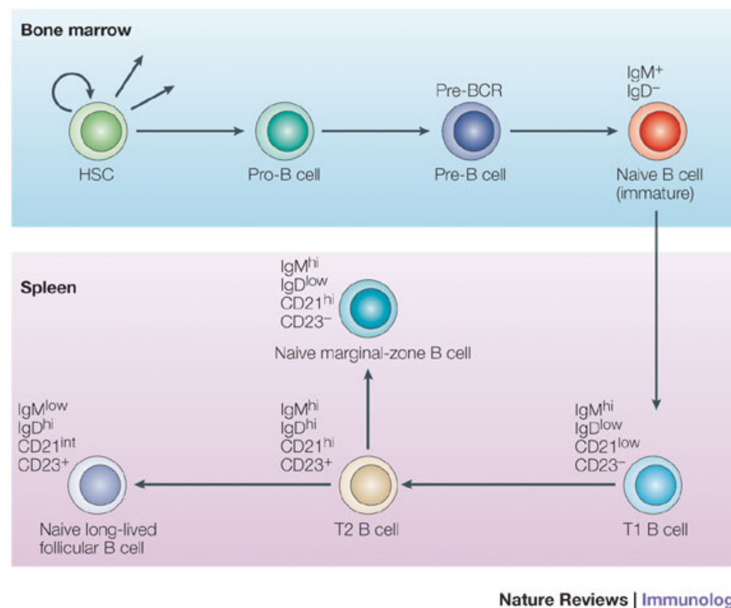



**Figure 9** ***Antigen independent B cell development***
Schematic view of B cells commitment starting from pluripotent stem cell in the bone marrow. Naïve B cells IgM+ exit from bone marrow and they mature in the spleen mainly as long-live follicular B cells and in a minor part as naïve marginal zone B cells which remain in the spleen as non-circulating cells. T1 and T2 B cells are two transitional stages before complete maturation. (Source: Shapiro-Shelef M. & Calame K., (2005). Regulation of plasma cell development. Nature Rev Immunol. 5, 230-242. Copyright © 2005, Rights Managed by Nature Publishing Group).

Here, the cells undergo further maturation and become follicular and marginal zone B cells. At this point, marginal zone B cells remain in the spleen as naïve non-circulating cells whereas follicular B cells may circulate in the periphery until they die or encounter antigens and undergo additional maturation. Upon contact with the foreign antigen, marginal zone B cells and subsequently follicular B cells differentiate into plasma cells, (mostly short-lived plasma cells), and some activated follicular B cells can form the germinal center, a specialized area which permit development of B cells memory through rounds of proliferation, cycles of somatic antigen receptor diversification (SHM) and class switch recombination of immunoglobulin (CSR) (McHeyzer-Williams LJ. et al., 2001). Before encountering the antigen, all the subtypes of mature B cells shown a small size, a high nuclear to cytoplasmic ratio and are in a poised metabolic status, where RNA and protein synthesis are maintained at basal levels (Shapiro-Shelef M. & Calame K., 2005). On the other hand, both short-lived and long-lived plasma cells are characterized by an increase in protein synthesis and RNA production to sustain cell division and clonal expansion (Rajewsky K. 1996). All of these steps can be recapitulated *in vitro* until cell death after plasma cells differentiation (**Figure 10**).

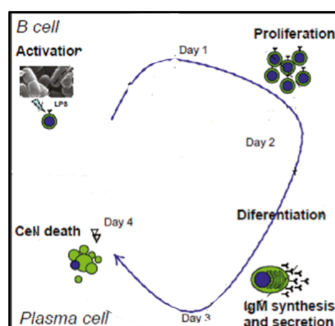



**Figure 10** ***Schematic representation of B cell fate from antigen activation to plasma cell differentiation in vitro.***
Clonal expansion, differentiation and cell death occurs within 4 days after stimulation.(Source: Modified from Garcia-Manteiga J M. et al Metabolomics of B to Plasma Cell Differentiation J. Proteome Res. 2011, 10, 4165–4176. Copyright © 2011, American Chemical Society)

B cells can be activated either via B-cell receptor in an antigen-specific manner, or through Toll-like receptors (TLRs) that recognize specific microbial products such as lipopolysaccharide (LPS). Upon ligation, TLRs and also BCR receptors, induce B cells proliferation and memory B cell development through activation of Rel/NF-κB transcription factors. In unstimulated cells, NF-κB dimers reside in the cytoplasm and are maintained inactive by inhibitory molecules, collectively termed inhibitors of κB (IķBs). NF-κB translocation into the nucleolus with subsequent binding to the DNA results from the activation of kinases that phosphorylate IķBs, signaling their ubiquitination and degradation (Karin M & Ben-Neriah Y., 2000). There are two distinct pathways (type 1 and type 2 NF-κb) regulating phosphorylation of IκB proteins that result in the release of specific and distinct Rel dimers (Zandi E. & Karin M 1999; Dejardin et al., 2002). CD40, a ligand of BCR receptors, controls type 1 and type 2 of NF-Kb signalling pathway, whereas TLRs engagement trigger only type 1 NF-kb (Zarnegar et al. 2004). Even if BCR and TLR activation induce similar cellular response there are some differences in gene activation programs ( Dadgostar et al., 2002; Zarnegar et al., 2004 Gerondakis et al., 2007). CD40L activation for example induces the type 2 NF-κB pathway and thus the expression of genes involved in adhesion, migration, and germinal center formation, a property that LPS lacks (Zarnegar et al., 2004). Whereas the signalling pathway cascades acting during B cell activation are very well described, a complete picture of the transcriptional mechanisms and epigenetic changes occurring before proliferation and functional immune response is still not available. Initial mitogenic stimulation of B cells leads to the induction of primary response genes (PRGs) which are usually turned on within minutes (Fowler et al.2013) such as the immediate early response genes *c-myc, c-fos* and *c-jun*. Very recently, a genome wide study compared the BCR and TLR –mediated activation response of B cells

and showed a partial overlap between the two transcriptional response at very early stages of activation. Remarkably, in this common signature there was the prevalence of known Myc induced genes (Fowler et al. 2015).

Also T cells undergo a growth phase upon stimulation of antigen receptors, accumulation of cell biomass and rapid proliferation associated with transcriptional metabolic reprogramming to increase the bioenergetics and the biosynthetic demand. It was shown that Myc drives this metabolic reprogramming in activated T lymphocytes coupling glutaminolysis to polyamine biosynthesis to enhanced the polyamine demand required for proliferation (Wang et al., 2011). Since these changes in T cell metabolism were observed within 3 to 10 hr of stimulation, the metabolic effects of Myc, either direct or indirect, were very rapid and indipendent to S-phase entry. Myc expression is also transient and it is demonstrated that one transcription factor, AP-4 part of its target genes, is induced to sustain T cells activation. in the'post Myc phase' through the expression of genes mainly involved in metabolism (Chou C. et al. 2014). Thus, Myc is a primary response gene rapidly induced upon lymphocyte activation. Altogether, the metabolic switch from quiescent to activated cells is controlled by a complex gene regulatory network, different combinations of transcription factors being required to maintain B-cell and plasma cell-specific programs.

In the simplest view, the key factors sustaining the B cell phenotype are Pax5, Bcl6, Bach2, PU.1 and IRF8, whereas the terminal differentiation is driven by IRF4, Blimp-1 and XBP1 (Shapiro-Shelef and Calame, 2005) (**Figure 11**). Each transcription factors is stage-specific and may repress those factors that are required for the alternative developmental state, creating mutually exclusive gene-expression programs. In this scenario, Myc shows different pattern of expression: it is expressed at very low levels in

mature B cells, is rapidly induced upon antigen activation, and then repressed during plasma cell differentiation (de Alboran et al 2001; Lin C. et al., 2000; Murn et al., 2009). *In vitro*, Myc-deleted B cells are impaired in the mitogenic respose upon CD40+ IL4 treatment with a delay in activation and cell cycle progression (de Alboran et al., 2001). Myc stimulates not only proliferation but also development of B cells through an increase in intracellular $Ca^{2+}$ (Habib et al., 2007) inducing thus the expression of Myc and $Ca^{2+}$-induced genes.

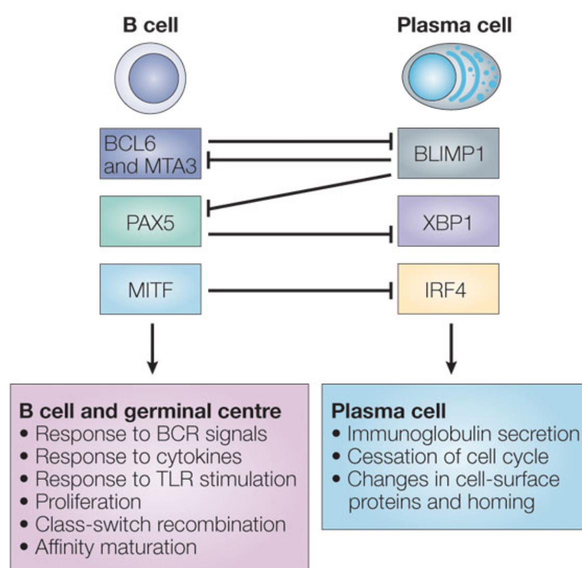



**Figure 11** ***Changes in the trascription factor networks from B cells to plasma cells.***
BCL-6 (B-cell lymphoma 6), MTA3 (metastasis-associated 1 family, member 3), MITF (microphthalmia-associated transcriptionfactor) and PAX5 (paired box protein 5) —defend B cell identity by repressing BLIMP1 (B-lymphocyte-induced maturation protein 1), XBP1 (X-box-binding protein 1) and IRF4 (interferon-regulatory factor 4). In plasma cells, BLIMP1 represses B-cell gene-expression programmes. This mutual repression prevents the unelicited formation of plasma cells in the germinal centre and prevents the reversion of plasma cells to a B-cell stage. (Source: Shapiro-Shelef M. & Calame K., (2005). Regulation of plasma cell development. Nature Rev Immunol. 5, 230-242. Copyright © 2005, Rights Managed by Nature Publishing Group).

In conclusion, Myc induction has an important role during lymphocyte activation (Quade et al., 1983; Eilers and Eisenman, 2008; Wang et al., 2011). In mitogen-stimulated B cells, P493 and Eμ-myc B cells, metabolic growth which includes cellular size and prothein synthesis increase is a very well described process concomitant to Myc-induced proliferation (de Alboran et al.2001; Iritani B & Eisenman 1999; Schuhmacher et al., 1999).

## 2.4 Myc regulated genes

Over the past two decades many studies aimed to identify the genes that mediate Myc biological activities in physiological and pathological contexts. According to many studies the functional categories mainly affected by Myc activity are cell growth (including cell size and biomass accumulation), RNA processing, ribosome biogenesis and protein synthesis, metabolic processes (such as glycolysis, glutaminolysis, amino acid and nucleotide biosynthesis).

### 2.4.1 Myc activated genes: role in growth, metabolism and RNA processing

Ectopic expression of Myc leads to activation of many mRNAs involved in different aspect of cell physiology, which will be separately discussed below.

-Ribosome biogenesis and protein synthesis. In different model systems, Myc promotes the RNAPolII-mediated transcription of mRNAs encoding various ribosomal proteins, which are part of a general, cell-type independent core Myc target gene signature (Ji et

al., 2011). Myc also induces the expression of translation initiation factors (such as eIF2A and eIF4E) and, in the absence of Max, RNAPolIII-mediated synthesis of tRNA (Rosenwald et al. 1993; Gomez-Roman et al. 2003).

Myc can also control directly the transcription of genes involved in mRNA cap methylation. The Myc target eIF4F, for example, is responsible for the recognition and enhanced translation of cap-methylated mRNAs contributing to the broad increase in protein synthesis (Jones et al., 1996; Cole and Cowling, 2008; Koromilas et al.,2009).

-Nucleotide biosynthesis. In several cell types such as B cells, fibroblasts and liver cells, Myc overexpression globally induces expression of genes involved in nucleotide biosynthesis (Liu et al., 2008). In serum-stimulated fibroblasts, for example, several MYC-dependent serum response genes encode key enzymes in purine and pyrimidine biosynthetic pathways, including ribonucleoside-diphosphate reductase subunit M2 (RRM2), amidophosphoribosyltransferase (PPAT), GAR transformylase (GART) and CAD (Perna et al., 2012). As shown in activated T lymphocytes, Myc can up regulates glycolytic enzyme such as LDH-A (lactate dehydrogenase) and GLUT1 (glucose transporter) providing important precursor also for nucleotide metabolism (Wang et al., 2011). Myc is also described as a master regulator of the de-novo purine biosynthetic pathway: phosphoribosyl pyrophosphate synthase (PRPS2) was translationally regulated by Myc whereas enzymes of PRPS2 pathway were transcriptionally activated by MYC in Eμ-myc B cells, most likely contributing to the global enhancement of RNA production (Sabò et al.2014). This was directly proven in an independent study showing that deletion of Prps2 in the same model eliminated the general increase in cellular RNA levels that is normally observed in pre-tumoral and tumoral B cell stages  (Cunningham et al., 2014).

-RNA processing and stability. Myc may also contribute to RNA processing and stability. It can directly induce expression of alternative splicing factors serine/arginine-rich splicing factor 1 (SRSF1) or interferes with the splicing machinery (Das et al., 2012). In this regard, during lymphomagenesis Myc can promote proper splicing of precursor mRNAs by up regulating protein arginine N-methyltransferase 5 (PRMT5) and other genes encoding for components of the core small nuclear ribonucleoprotein particle (snRNP) (Koh C.M .et al.2015). In addition, MYC can also regulate RNA stability factors such as nuclear-interacting protein 1 (SNIP1). It was shown in various cell lines that SNIP1 might interfere with the transcriptional activation of some Myc target genes regulating their mRNA stability (Fujii, M. et al. 2006).

Our data confirm the Myc-dependent activation of many of these genes in B-cells.

### 2.4.2 Myc-repressed genes

The first identified target genes of Myc-mediated repression was *c-myc* itself, suggesting a negative auto-regulatory feedback (Penn et al., 1990). Myc can down-regulate either mRNAs, as described below, and/or specific miRNAs (Jackstadt R & Hermeking H., 2014).

-Negative regulators of proliferation. Myc promotes cell cycle progression through down-regulation of cell cycle-inhibitory genes such as CDK1A ($p21^{CIP1}$), CDKN2B ($p15^{INK4b}$) (Gartel et al.,2001; Staller et al., 2001). The mechanism of repression is based on the ability of Myc to prevent Miz1 from activating transcription of these genes.

-Cell adhesion molecules. Myc can repress a group of genes involved in cell adhesion (Inghirami et al. 1990) as the integrin β1 (Itgb1), a subunit of the integrin complex. Since this integrin mediate cell-cell interactions as well as contact to the extracellular matrix, its dowregulation may facilitate the exit of stem cells from the niche and subsequent differentation (Herkert B. & Eilers M., 2010).

### 2.4.3 Myc target genes as therapeutic opportunities

Myc expression is estimated to be deregulated in up to 70% of human cancers. In order to find an effective therapeutic treatment for these Myc overexpressing tumours, many strategies have been proposed: inhibition of Myc expression, interruption of Myc-Max dimerization or DNA binding and also the possibility to interfere with key Myc target genes or transcriptional co-factors. More than ten years ago, a dominant negative form of Myc (called Omomyc) was generated in order to block Myc driven transcriptional activation (Soucek et al 1998). It was shown that Omomyc could reverse Myc-induced tumorigenesis *in vitro* and *in vivo* (Soucek et al 2002; Soucek, Nasi & Evan 2004). While Omomyc was shown to homodimerize with all three Myc proteins (c-Myc, N-myc, L-myc) competing with the binding with Max and sequestering Myc in complexes with low DNA affinity (Soucek et al 1998), its precise mode of action *in vivo* remains to be addressed.

Considering that Omomyc or Myc inhibitory drugs can lead to side effects such as proliferation arrest of normal tissues (Soucek et al., 2008), other strategies have been investigated to precisely affect selected Myc functions in tumour cells. In this regard, Myc-regulated genes such as lactate deidrogenase (LDHA), ornithine decarboxylase (ODC) or glutaminase (GLS) have been targeted by shRNAs or drug-like molecules in vivo. In

particular, shRNA-based LDH-A knockdown in different tumors cell lines compromised the ability of these cells to proliferate under hypoxia, promoting a decrease in tumorigenesis (Fantin et al 2006).

Since also *miRNAs* are very well known to be Myc targets, some studies focused on their targeting. The expression and activity of Myc itself are under the *control of miRNAs* and different classes of miRNAs can be induced or repressed by Myc (*Jackstadt R & Hermeking* H., 2014). It was shown that expression of miRNA-26a, which is a Myc, regulated gene, induce inhibition of liver cancer cells in vitro and in vivo (Kota et al.2009).

In conclusion, we underlined the importance of a putative Myc core signature in normal cells and we identified also in stimulated B cells Myc regulated genes associated with the described pathways. Since these genes that impinge on the normal physiology, are often deregulated in cancer, their further characterization will be very useful for the treatment of Myc-driven cancers.

## 2.5 Aim

Myc plays a central role in the activation of B cells in response to growth-stimulatory cues. However, the gene expression programs mobilized by Myc under those circumstances remain largely unknown. The aim of this work was to unravel these programs following LPS-mediated activation of primary mouse B cells, by combining gene expression profiling with the mapping of Myc-binding sites along the genome. We took advantage of a conditional knockout allele of the c-*myc* gene, allowing the rigorous identification of Myc-dependent events, and of advanced RNA- and chromatin-profiling techniques based on next-generation sequencing (RNA-seq and ChIP-seq). Having provided us with a complete Myc-dependent gene expression program in activated B-cells, our work sets the basis for a functional characterization of critical downstream effectors in the same biological setting.

# 3. Materials & Methods

## 3.1 Mouse strains

*c-myc* $^{flox/flox}$ mice in the C57BL/6 background were obtained from the Trumpp Laboratory (Trumpp et al., 2001). Mice at 7–10 weeks of age were used.

## 3.2 Primary mouse B cells

Naïve mouse B-cells were isolated with the B cell isolation kit (MACS Miltenyi Biotech Cat. no. 130-090-862) from the spleen of wt and homozygous c-*myc*$^{f/f}$ conditional knockout animals (Trumpp et al 2001). After smashing of spleens and some steps of filtering of the mixed cell population, erithroid cells were lysates. Progressively cells not belonging to the B cell lineage, i.e, T cells, macrophages, dendritic cells or granulocytes were labeled by using a cocktail of biotin-conjugated antibodies against CD-43, CD4 and Ter-119 and then captured with an anti-biotin monoclonal antibodies conjugated to magnetic Micro Beads to be depleted from the cell suspension.

After isolation, splenocytes were exposed to a recombinant Tat-Cre protein (50μg/ml for 1h in optimem +1% fetal bovine serum) (Peitz et al., 2002) in order to induce deletion of the c-*myc*$^{f/f}$ allele. Tat-cre protein was homemade purified according to the protocol published in Peitz et al.2002. After 1h of incubation at 37°C Tat-cre was washed out and cells were then grown in suspension in DMEM medium (Dulbecco's Modified Eagle Medium) and IMDM medium (Iscove's Modified Dulbecco's Medium) in ratio 1:1 containing 10% fetal calf serum (FCS) (Globefarm Ltd, Cranleigh, UK), 2 mM L-glutamine (Invitrogen Life Technologies, Paisley, UK), 1% of non-essential amino acids (NEAA), 1%

penicillin/streptomycin and 25 uM β mercaptoethanol (Gerondakis et al., 2007). 12h after seeding, splenocytes were stimulated with lipopolysaccharide LPS (50ug/ml; SIGMA L6237) to induce cell activation.

## 3.3 Proliferation and Cell Size analysis

Starting from a cell density of 500000 cells/ml, cell proliferation was monitored counting the cells with Trypan Blue to exclude dead cells. To measure cell size, 500000 live cells were resuspended in 500 μl of PBS with Propidium iodide (PI) to exclude dead cells from the analysis and 40000 total events were collected using a FACsCalibur machine (Becton Dickinson). Data were then analyzed by using FlowJo software (TreeStar) and the mean of PI negative population scored.

## 3.4 Caspase 3/7 assay and Trypan blue exclusion assay

In order to evaluate apoptosis, caspase activity was measured using the Caspase-Glo 3/7 luminescence kit (Promega) with Glomax luminometer following manufacturer's instruction. The assay provides a proluminescent caspase-3/7 DEVD-aminoluciferin substrate and luciferase in a reagent optimized for caspase-3/7 activity, luciferase activity and cell lysis. Adding the single Caspase-Glo® 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate. This liberates free aminoluciferin, which is consumed by the luciferase, generating a "glow-type" luminescent signal. The signal obtained, normalized to the cell numbers, is proportional to caspase-3/7 activity given a measure of apoptosis cell death. The cells were in parallel counted with Trypan blue to exclude dead cells and calculate the percentages of death.

## 3.5 Cell cycle analysis and cell sorting

Cells were incubated with 33 µM BrdU for a pulse labeling of 30 min. Cells were then harvested, washed with PBS and ice-cold ethanol-fixed. Upon DNA denaturation using 2N HCl, cells were stained with an anti-BrdU primary antibody (BD Biosciences) and anti-mouse FITC conjugated secondary antibody (Jackson Immunoresearch). DNA was stained by resuspending the cells in 2.5 µg/ml Propidium Iodide (Sigma) overnight at 4°C before FACS analysis. All samples were acquired on a FACS Canto II (BD Biosciences) flow cytometer. At least 15,000 events were acquired and the analysis was performed using FlowJo X software. For sorting, cells were resuspended at the appropriate time points in Macs Buffer (0.5 % BSA, 2mM EDTA in PBS) and sorted for size at the FAcsAir of our Campus service facility.

## 3.6 Markers staining with FACS

Cells were stained with the appropriate antibodies (listed below) in 1%BSA in PBS and incubated for 1h at 4°C:

- IgM APC, B220 eFluo450, Cd19 PE-Cy7, CD44 PE (1:200 dilution), CD38 APC (Biolegend; 1:400 dilution), CD138 Bv510 (BD; 1:100 dilution)

Afterwards, cells were fixed in 1% FA in PBS for 10’ at room temperature, washed with PBS and stored at 4°C before FACS acquisition.  All samples were then analyzed with FlowJo X software.

## 3.7 Immunoblot analysis

5 x$10^6$ B-cells were lysed with RIPA Buffer (20 mM HEPES at pH 7.5, 300 mM NaCl, 5 mM EDTA, 10% Glycerol, 1% Triton X-100, supplemented with protease inhibitors Mini, Roche and phosphatase inhibitors 0.4 mM Ortovanadate, 10 mM NaF) and sonicated. Cleared lysates were electrophoresed and immunoblotted with the indicated primary antibodies: c-Myc Y69 (ab32072) from Abcam, Vinculin (V9264) from Sigma, total H3 (ab1791) from Abcam, H3panAc (06-599) and H4panAc (06-866) from Millipore. We also performed histone acid extraction as described by abcam protocol: cell lysate was obtained with Triton extraction buffer and o/n HCl incubation was then performed to extract histones. Chemiluminescent detection, after incubation of the membranes with appropriate secondary antibodies, was done through a CCD camera using the ChemiDoc System (Bio-Rad). Quantification of protein levels was done using the Image Lab Software (Bio-Rad, version 4.0).

## 3.8 Isolation genomic DNA

Cells pellet (1.5*10^7) were collected at different time points (0h, 24h, 48h, 72h post LPS stimulation) and DNA was extracted with the Nucleospin® tissue kit. The genomic DNA was finally eluted in 50 µl of BE buffer (5mM Tris/HCl, pH 8.5). The analysis of the *c-myc* deletion efficiency was performed on 10 ng of genomic DNA isolated from wt or *c-myc* Δ cells using quantitative PCR with the primers pair 5'flox-3'flox (Trumpp et al, 2001). Data were normalized to Nucleolin amplicon.

## 3.9 RNA extraction and analysis (RT-qPCR and RNAseq)

Total RNA (at least from 2.5* 10^6 cells) was purified onto RNeasy columns (Qiagen) and treated on-column with DNase (Qiagen). Complementary DNA (cDNA) was prepared using ImProm-II™ reverse transcription kit (Promega) and 10 ng of cDNA were used for each real-time PCR reaction. cDNA was detected by fast SyberGreen Master Mix (Applied Biosystems) on CFX96 Touch™ Real-Time PCR Detection System (Biorad). We normalized gene expression to TBP and expressed values relative to control using the ddCT method. For quantitative PCR primers, see primers list below.

Another method highly accurate for quantifying expression levels as determined by qPCR is RNA-seq; the last permits not only to look at changes in gene expression during development or under different conditions but also to catalogue all species of transcripts, mRNAs, small RNAs and non-coding RNAs, alternative gene spliced transcripts, post-transcriptional modifications, gene fusion and mutations/SNPs. Briefly, total RNA is converted to a library of cDNA fragments with adaptors attached at one or both ends. Each molecule is then sequenced in a high-throughput manner to obtain short sequences, called reads from one (single-end sequencing) or both ends (pair-end sequencing). The reads, typically 30-400 bp length compatible to the sequencing technology, are mapped on a reference genome to reveal a transcriptional map where the number of reads aligned on each gene, called counts, gives a measure of its level of expression (**Figure 12**) (Trapnell et al., 2010; Wang et al., 2009, Morin et al., 2008).

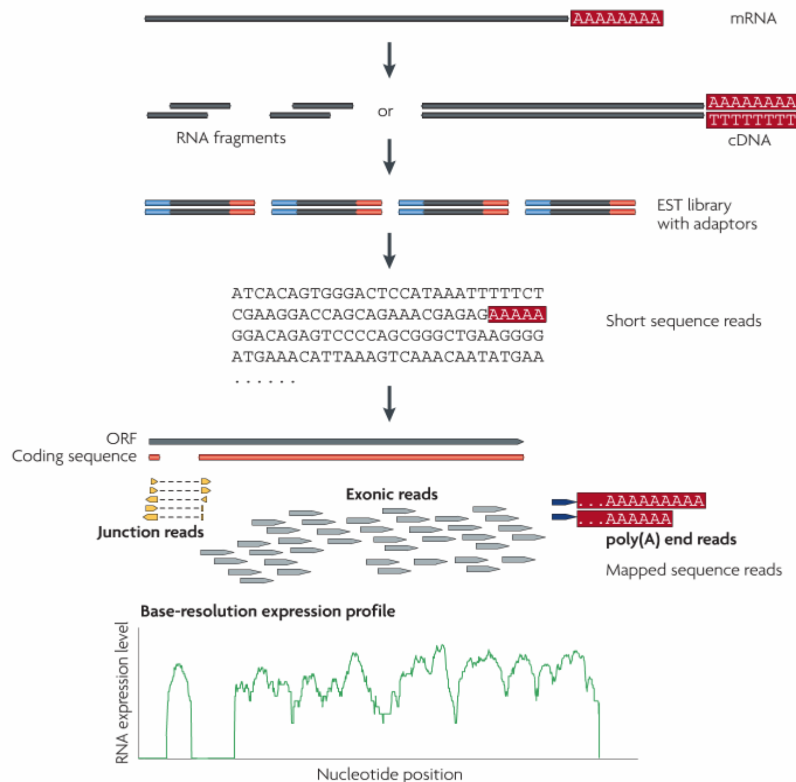



**Figure 12** ***Steps of a typical RNA-seq experiment.***
Long RNAs are first converted into a library of cDNA fragments through either RNA fragmentation or DNA fragmentation, then adaptors (blue) are added to each cDNA fragment and short sequence are obtained from each cDNA using high-throughput sequencing technology. Subsequently the sequence reads are aligned with the reference genome or transciptome , and classified as three types: exonic reads, junction reads and poly(A) end-reads. These three types are used to generate a base-resolution expression profile for each gene, as illustrated at the bottom; yeast ORF with one intron is shown.(Source: Wang Z. et al., Nature review Genetics, (2009); Copyright © 2009, Rights Managed by Nature Publishing Group).

In this work, for the RNA-seq experiment, total RNA from $8*10^6$ B cells was purified and 0.5 μg were then treated with Ribozero rRNA removal kit (Epicentre) and EtOH precipitated. RNA quality and removal of rRNA were checked with the Agilent 2100 Bioanalyser (Agilent Technologies). Libraries for RNA-Seq were then prepared with the TruSeq RNA Sample Prep Kits v2 (Illumina) following manufacturer instruction (except for skipping the first step of mRNA purification with poly-T oligo-attached magnetic bead) with the paired-end option. 50bp reads were obtained from the Illumina sequencer. The

RNAseq experiment was performed in 3 technical replicates for time 0h and in single for all the other samples.

## 3.10 Chromatin Immunoprecipitation and sequencing

Splenic B cells were resuspended in PBS at room temperature and fixed by addition of 1% formaldehyde for 10 min. Fixation was stopped by addition of 0.125 M glycine. Cells were washed in PBS, resuspended in SDS buffer (50 mM Tris at pH 8.1, 0.5% SDS, 100 mM NaCl, 5 mM EDTA, and protease inhibitors) and stored at -80 °C before further processing for ChIP. Chromatin was sonicated and processed as described in (Frank et al., 2001) except for blocking protein A-Sepharose beads with tRNA (Sigma) instead of salmon sperm and purifying immunoprecipitated DNA through Qiaquick columns (Qiagen) instead of phenol-cloroform extraction. We combine chromatin immunoprecipitation with high-throughput DNA sequencing (ChIP-seq) in order to obtain high resolution and genome-wide analysis of DNA-protein interactions. For ChIP-Seq analysis of Myc and RNAPol2 lysates from $30x10^6$ B-cells or $50X10^6$ were immunoprecipitated with 10 µg of the corresponding antibody (see below). Immunoprecipitated DNA, after several washes, was eluted in TE-2% SDS and crosslinks were reversed by incubation overnight at 65 °C. DNA was then purified by Qiaquick columns (Qiagen) and quantified using Qubit ™ dsDNA HS Assay kits (Invitrogen). 1.5-2 ng ChIP DNA was end-repaired, A-tailed, ligated to the sequencing adapters and amplified by 17-cycles of PCR, size selected (200-300bp) according with TruSeq ChIP Sample Prep Kit (Illumina). We run Agilent 2100 Bioanalyser (Agilent Technologies) to evaluate ChIP-seq library quality checking for size distribution and contamination by adapter primers and then proceed with HiSeq2000 sequencing

## 3.11 Antibodies for ChIP

The following antibodies were used for ChIP: Myc N262 (Santa Cruz, sc-764) and RNAPII N20 (Santa Cruz, sc-899).

## 3.12 List of Primers

Primers for ChIP and mRNA analysis were previously designed in the lab by using computer assisted primer design software (Primer 3). The list of primers used is below:

**Primers for ChIP:**

| **Gene** | **Forward** | **Reverse** |
|---|---|---|
| AchR | AGTGCCCCCTGCTGTCAGT | CCCTTTCCTGGTGCCAAGA |
| Smyd2 | CCGCGTACCTGGCGAAGC | GCCTGCGTGCTCACCGTG |
| Ncl | GGCGTGGTGACTCCACGT | CGAAATCACCTCTTAAAGCAGA |
| pus 7 | GCTGCACCGCGTGGAGAC | GGCTGGTGGGATAACCCGT |

**Primers for RT-PCR:**

| **Gene** | **Forward** | **Reverse** |
|---|---|---|
| TBP | TAATCCCAAGCGATTTGCTG | CAGTTGTCCGTGGCTCTCTT |
| Myc | TTTTTGTCTATTTGGGGACAGTG | CATCGTCGTGGCTGTCTG |
| Smyd2 | TGGTTGTTTTGGGGGAGAACT | AGCTTGTCCAGATGTGACTCA |
| Gart | CGTCATTGCTGGAATTGCT | TTGGGCATCTCTGCTGTCT |
| Ncl | GGCGTGGTGACTCCACGT | CGAAATCACCTCTTAAAGCAGA |
| Xbp1 | AAGAACACGCTTGGGAATGG | ACTCCCCTTGGCCTCCAC |
| BCL6 | CTGCAGATGGAGCATGTTGT | CACCCGGGAGTATTTCTCAG |
| Blimp1 | TGGCAAGATCAAGTATGAGTGC | CCAAGTAGTGTTTCTGCAGGTG |
| junb | ATCAGCTACCTCCCACATGCA | TACGGTCTGCGGTTCCTCTTT |
| Ikba | CTTGGCTGTGATCACCAACCAG | CGAAACCAGGTCAGGATTCTGC |
| Slc16a1 | GGATATCATCTATAATGTTGGCTGTC | GCTGCCGTATTTATTCACCAA |

**Primers for PCR on gDNA:**

| **Gene** | **Forward** | **Reverse** |
|---|---|---|
| myc fl/fl | TCTAGACTTGCTTCCCTTGCTGT | TTCCTGTTGGTGAAGTTCACGT |
| myc Δ | AAATAGTGATCGTAGTAAAATTTAGCCTG | ACCGTTCTCCTTAGCTCTCACG |
| Ncl | GGCGTGGTGACTCCACGT | CGAAATCACCTCTTAAAGCAGCA |

**Primers genotyping:**

| **Gene** | **Forward** | **Reverse** |
|---|---|---|
| myc | CACCGCCTACATCCTGTCCATTC | TACAGTCCCAAAGCCCCAGCCAAG |

## 3.14 Computational analysis

### 3.14.1 Next generation sequencing data filtering and quality assessment

ChIP-seq and RNA-seq NGS reads sequenced with the Illumina HiSeq2000 were filtered using the fastq_quality_trimmer and fastq_masker tools of the FASTX-Toolkit suite (http://hannonlab.cshl.edu/fastx_toolkit/). Their quality was evaluated and confirmed using the FastQC application: (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Then we use our homemade pipeline called HTS flow for analysis of both ChIP and RNAseq data. The pipeline include primary analysis which means quality control of the raw reads followed by filtering and alignment to the reference genome and secondary analysis which concerns differential gene expression, peak calling or footprint calling.

### 3.14.2 ChIP-seq data analysis

ChIP-seq NGS reads were aligned to the mouse reference genomes (mm9) through the BWA aligner using default settings (Li and Durbin, 2009). After this,, peaks were called using the MACS software. Only peaks with p-value <1e-8 were retained. Normalized reads

count within a genomic region were determined as the number of reads per million of library reads (total number of aligned reads).

MACS was also used to perform saturation analysis (as a control of false negatives) and to determine an estimated false discovery rate (as a control of false positives) for each experiment. In the saturation analysis, the fraction of peaks confirmed with 80% of the reads was determined. False discovery rate was determined as the proportion of negative vs positive peaks where negative peaks were identified by calling MACS on the input samples, using the ChIP as reference.

Peak enrichment was determined as log2 ($ChIP_w$ - $input_w$), where $ChIP_w$ and $input_w$ are the normalized counts of reads in the peak region in the ChIP and in the corresponding input.

Peaks were mapped to reference genes and annotated as intragenic, intergenic, or promoter on the basis of the genomic position of each peak's midpoint:

-*promoter*: the peak position is within -2Kb upstream and 1Kb downstream from an annotated refgene start coordinate or TSS

-*Intragenic*: the peak position is inside an annotated refgene (> 1 Kb from the TSS to its 3' end).

-*Intergenic*: the peak position doesn't match neither the criterion for being intragenic nor promoter.

Qualitative and quantitative heatmaps of ChIP-seq enrichment for the indicated factor or modification were determined using compEpiTools package, a tool for computational epigenomics developed for the analysis, integration and simultaneous visualization of various epigenomics data types across multiple genomic regions in multiple samples.

To investigate if Myc binding intensity could affect regulation of gene expression we stratified Myc peak enrichments in 3 quantiles:

-for the 2h LPS time point, log2 enrichment was divided in: low intensity if lower or equal to 7.71, medium intensity if between 7.71 and 10. 05, high intensity if higher than 10.05

- for the 4h LPS time point, log2 enrichment was divided in: low intensity if lower or equal to 7.52, medium intensity if between 7.52 and 9.84, high intensity if higher than 9.84

- for the 8h LPS time point, log2 enrichment was divided in: low intensity if lower or equal to 7.11, medium intensity if between 7.11 and 9.63, high intensity if higher than 9.63.

### 3.14.3 RNA-seq data analysis

NGS reads were aligned to the mm9 mouse reference genome using the TopHat aligner (version 2.0.6) with default parameters (Trapnell et al., 2009). Read counts were associated to each exon using the HTSeq software (http://www.huber.embl.de/users/anders/HTSeq/doc/overview.html). The expression of a transcript X (eRPKM) was estimated as the reads per kilobase over the total number of aligned reads considering only exonic reads:

$$RPKM(X) = \frac{10^9 \cdot r(X_{Exon})}{R_{Exon} \cdot s(X_{Exon})}$$

where $r(X_{Exon})$ is the number of reads mapped to exons of X , $R_{Exon}$ is the sum of reads mapped to all exons (of all transcripts) in the experiment, and $s(X_{Exon})$ is the total length (in kilobases) of exons in X. We estimated the expression of a gene with more than one isoform as the mean of the expression of its isoforms.

### 3.14.3i Identification of differentially expressed genes

Differentially expressed genes (DEGs) were identified using the Bioconductor Deseq2 package. In order to call DEGs when multiple isoforms are present, the rounded mean of counts over the isoforms is used. Considering only protein coding genes and after removing the very low expressed genes (that never reached eRPKM of 1 in any condition analyzed), we obtained a set of 11614 expressed genes and we defined DEGs as genes whose q-value relative to the control is lower than 0.05.

The different categories of Myc dependent LPS response and Myc independent LPS response, were identified as following:

- Myc-dependent genes were defined as: regulatory group 1 or Myc-dependent induced (DEGs in wt OR myc$^{\Delta/\Delta}$ cells with qvalue<=0.05 & log2FCwt>0.58 & log2FCwt>(log2FCflox+0.58) which means genes significatively more up in wt than in *c-myc*$^{\Delta/\Delta}$ cells); regulatory group 2 (DEGs in wt OR *c-myc* $^{\Delta/\Delta}$ cells with qvalue<=0.05& log2FCwt>0.58 & log2FCwt<(log2FCflox-0.58) which are genes significatively more up in c-myc$^{\Delta/\Delta}$ than wt cells); regulatory group 3 or Myc-dependent repressed (DEGs in wt OR myc $^{\Delta/\Delta}$ cells with qvalue<=0.05 & log2FC<-0.58 & log2FCwt<(log2FCflox-0.58) which are genes significatively more down in wt than in *c-myc*$^{\Delta/\Delta}$ cells); regulatory group 4 (DEGs in wt OR *c-myc*$^{\Delta/\Delta}$ cells with qvalue<=0.05 & log2FCwt<-0.58 & log2FCwt>(log2FCflox+0.58) which are genes significatively more down in myc$^{\Delta/\Delta}$ than in wt cells).

- Myc-independent genes were defined as DEGs in wt OR *c-myc* $^{\Delta/\Delta}$ cells with the same fold change, but considering only fold changes bigger than 0.58 and +- 0.3 as threshold:

-regulatory group 5 or Myc-independent induced (DEGs in wt OR *c-myc*$^{\Delta/\Delta}$ cells with qvalue<=0.05 & log2FCwt>0.58 & log2FCflox>0.58 & log2FCwt<=(log2FCflox+0.3) & log2FCwt>=(log2FCflox-0.3) which are genes positively regulated not in a myc dependent manner);

-regulatory group 6 or Myc-independent down (DEGs in wt OR *c-myc*$^{\Delta/\Delta}$ cells with qvalue<=0.05 & log2FC<-0.58 & log2FC<-0.58 & log2FCwt<=(log2FCflox+0.3) & log2FCwt>=(log2FCflox-0.3) which are genes negatively regulated not in a myc dependent manner).

- No_DEGs (qvalue>0.05 in wt AND *c-myc* $^{\Delta/\Delta}$ & -0.4<log2FCwt<0.4 & -0.4<log2FCflox<0.4 which are genes that didn't change their expression in the two fenotypes).

### 3.14.4 Stalling index analysis

In order to study promoter-proximal pausing of Pol II, a post-initiation regulatory event, we calculate the RNA polymerase II stalling index (SI, also called elongation rate) (Rahl et al., 2010; Frank et al., 2001). It is the ratio between the read counts on the promoter (TSS ±300 bp interval) and the read counts in the gene body (the interval between TSS +301 and 3,000 bp after the TSS). These values were normalized both to library size (total number of reads) and to the length of the interval, and only genes with GB>600 bp and with a RNAPII ChIP-seq peak in the region [TSS - 2,000; TSS + 1,000 bp] were considered.

### 3.14.5 Motif analysis

To identify motifs of TFs, the enrichment of position weight matrices (PWMs) was computed on the set of sequences corresponding to the +/-75bp regions around the summit of Myc peaks on promoters of the genes of each different category of DEGs. In order to find a significant enrichment (zscore at least ≥3) we considered a genomic background characterized by sequences at the peaks of Myc on promoters corresponding to not regulated genes (noDEGs).

The presence of canonical and non canonical Eboxes (Perna et al.,2012) was also identified at genes with a Myc peaks on promoters in the region +-75 bp around the peak summit.

## 3.15 Gene ontology and gene set enrichment analysis (GSEA)

Functional annotation analysis to determine enriched Gene Ontology was performed using Molecular signature database (MsigDB) of GSEA Broad Institute or DAVID (Huang

et al., 2008) . Pre-ranked Gene set enrichment analysis was also performed (Subramanian et al., 2005).

### 3.16 Other bioinformatic and statistical analysis

Differentially expressed genes were also analyzed by Ingenuity Pathway Analysis (IPA) version 9.0 (Redwood City, CA, http://www.ingenuity.com) software. Briefly, a list of genes with corresponding expression level were uploaded and core analysis was performed in IPA. Canonical pathways obtained in this study were identified from the IPA library based on Fisher's Exact Test P-value.

Bioinformatic and statistical analysis, including heatmaps of ChIP-seq data and plots for DEGs analysis of RNA-seq and other visual representation of the data were performed using R with Bioconductor (J Zhang et al 2004) and compEpiTool packages.

All the experiments, except for ChIP and RNAseq, were performed in biological duplicates or triplicates. Two tailed-Student t test was used to calculate P value. Significant values are reported in the figure legends.

# 4 Results

## 4.1 An *in vitro* model for conditional deletion of c-Myc in B cells

In order to map Myc-dependent transcriptional and genomic responses in a physiological context, we took advantage of primary mouse B cells homozygous for a conditional *knockout* allele bearing loxP sites in the first intron and in the 3'-untranslated region of the *c-myc* gene (Trumpp et al., 2001) (**Figure 13a** henceforth *c-myc* $^{f/f}$ cells). Using a strategy analogous to that followed in our laboratory for the screening of Myc dependent serum response (MDSR) genes in fibroblast (Perna et al.2012), conditional deletion of c-*myc* was induced before cell activation by lipopolysaccharide (LPS) stimulation (**Figure 13b)**.

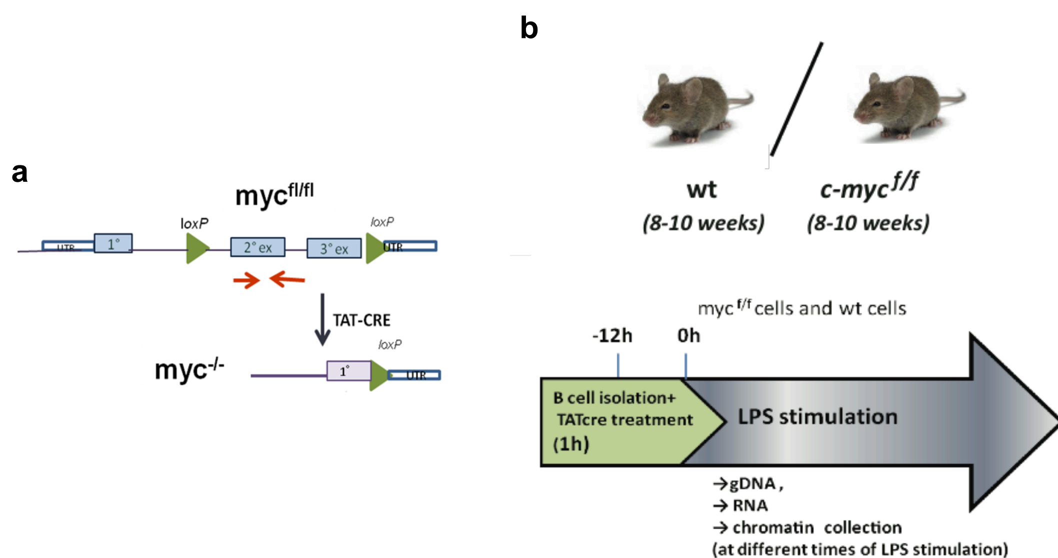



**Figure 13** ***Schematic representation of the experimental approach*.**
**a**) Design of the genomic position of the primers designed to detect the presence of deleted (myc $^{-/-}$) and non-deleted (myc $^{fl/fl}$) *c-myc* allele. **b**) Splenic wt and *c-myc* $^{f/f}$ B cells isolated from mice are treated with TAT-cre for 1 h in optimem with 1% of serum. After 12h, the cells are stimulated with LPS and gDNA, RNA and chromatin are collected at various time points for profiling.

Cells were isolated from 8-10 weeks old wt and homozygous *c-myc* $^{f/f}$ mice. Mature splenic B cells were purified through depletion of all other white blood cells, dendritic and erithroid cells ("non B cells") present in the spleen. As assessed by flow cytometry (FACS), the B cell fractions were in large part (85%) B220 and IgM positive, while the discarded cells were negative for both markers (**Figure 14**). B cells were treated *in vitro* with a recombinant TAT-cre protein to induce deletion of *c-myc* allele and then plated in mitogen free medium. After 12h, the quiescent cells were stimulated with LPS to induce cellular activation and re-entry into the cell cycle (Kelly et al., 1983): different time points of stimulation were then considered to compare their transcriptional and epigenetic profiles.

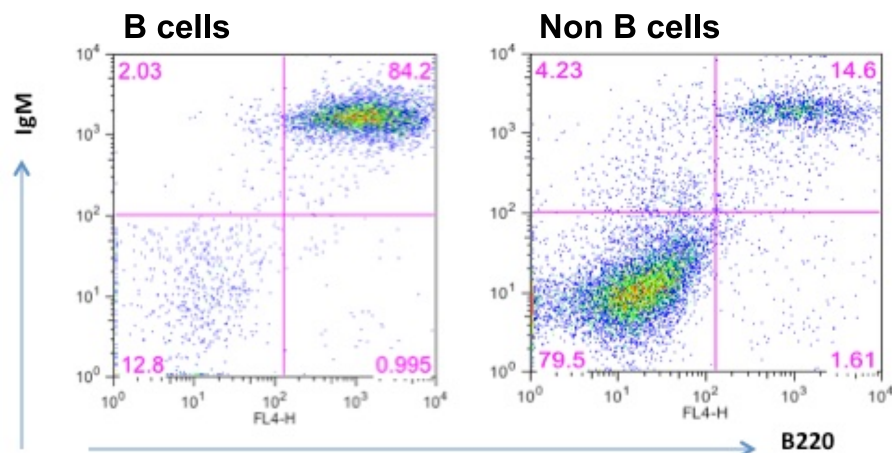



**Figure 14** ***Isolation of mature B cells.***
FACS profile of purified splenic mature B cells used in this study (on the left) respect to the other lymphoid cells discarded (on the right).

TAT-cre is a fusion protein in which an 11–residue domain of HIV TAT protein is juxtaposed to the cre recombinase in order to make it able to pass the plasma membrane, reach the nucleus and mediate deletion of loxp-flanked targets (Capasso et al., 2009; Joshi et al., 2002). Since its effectiveness depends on its uptake by target cells, we optimized the conditions of TAT-cre delivery in our system.

We treated *c-myc* $^{fl/fl}$ B cells with different TAT-cre concentration and for different times of exposure (**Figure 15**). Since we also tested in the same experiment the toxicity of the fusion protein through Trypan blue direct counting, we observed that more than one hour of treatment originated an increase in cell death compared to untreated cells explaining the inadequate efficiency of deletion in the remaining alive cell population. Approximately 80% of deletion was instead achieved after one hour of treatment already at the lowest concentration without a toxicity effect compared to untreated cells.

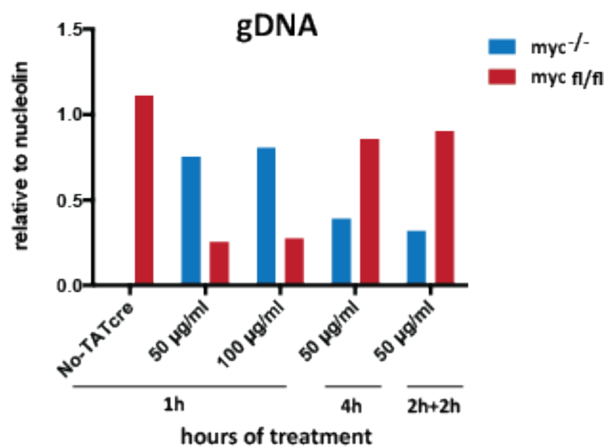



**Figure 15** ***Setting up of the TAT-cre treatment conditions in terms of time exposure.***
qPCR of gDNA showing the levels of myc $^{-/-}$ and myc $^{fl/fl}$ alleles relative to the nucleolin amplicon, in *c-myc* $^{fl/fl}$ B cells treated with 100 or 50 µg of TAT-cre for 1h, 4h or a double shot of 2h of incubation in optimem plus 1% of serum. Negative control (cells not exposed to the TAT-cre protein, No TAT-cre) is also shown.

Since it was shown that fetal calf serum (FCS) and bovine serum albumin (BSA) inhibited the efficiency of TAT-cre deletion in erythroblastic leukemia cells (Joshi et al., 2002), we also tested different concentration of these reagents in optimem during TAT-cre incubation with our cells. Coating with BSA or serum before protein treatment and addition of 1% of serum during incubation were equivalent strategy in term of deletion efficiency. For practical reasons we decided to use as standard condition TAT-cre incubation with 1% of serum in optimem, a good compromise in terms of deletion efficiency and lack of cell attachment to the plastic (**Figure 16**).

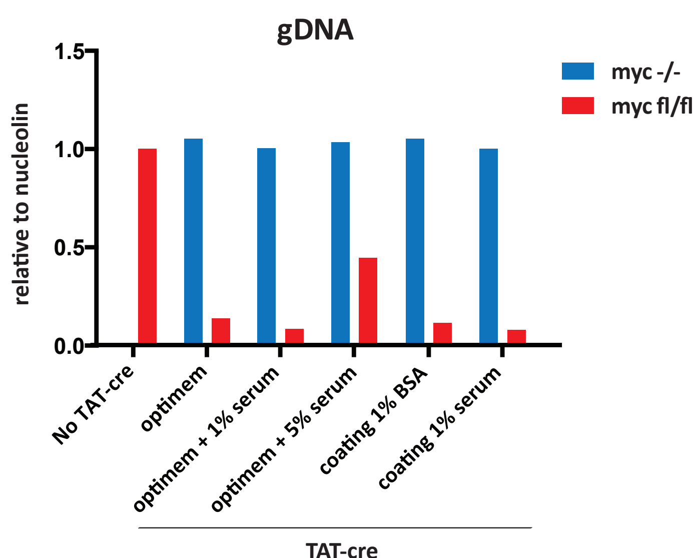



**Figure 16** ***Setting up of the TAT-cre treatment in terms of conditions of incubation.***
qPCR of gDNA showing the levels of myc $^{-/-}$ and myc $^{fl/fl}$ alleles relative to the nucleolin amplicon, in *c-myc* $^{fl/fl}$ B cells treated with 50 µg of TAT-cre for 1h in optimem, in optimem plus 1% of serum, optimem plus 5% of serum and in optimem after coating of the falcons with 1% of BSA or 1% of serum. Negative control (cells not exposed to the TAT-cre protein No TAT-cre) is also shown.

Considering also that the TAT-cre protein is purified from bacteria (Capasso et al., 2009) and may thus be contaminated with bacterial lipopolysaccharides, we checked whether it

could induce an inflammatory response in B cells. We verified that immediately after TAT-cre treatment, some LPS responsive genes were induced (*ccl5, ikβα, junb*) in a similar extent as after LPS stimulation (the positive control) but in a transient manner (**Figure 17**). 12h after TAT-cre treatment, when we started the LPS treatment, these genes have returned at a basal level and were still LPS responsive (**Figure 17**; **Figure 18**).

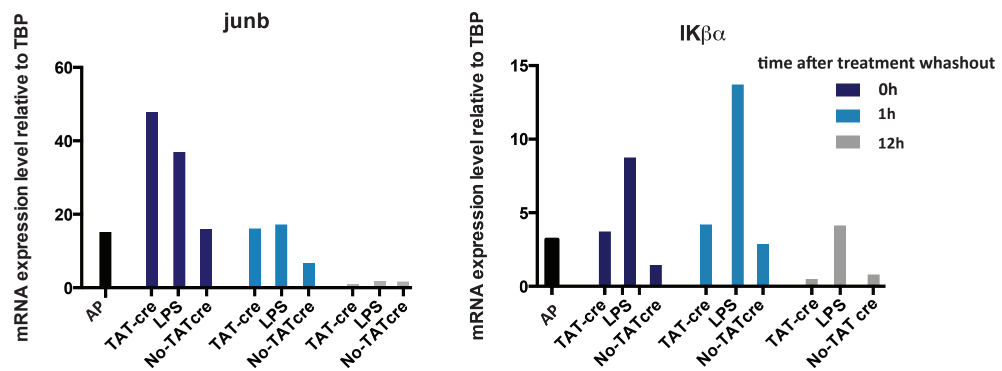



**Figure 17** ***LPS target genes transcriptional response after TAT-cre treatment.***
RT-qPCR of *junb* and *Ikβα* is reported. mRNA expression levels normalized to TBP are shown for control cells taken immediately after purification (AP) , cell taken immediately after and whashout (0h), or 1 or12 h after washout (the later corresponding to the time at which we wanted to activate cells with LPS). Treatments were: TAT-cre, No TAT-cre (50% glycerol) or LPS (50 µg/ml LPS) for 1 hour incubation.

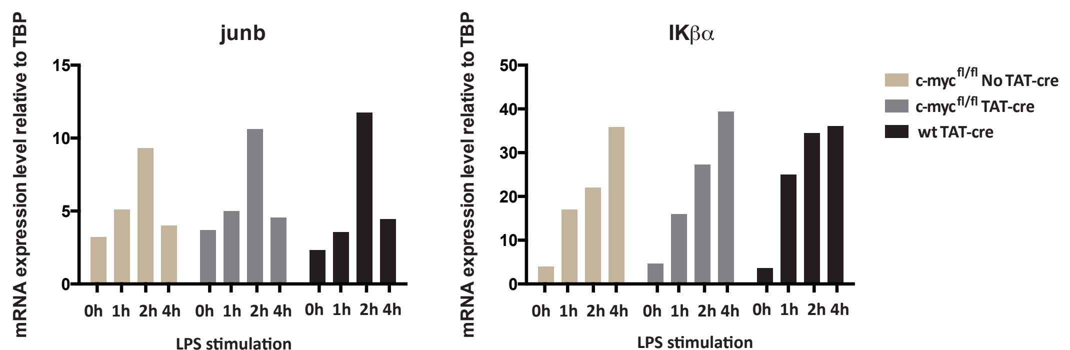



**Figure 18** ***Active transcriptional response of LPS target genes.***
RT-qPCR of *junb* and *Ikβα* is reported. mRNA expression levels normalized to TBP are shown for in *c-myc* $^{fl/fl}$ cells treated or not with TAT-cre and wt cells TAT-cre treated. RNA was collected 12h after treatment (0h LPS) and 1,2 and 4h after LPS stimulation.

On the basis of these observations we decided to always use as control wt B cells treated with TAT-cre, instead of untreated c-myc$^{f/f}$ cells, to take into account possible unspecific effects of TAT-cre. Therefore, we decided to treat c-myc $^{f/f}$ and wt cells with 50 µg/ml of TAT-cre in agitation at 37° C for one hour in optimem plus 1% serum.

qPCR analysis of genomic DNA indicated that the *c-myc* $^{f/f}$ allele was deleted in ca, 80% of the cells 12h after TAT-cre treatment (c-*myc*$^{\Delta/\Delta}$ cells: t0 of LPS treatment) (**Figure 19**). Over the time (at 48 and 72h) we observed a gradual loss of the c-*myc*$^{\Delta/\Delta}$ alleles in favour of the undeleted one similarly to what already reported in previous studies on *c-myc* deletion in B cells *in vivo* (de Alborán et al., 2001). This was most probably due to the counterselection of myc deleted cells in the overall population that continue to divide overcoming the myc deleted cells.

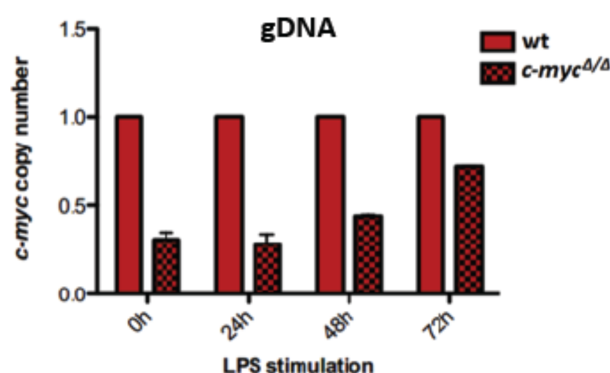



**Figure 19** ***Efficiency of c-myc deletion at genomic DNA level.***
q-PCR analysis of gDNA to assess *c-myc* copy number in wt and *c-myc* $^{\Delta/\Delta}$ cells. The data are normalized to *nucleolin* amplicon and represent the average ± s.d. of 3 independent experiments.

Consistent with the efficiency of deletion measured on the gDNA, we also observed reduced *c-myc* mRNA and protein accumulation upon LPS stimulation in the floxed

population (**Figure 20; Figure 21**). In control cells, we observed a peak of *c-myc* mRNA and protein induction at 2h of LPS stimulation, followed by a progressive decrease over. It 's well established, indeed, that a mitogenic stimulus rapidly induces the expression of *c-myc* favoring B cells to re-enter in G1-S phase of the cell cycle (Kelly et al., 1983). Afterwards, with the consequent transition from B cells to plasma cells, others transcription factors start to play important roles in controlling each phase of plasma cells development. Not surprisingly, one way by which the master transcription factor Blimp-1 promotes generation of plasma cells is the repression of c-Myc, thereby allowing the B cell to exit the cell cycle and undergo terminal differentiation (Lin Y et al.1997, Yu J et al., 2000).

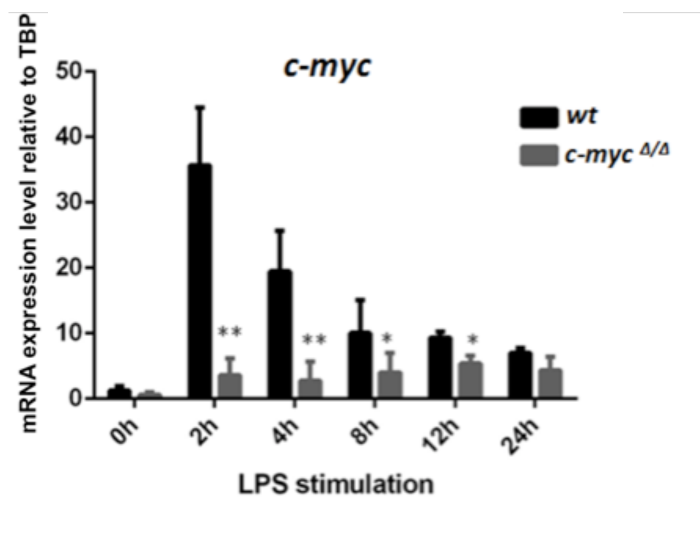



**Figure 20** ***Myc expression in wt and c-myc*** $^{\Delta/\Delta}$ ***cells.*** *c-myc* mRNA level were measured by RT-qPCR in control and deleted cells followed by LPS stimulation as indicated. The data were normalized to TBP expression. The average ± s.d. of 3 independent experiments is shown, *P<0.05, **P<0.001.

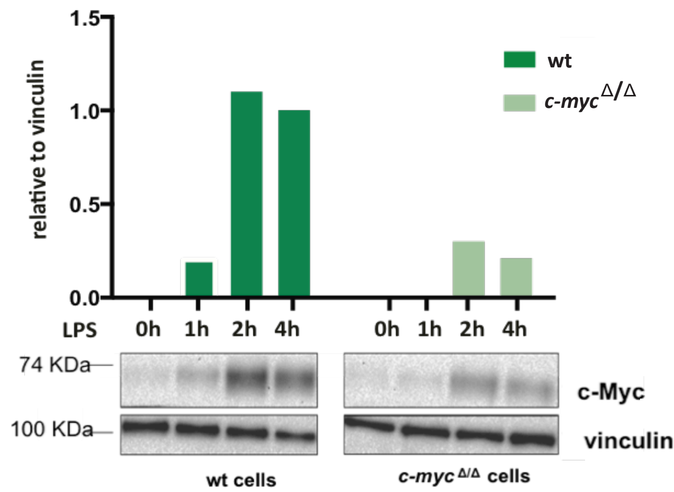



**Figure 21** ***Myc protein levels in wt and c-myc deleted B cells.***
Immunoblot of cell extracts from wt and c-myc $^{\Delta/\Delta}$ B cells at 0, 1, 2, 4h post LPS-activation and corresponding quantification are shown.

In conclusion, we set up a clean model system to perform loss of function experiments to investigate the role of c-Myc in activated B cells at short time points (up to 48h). Besides, since RT-qPCR analysis showed that N-myc and L-myc are not expressed in B cells in that condition (**Figure 22**) we can attribute any phenotypic effects observable only to *c-myc* deletion.

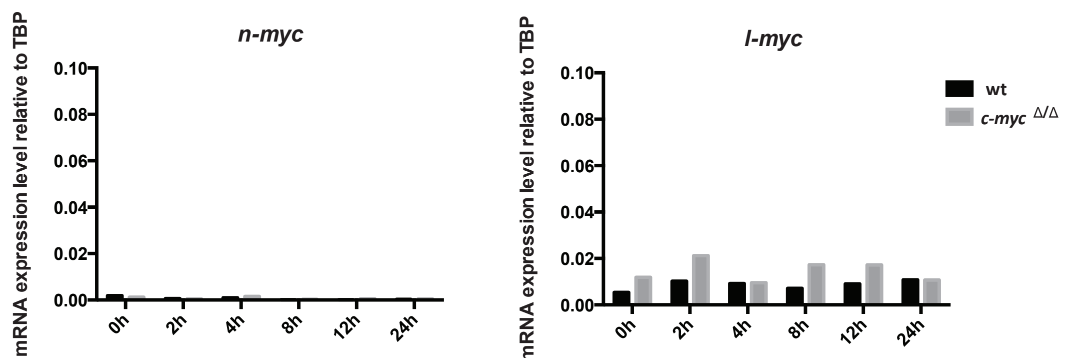



**Figure 22** ***N-myc and L-myc expression in B activated cells.***
*n-myc and l-myc* mRNA level were measured by RT-qPCR in control and deleted cells followed by LPS stimulation as indicated. The data were normalized to TBP expression.

## 4.2 Impaired mitogenic response of c-*myc*$^{\Delta/\Delta}$ cells

A vast amount of published data points to Myc as a master regulator of cell proliferation. Deletion of the *c-myc* gene in RAT1a fibroblasts produced a significant delay in S phase entry resulting in a prolonged cellular doubling time while, on the contrary, ectopic Myc expression in non-proliferating fibroblasts induced S phase entry and shorter doubling time (Mateyak et al., 1997; Palmieri et al., 1983). In B cells specifically, de Alborán's lab showed that activation and proliferation upon CD40+IL4 treatment is *c-myc* dependent (de Alborán et al., 2001). We thus decided to check if there was an impairment of the proliferative response in *c-myc*$^{\Delta/\Delta}$ B cells stimulated with LPS. Direct cell counting showed a clear reduction in proliferation of *c-myc*$^{\Delta/\Delta}$ cells compared to wt cells (**Figure 23**). Proliferation in *c-myc*$^{\Delta/\Delta}$ cultures can be mainly explained by the outgrowth of non-deleted cells, as shown by the increased frequency of the undeleted *c-myc*$^{f/f}$ allele at 48h and 72h post LPS stimulation (**Figure 19**).

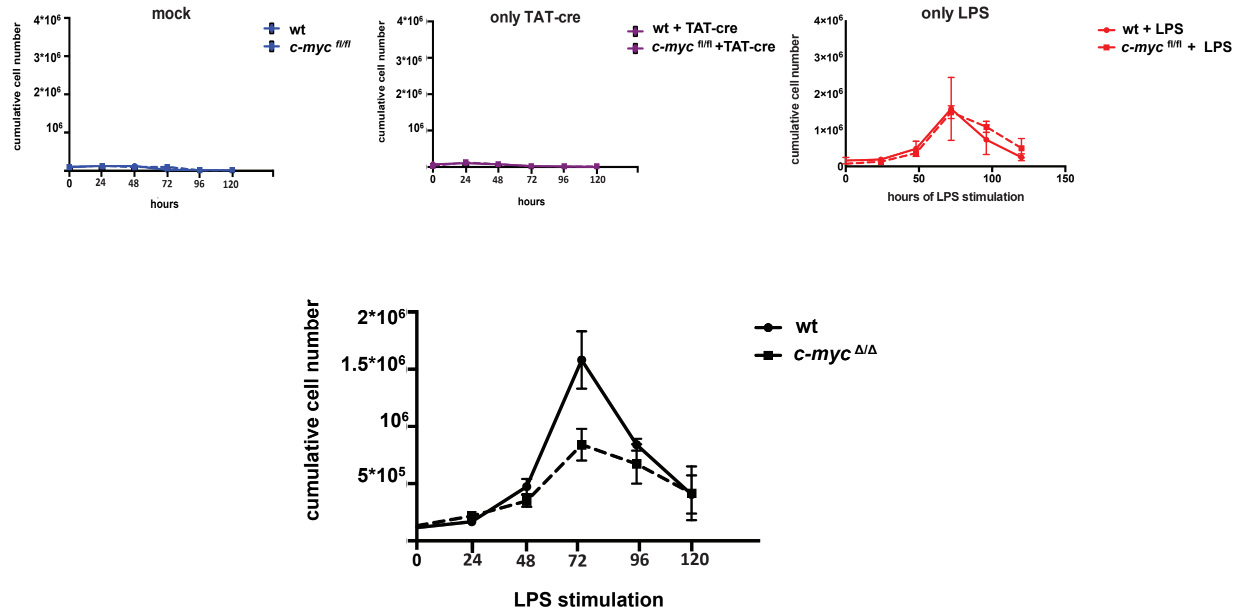



**Figure 23** ***Proliferation of Myc-deficient B cells is impaired compared to wt B cells.***
Growth curves of wt and fl/fl cells upon different treatments. In the upper part 3 control curves are shown: wt and *c-myc* $^{fl/fl}$ cells without any treatment, wt and *c-myc* $^{fl/fl}$ cells after TAT-cre only or LPS only. Below, growth curves of wt and fl/fl cells treated with TAT-cre stimulated in culture with LPS are shown. The average ± s.d. of 3 independent experiments is reported.

We further addressed if the reduced growth rate of *c-myc*$^{\Delta/\Delta}$ cells was due to increased apoptosis, decreased proliferation or both. Caspase activity measurement and Trypan blue exclusion assay showed, even if in different extent, that wt cells start to die at 72h of activation and then undergo a massive cell death. On the contrary, *c-myc*$^{\Delta/\Delta}$ cells do not display the same behavior as previously published (de Alborán et al., 2004; Murn et al., 2009) (**Figure 24**). de Alboran and colleagues propose a possible explanation for this phenotype, which remains to be confirmed in the current system. Since CD95/CD95L is induced in mitogenic activated B cells and leads to susceptibility to apoptosis via CD95 pathway, it was shown that c-*myc*$^{\Delta/\Delta}$ cells express low surface CD95 and CD95L levels and when treated with anti-CD95 antibody or staurisporine show resistance to CD95-induced apoptosis than control cells. In addition, non-activated c-Myc-deficient B-lymphocytes

were observed to be more resistant to cell death that an activated wt cells (de Alborán et al., 2004) arguing for an important Myc role on this process.

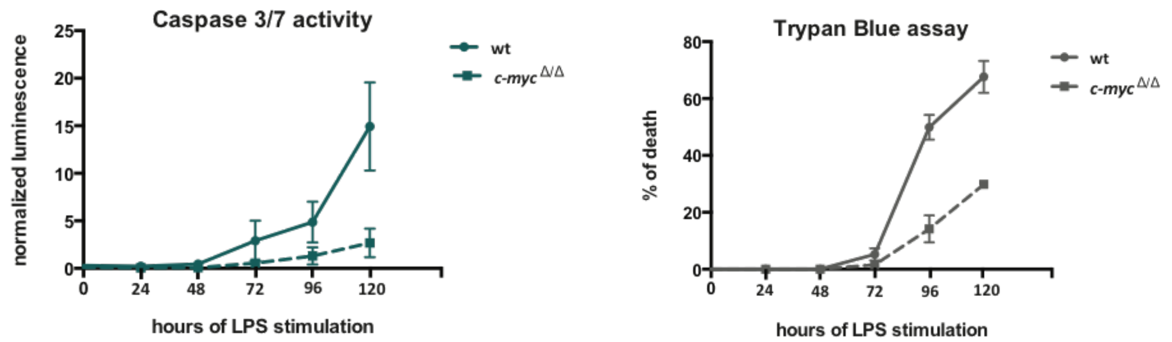



**Figure 24** ***Myc-deficient B cells are less prone to apoptosis.***
Left: Caspase 3/7 activity as a measure of apoptotic cell death in normal and *c-myc* $^{\Delta/\Delta}$ cells; Right: percentage of dead cells counted by Trypan blue. The average ± s.d. of 3 independent experiments is reported.

We then checked if Myc-deleted cells might have a proliferative defect through cell cycle analysis. Following a pulse of BrDU labelling in wt and *c-myc*$^{\Delta/\Delta}$ cells at different time points of LPS stimulation we observed by FACS analysis an accumulation of *c-myc*$^{\Delta/\Delta}$ cells in G0-G1 phases (**Figure 25 b**) with a concomitant delay in S phase entry resulting in the decrease rate of proliferation (**Figure 23; Figure 25**). In fact wt B cells start to enter in S phase after 12h of LPS stimulation, while *c-myc*$^{\Delta/\Delta}$ cells accumulate more in G1 phase and incorporate less BrDU indicating that are not prone to duplicate and progress into the cell cycle as wt cells (**Figure 25 a**).

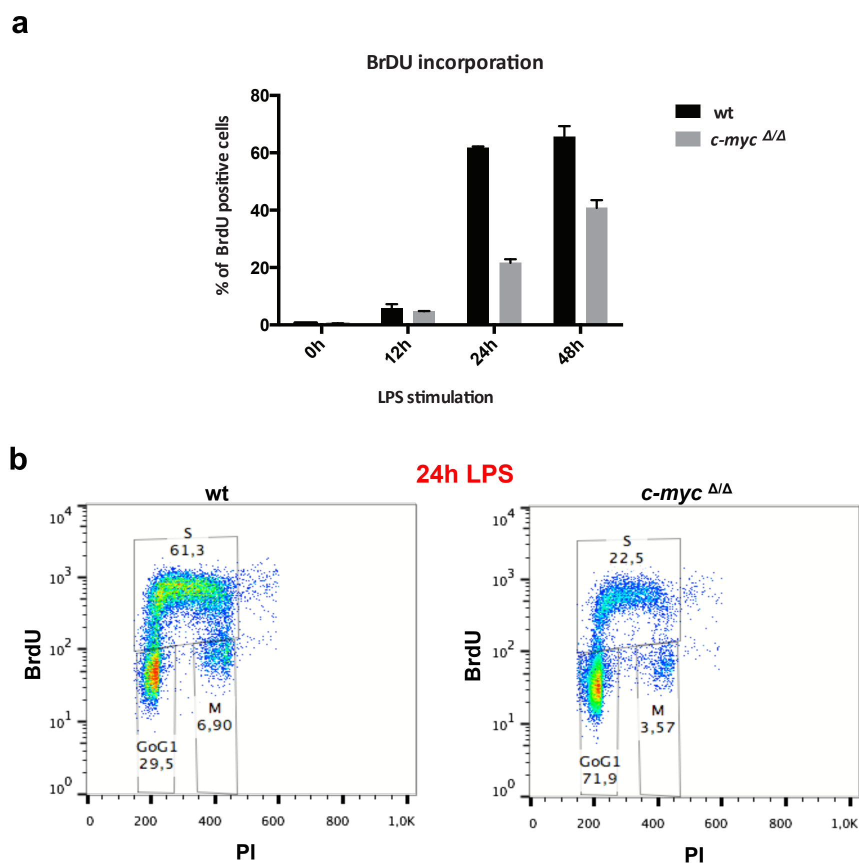



**Figure 25** ***Conditional deletion of c-myc in quiescent B cells impairs cell-cycle re-entry.*** a) Percentages of cells in S-phase at 0, 12, 24 and 48h of LPS stimulation in wt and *c-myc* $^{\Delta/\Delta}$ cells is shown. BrdU was added to the culture medium for 30 min at each time points and incorporation was assessed by flow cytometry. The average ± s.d. of two independent experiments is reported. b) FACS profile of wt and *c-myc* $^{\Delta/\Delta}$ cells at 24h after LPS stimulation is reported as example. Cells after BrdU incorporation and ethanol fixation were stained with anti-BrdU detected via FITC labeled antibody and also with Propidium iodide (PI) for visualization of total DNA content. Percentages of cells in each phase of cycle (Go-G1, S, M) were reported.

## 4.3 Impact of Myc on cell size, RNA content and plasma cell differentiation

In normal cells such as fibroblasts and naïve T cells, mitogenic growth factor signaling promotes cell activation through the induction of Myc expression, which in turn enhances the transcription of metabolic and proliferation-associated genes (Eilers and Eisenman, 2008; Wang R et al., 2011). Upon stimulation of antigen receptors, quiescent naïve B cells,

similarly to T cells, undergo metabolic activation with a dramatic increase in RNA production and protein synthesis. This active metabolic state sustains cell division, clonal expansion and differentiation in plasma cells or long-lived memory cells (Rajewsky, 1996). c-Myc has a fundamental role in this mitogen-induced growth of mammalian cells (Grumont et al., 2002; Eisenman, 2001; Schuhmacher et al., 1999). Since it has been proposed that the increase in cellular RNA content that can be observed during B cell activation (Kouzine et al., 2013) could be due to the positive effect that Myc exerts on the transcription of all expressed genes (the so-called "transcriptional amplification") (Nie et al., 2012), we decided to measure the RNA content per cell in wild type and *c-myc* $^{\Delta/\Delta}$ cells. We did find a massive increase in total RNA after 24 and 48h of LPS stimulation in wild type cells that was less pronounced in *c-myc* $^{\Delta/\Delta}$ cells but we could not score a significant difference in the first 12h as previously reported (Nie et al., 2012) (**Figure 26**).

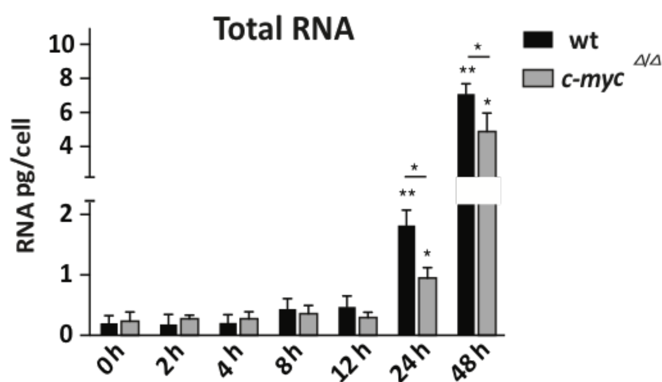



**Figure 26** ***Increase in RNA levels during B cells activation.***
Levels of total RNA in wt and *c-myc* $^{\Delta/\Delta}$ B cells at different time points upon LPS stimulation. The average ± s.d., of 3 independent experiments is shown, *P<0.05, **P<0.001. Adapted from Sabò et al. 2014.

At the same time, we also observed an increase in cell size during LPS treatment in a Myc-dependent manner. RNA content does not show a significant increase within the first 12 hours of LPS stimulation, whereas the enhancement in forward scatter (as measure of cell size) resembles a more gradual process starting soon after Myc induction (**Figure 27**).

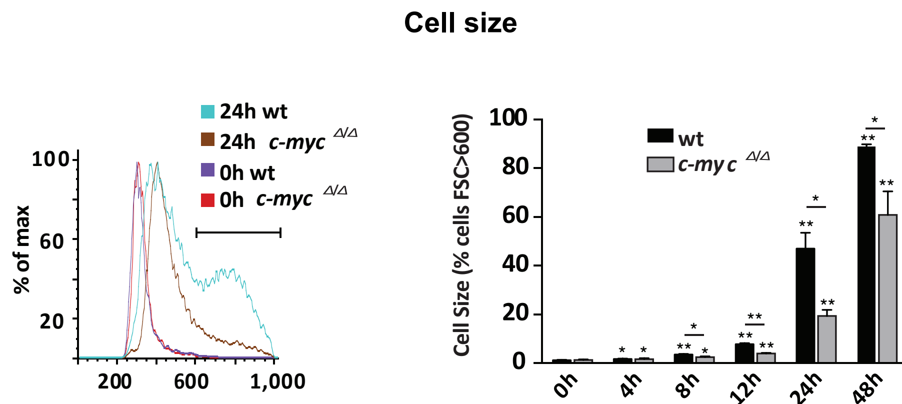



**Figure 27** ***Increase in cell size during B cells activation.***
Left: Distribution of FSC values of wt and *c-myc* $^{\Delta/\Delta}$ B cells as measured by FACS. Right: Percentage of cells with forward scatter bigger than 600 was reported. The average ± s.d., of 3 independent experiments is shown, *P<0.05, **P<0.001. Adapted from Sabò et al. 2014. Below the quantification graph, images of wild type B cells resting or activated for 48h are shown under 40X magnification of canonical optical microscope.

At 48h, the differences in RNA content and cell size between wt and *c-myc*$^{\Delta/\Delta}$ cells are reduced compared to 24h, due to recovery of the *c-myc*$^{\Delta/\Delta}$ cells. Since we know that there is counter-selection of deleted cells at later time points, we decided to sort myc deleted and wt cells according to their size and then check the genotype on the sorted cells. At 24 and 48h of LPS stimulation, the activated cells are bigger compared to resting condition and are mainly *c-myc* wt even when sorted from the *c-myc* $^{\Delta/\Delta}$ population suggesting that they are most probably escapers. On the other hand, we found that resting cells are

smaller and mainly *c-myc* deleted; only a minor part (ca. 24% at 48h LPS) of activated, big cells, are actually myc knockout. (**Figure 28; Figure 29**).

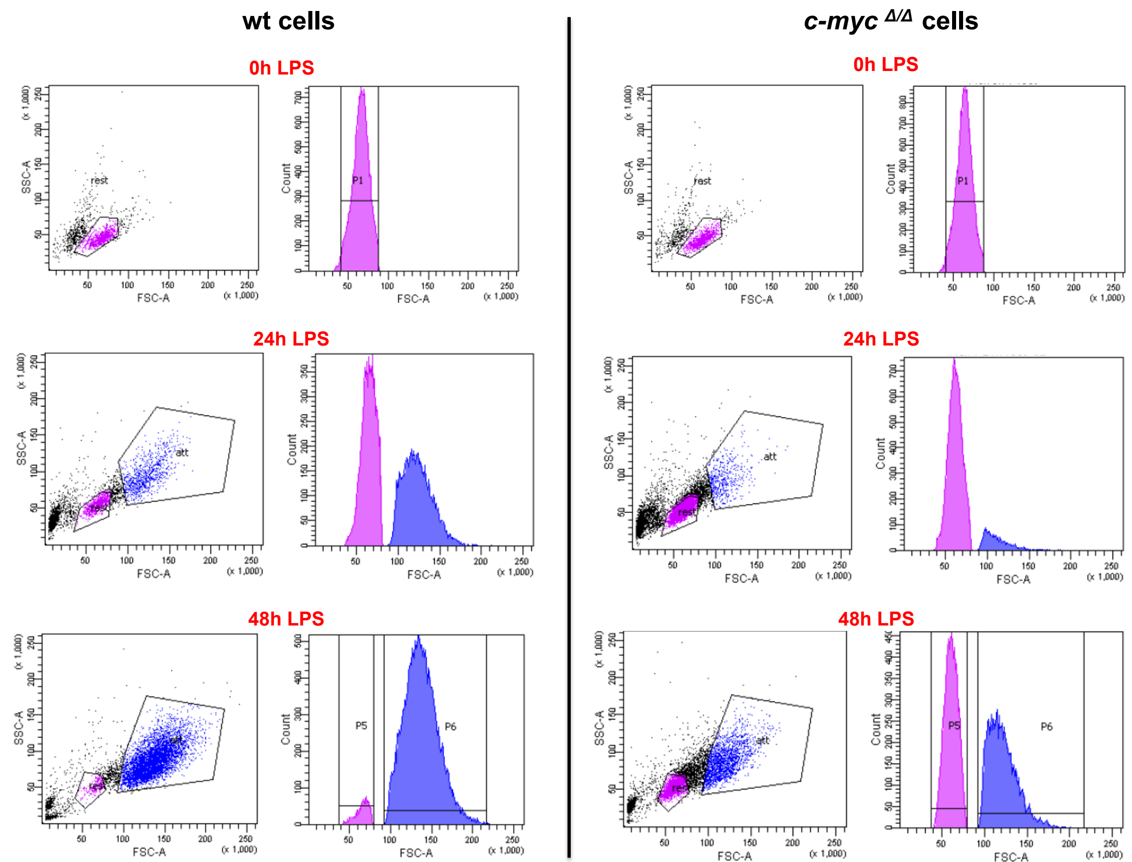



**Figure 28** ***Activation of wt and c-myc*** $^{\Delta/\Delta}$ ***cells.***

FACS analysis of sorted mature B cells from wt (on the left) and *c-myc* $^{\Delta/\Delta}$ (on the right) mice based on cell size (forward scatter) and internal complexity (side scatter). Cells were sorted before or after LPS stimulation (0, 24 and 48h LPS). Gate of activated cells (in blue) is reported as att; while rest stands for resting cells (in pink).

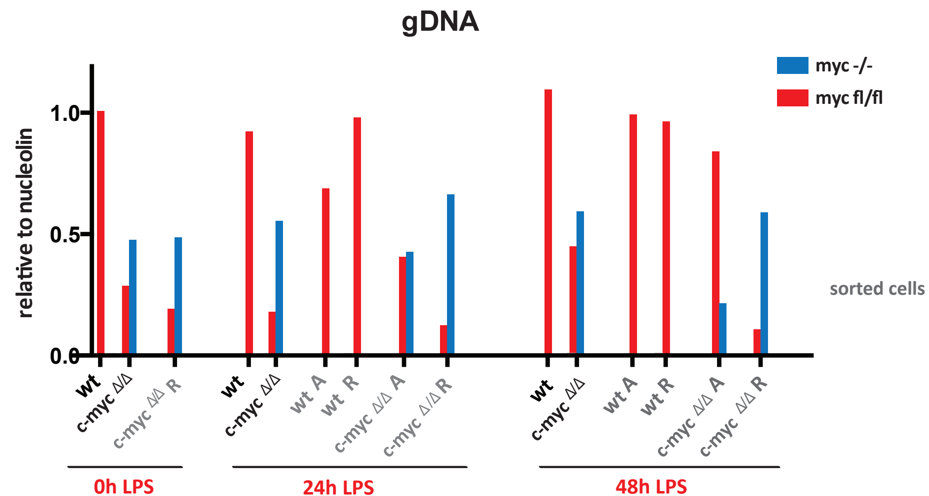



**Figure 29** ***Genomic analysis of myc level in different sorted cells populations.***
Relative amount of amplicon c-myc fl/fl and myc -/- genomic DNA measured by quantitative RT–PCR in wt and, *c-myc* $^{\Delta/\Delta}$ cells before sorting (labels in black) and after sorting (labels in grey) after 0,24 and 48h of LPS stimulation: wt and *c-myc* $^{\Delta/\Delta}$ A (activated), wt and *c-myc* $^{\Delta/\Delta}$ R (resting) sorted cells are shown. Values were normalized to a PCR amplicon in the Nucleolin locus.

In conclusion, upon LPS-mediated activation, B cells increase in size and RNA content in a Myc-dependent manner. What we cannot conclude from this data is whether the role of Myc here is to amplify the expression of all active genes and thus make the cells to produce more RNA and get bigger, or whether it regulates key genes involved in cell cycle entry, cell growth, metabolism and other cellular features that may then feed back on the global RNA production (Sabò et al., 2014). In the latter scenario, the reduced amount in RNA content and cell size in Myc-deleted cells would actually follow from defects in cellular activation. Although Myc induction has a fundamental role in proliferation of B cells, Myc repression was also reported to be a necessary step for terminal plasma cell differentiation (Lin K. et al., 2000; Lin Y et al., 1997). We thus analyzed by qPCR the expression of 3 genes associated to plasma cells differentiation (*Blimp1*, *Xbp1*, *Bcl6*) in wt and *c-myc* $^{\Delta/\Delta}$ cells. We found that while *Blimp1* was more induced in deleted cells, Xbp1 was not affected by Myc and *Bcl6* was less repressed in deleted cells (**Figure 30**). In

conclusion, we could at least exclude any important role of Myc on plasma cell differentation.

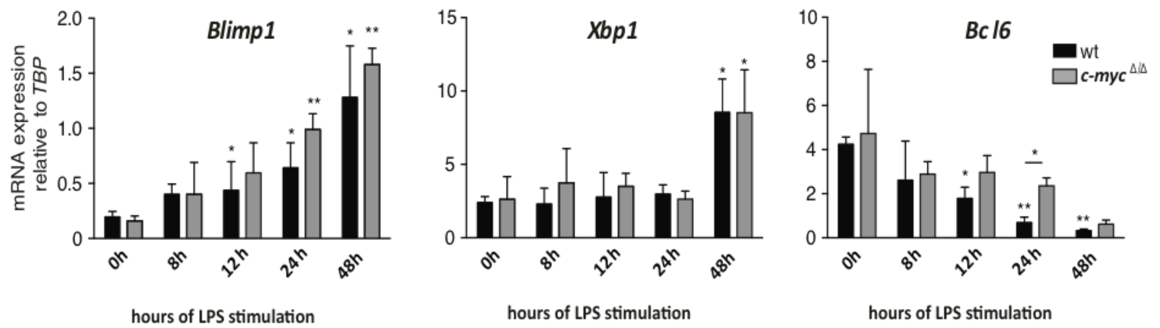



**Figure 30 Gene *expression quantification of markers of plasma cell in wt and c-myc* $^{\Delta/\Delta}$*cells.***
Quantitative qPCR of 3 genes regulated during plasma cells differentiation (*Blimp1, Xbp1, Bcl6*) in wt and *c-myc* $^{\Delta/\Delta}$ cells at different time points after LPS stimulation. The data were normalized to TBP. The average ± s.d., of 3 independent experiments is shown, *P<0.05, **P<0.001. Adapted from Sabò et al. 2014.

In addition, FACS analysis of surface markers of plasma cell differentiation (CD44, CD138 and CD38) revealed changes in the level of CD38 and no differences in the distribution of the other markers between the two genotypes (**Figure 31**). We then concluded that *c-myc* $^{\Delta/\Delta}$ cells are still undergoing plasma cell differentiation.

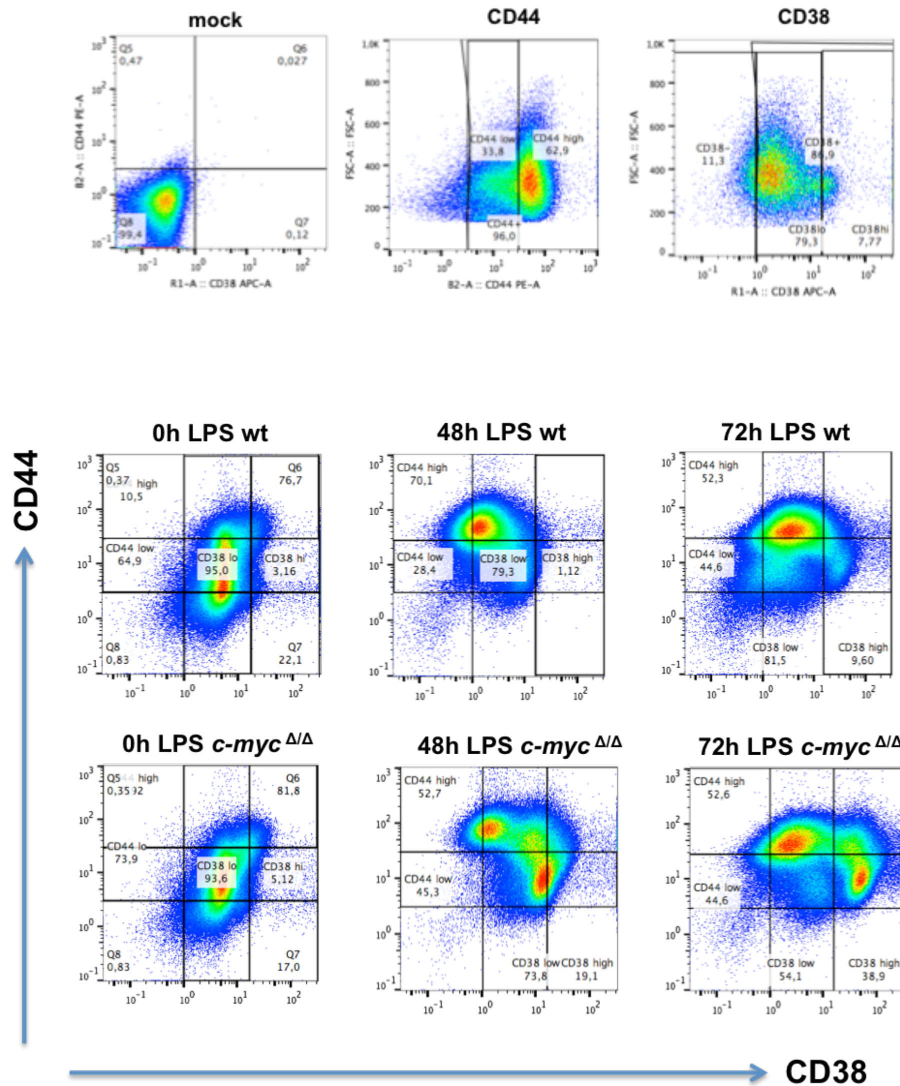



**Figure 31** ***Expression of plasma cells differentiation surface markers in wt and c-myc $^{\Delta/\Delta}$ cells by FACS***. Upper part (from left to right): mock staining, single staining for CD44 and CD38 with indicated gates for low and high intensity. Bottom part: Double staining of CD44 and CD38 in wt and *c-myc* $^{\Delta/\Delta}$ B cells at 48 and 72h after LPS stimulation.

## 4.4 Characterization of the Myc-dependent transcriptional response to LPS stimulation

To determine whether Myc binding at promoters implicates direct transcriptional regulation of the corresponding genes, we decided to profile total RNA by RNA-seq in wt and *c-myc*$^{\Delta/\Delta}$ cells at 0, 2, 4, 8 hr after LPS stimulation. As a preliminary control, we

monitored the expression of three Myc-dependent serum response (MDSR) genes previously identified in fibroblasts, *Gart, Smyd2* and *Ncl* (Perna et al., 2012). We used RT-qPCR to profile mRNA expression in control and *c-myc* deleted B cells following LPS stimulation (**Figure 32**). We observed impairment in the LPS-dependent induction of these genes in c-*myc* $^{\Delta/\Delta}$ *relative* to control. This suggests that, as in fibroblasts, the activation of these genes by mitogenic is Myc-dependent in B-cells. We thus proceeded with the full transcriptional profiling of the Myc-dependent response.

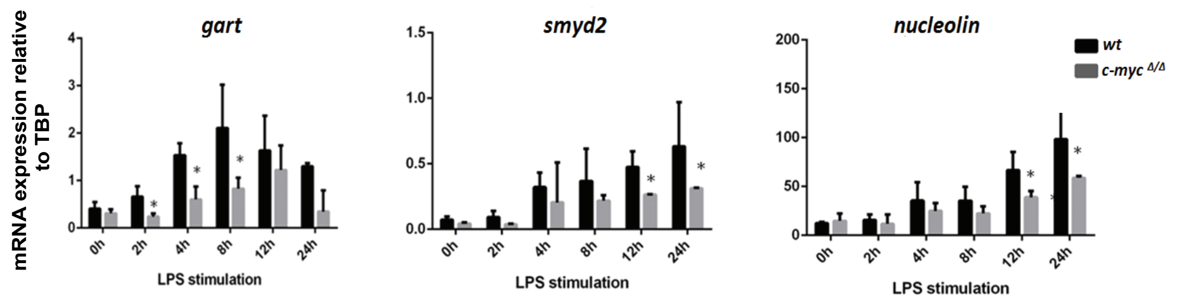



**Figure 32** ***c-myc deletion impaired the transcriptional activation of known Myc target genes in response to LPS.*** RT-qPCR of the 3 genes previously shown to be dependent on Myc for their induction by serum (Perna et al. 2012) in wt and *c-myc* $^{\Delta/\Delta}$ cells. The data were normalized to TBP expression. The average ± s.d. of 3 independent experiments is shown, *P<0.05, **P<0.001.

We profiled total RNA and considering the importance of data normalization highlight from authors of the amplifier model, we extracted RNA form the same amount of cells. Within 8h of LPS stimulation we didn't observe any RNA transcriptional amplification reasoning that a normalization of the data per cell equivalent (Lovén et al., 2012; Lin C et al., 2012; Nie et al., 2012) was not required.

We identified differentially expressed genes (DEGs) (q-value lower then 005) at each time point of LPS stimulation (for details see Materials and Methods) (**Figure 33**). The total numbers of DEGs increased during the time course as an expected effect of B cells activation.

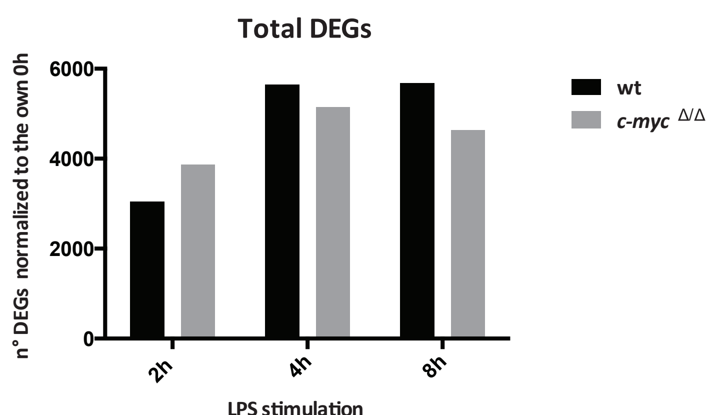
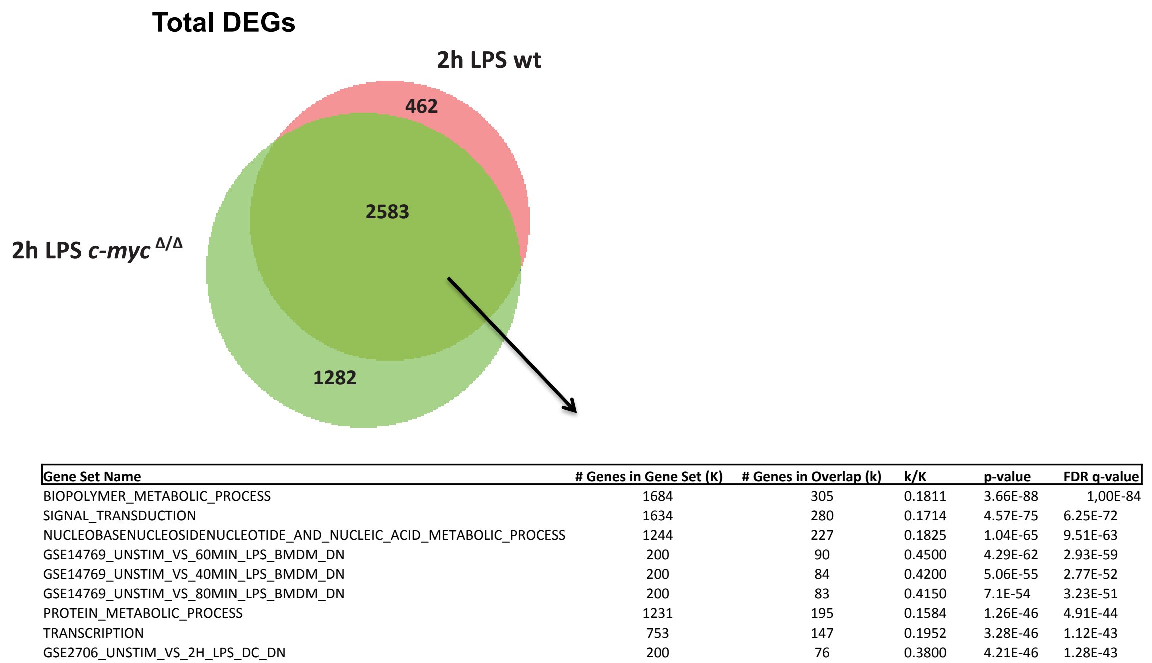



**Figure 33** ***Number of differentially expressed genes relative to untreated cells wt and c-myc*** $^{\Delta/\Delta}$ ***B cells.*** Total number of differentially expressed genes (DEGs) on the basis of q-value <0.05 at 2, 4, 8, after LPS stimulation respect to t0 in wt and *c-myc* cells.

Quiescent and transcriptionally poised B cells undergo a global transcription reprogramming soon after mitogenic activation occurs (Chen & Allfrey 1987; Fowler et al., 2013). Thus, we first checked wheter B cells transcriptionally responded to LPS irrespective to their genotype: both in wt and *c-myc* $^{\Delta/\Delta}$ 2h of mitogenic stimulation was enough to generate a similar transcriptional response that included genes involved in signal transduction processes such as the expected TLR signaling pathways with robust P values (**Figure 34**).




| Gene Set Name | # Genes in Gene Set (K) | # Genes in Overlap (k) | k/K | p-value | FDR q-value |
|---|---|---|---|---|---|
| BIOPOLYMER_METABOLIC_PROCESS | 1684 | 305 | 0.1811 | 3.66E-88 | 1,00E-84 |
| SIGNAL_TRANSDUCTION | 1634 | 280 | 0.1714 | 4.57E-75 | 6.25E-72 |
| NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS | 1244 | 227 | 0.1825 | 1.04E-65 | 9.51E-63 |
| GSE14769_UNSTIM_VS_60MIN_LPS_BMDM_DN | 200 | 90 | 0.4500 | 4.29E-62 | 2.93E-59 |
| GSE14769_UNSTIM_VS_40MIN_LPS_BMDM_DN | 200 | 84 | 0.4200 | 5.06E-55 | 2.77E-52 |
| GSE14769_UNSTIM_VS_80MIN_LPS_BMDM_DN | 200 | 83 | 0.4150 | 7.1E-54 | 3.23E-51 |
| PROTEIN_METABOLIC_PROCESS | 1231 | 195 | 0.1584 | 1.26E-46 | 4.91E-44 |
| TRANSCRIPTION | 753 | 147 | 0.1952 | 3.28E-46 | 1.12E-43 |
| GSE2706_UNSTIM_VS_2H_LPS_DC_DN | 200 | 76 | 0.3800 | 4.21E-46 | 1.28E-43 |

**Figure 34** ***Common differentially expressed genes in wt and c-myc $^{\Delta/\Delta}$ at 2h LPS.***
Total numbers of DEGs at 2h in wt and *c-myc* $^{\Delta/\Delta}$ respect to the own time 0 are compared via Venn Diagram and the gene ontology of common genes is reported below. GO terms in biological process ontology and immunological signature are considered .GO terms significantly enriched (FDR value < 0.05) were reported. Genes set with activated genes at 40,60, 80 and 120 minutes respect to unstimulated bone marrow derived macrophages (e.g UNSTIM_VS_60MIN_LPS_BMDM_DN) are enriched.

To investigate the transcriptional signature that distinguishes wt from *c-myc* $^{\Delta/\Delta}$ cells, we came along a further analysis of DEGs. We defined 4 LPS-associated regulatory patterns influenced by the loss of Myc (Myc-dependent LPS response genes) (Perna et al., 2012), where the vast majority is Myc-dependent LPS induced genes (regulatory group 1) and Myc-dependent LPS repressed (regulatory group 3). In a minor number of cases, Myc deletion has the opposite effect, augmenting either activation (regulatory class 2) or repression (regulatory class 4) by LPS. Other mRNAs showed a Myc-independent response to LPS, either induced (regulatory class 5) or repressed (regulatory class 6) (**Figure 35**) (for details, see Materials and Methods).

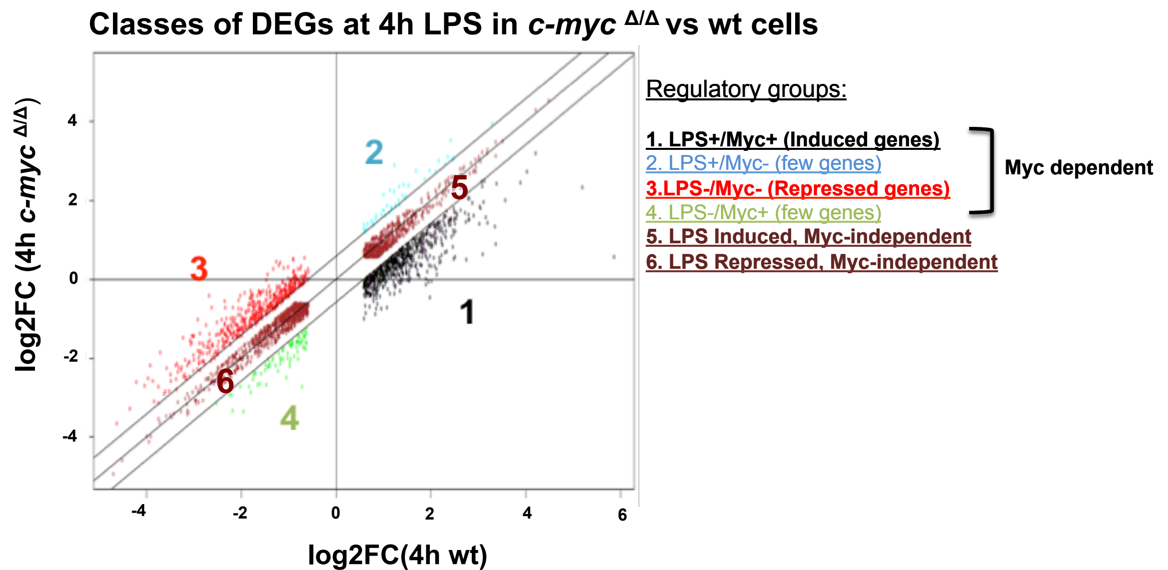



**Figure 35** ***Identification of categories of differentially expressed genes at 4h after LPS stimulation***. Different categories of Myc-dependent and Myc-independent LPS response genes were identified at each time point. This scatter plot is relative to 4h of LPS stimulation (as an example of the analysis we performed at each time point) with the different categories of genes represented in different colors. Log2 of the ration between gene expression at 4h and gene expression at 0h in wt (x-axis) or *c-myc* $^{\Delta/\Delta}$ (the y-axis) B cells is plotted.

In order to identify a core of Myc dependent genes we compared the lists of Myc dependent induced and repressed during LPS stimulation. Venn diagrams showed only a mild overlap among the genes identified at the different time points (**Figure 36**). That could be due to the dynamic changes of the transcriptional response: genes induced or repressed at a certain time points are no more regulated at the subsequent one because they return to the original baseline level, as classically occurring during the inflammation response (Fowler et al., 2003). Another possibility, not mutually exclusive with the previous one, would be that even if the trend is present at all time points it reaches statistical significance only at one.

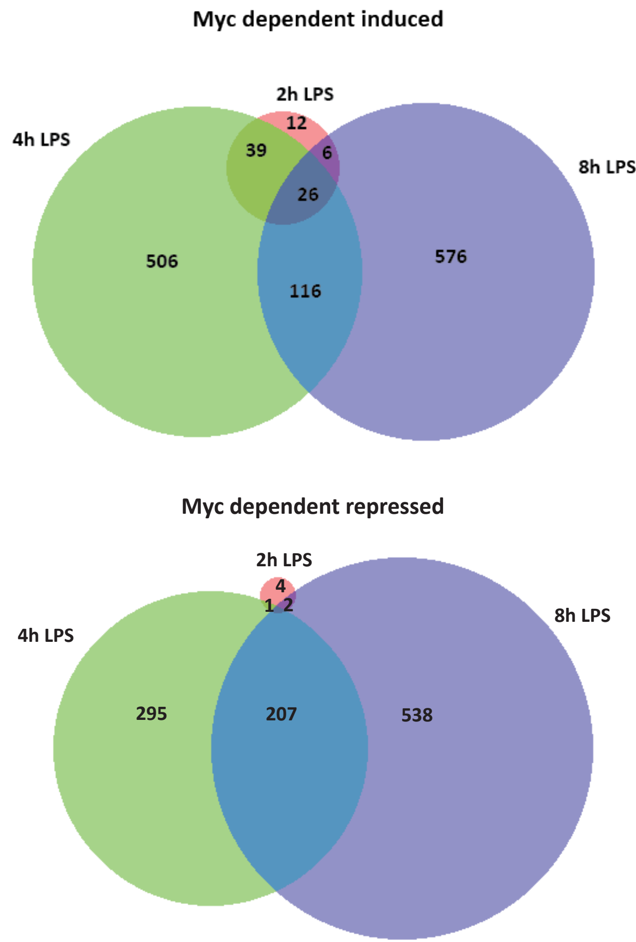



***Figure 36 Overlap among genes Myc dependent induced and represssed during LPS stimulation***. Genes Myc dependent induced (on the left) and repressed (on the right) at each time point (2h,4h,8h LPS) were compared via Venn Diagrams.

In order to verify this hypothesis we built heatmaps that show the changes in gene expression relative to untreated cells for the two main categories of DEGs Myc dependent induced and repressed reported in the **Figure 36** and also for Myc independent induced or repressed genes (**Figure 37; Figure 38**). The heatmaps pointed out indeed that the transcriptional response of genes changes dynamically during the time course in a Myc dependent or independent manner (**Figure 37; Figure 38**). We observed genes more induced or repressed in a transient way and groups of genes with a progressive trend of positive or negative regulation. On the other hand, we cannot exclude that some of these

genes were not differentially expressed in other replicative experiments. In this regard, validation of these regulatory groups of genes trough qPCR or Nanostring would be a fundamental step.

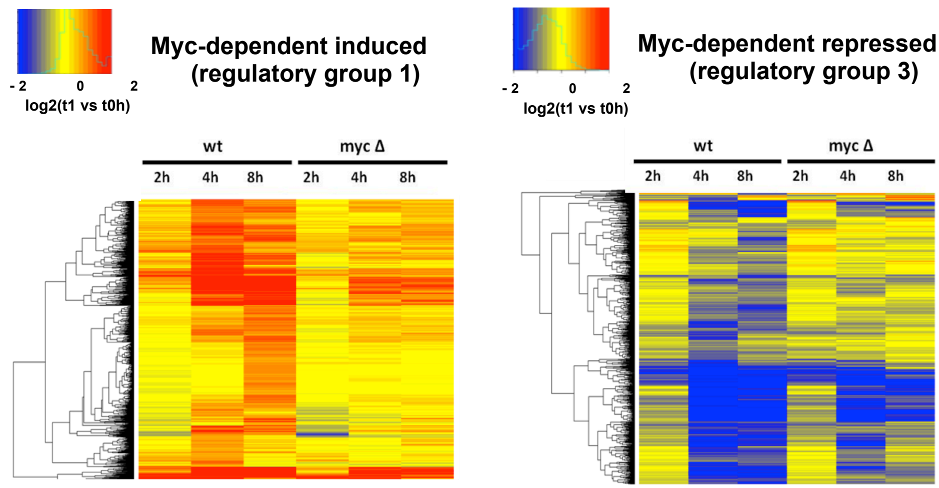



**Figure 37** ***Heatmaps of the Myc dependent genes identified in LPS stimulated B cells.***
Heatmap of the log2(FC) (log2 of fold-change) values of all Myc dependent LPS induced (left) and repressed (right) genes in at least one time point between 2, 4 and 8h of LPS treatment. The genes are clustered hierarchically on the basis of similarities in the relative expression level.

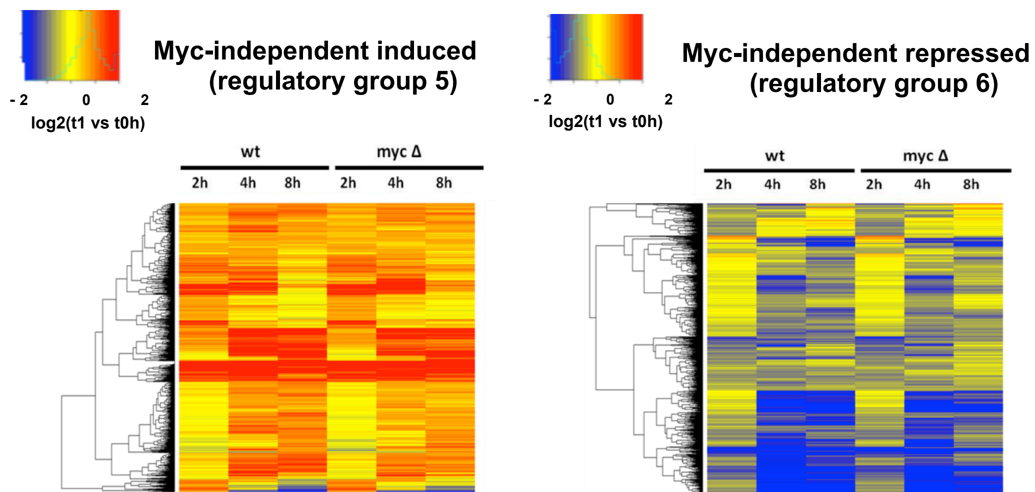



**Figure 38** ***Heatmaps of Myc independent genes identified in LPS stimulated B cells.***
Heatmap of the log2(FC) (log2 of fold-change) values of all Myc independent LPS induced (left) and repressed (right) genes in at least one time point between 2, 4 and 8h of LPS treatment. The genes are clustered hierarchically on the basis of similarities in the relative expression level.

## 4.5 Genome-wide analysis of Myc binding following LPS stimulation

We decided to map the Myc-dependent transcriptional response underlying the different behavior of Myc wt and deleted cells in response to LPS. First, we investigated the relationship between Myc levels, which in this physiological setting rise from a very low baseline to very high (Kelly et al., 1983), and its genomic occupancy. A careful analysis of ChIP-seq experiments on different cells types have shown that Myc binding to the genome is proportional to its expression level: when highly induced or overexpressed, Myc binding to promoters and distal sites increase in terms of numbers and intensity and at the highest level, Myc can be cross-linked at virtually all active promoters and enhancers in the genome, the already mentioned phenomenon called 'invasion' (Sabò et al., 2014; Sabò & Amati 2014; Kress et al., 2015). To investigate Myc binding on the genome of B cells, we then performed chromatin immunoprecipitation coupled to high-throughput sequencing (ChIP-Seq) with a specific c-Myc antibody at 0, 2, 4, 8 hours after LPS-mediated induction. Initial analysis by ChIP-qPCR showed that Myc binding to the promoters of previously identified target genes reached a maximum at 2h after LPS stimulation and was not present on a negative control region (AchR) (**Figure 39**). As a control, we also performed ChIP with the c-Myc antibody in *c-myc*$^{\Delta/\Delta}$ cells and with a non-specific IgG in wild type cells: in both cases, we did not retrieve any significant enrichment on the target amplicons (**Figure 39**).

The genome-wide analysis of the recovered DNA after Myc ChIP yielded ca. 2000 binding sites in resting wild-type B cells rising up to around 22000 after 2h of LPS stimulation and remaining similar in number at 4 and 8 h. Almost all Myc peaks, in resting condition, were proximal (-2 to +1 Kb) to an annotated transcription start site (TSS), which is the interval we hereby annotate as 'promoter'.

In stimulated cells, the new binding sites were not only on promoters, but also on distal sites, with an equal proportion of intra- and extragenic locations (**Figure 40**). Almost all Myc peaks identified in control samples (0h LPS) were retrieved in the LPS-stimulated samples and almost all the peaks identified at each time point of stimulation are contained in the previous one (**Figure 41**).

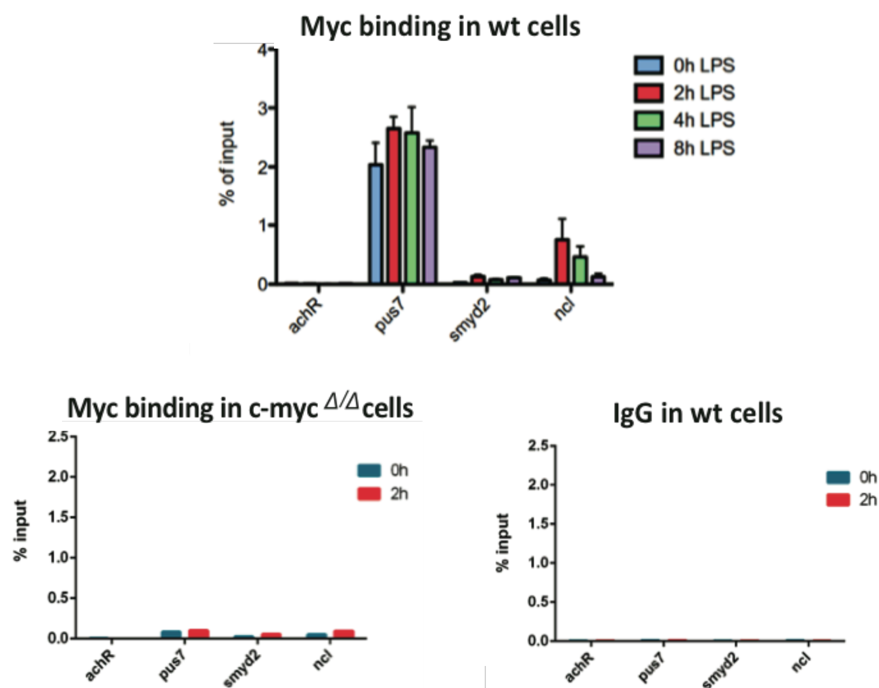



**Figure 39** ***Myc binding to the promoters of target genes increases upon LPS stimulation.***
ChIP-qPCR of Myc binding to promoters of selected bound (*pus7*, *smyd2*, *ncl*) and unbound (*AchR)* genes. The average ± s.d., of 3 independent experiments is shown. As controls, ChIP-qPCR with the anti-Myc antibody in *c-myc* $^{\Delta/\Delta}$ cells and with the normal rabbit IgGs in wt cells at 0 and 2h after LPS stimulation is shown.

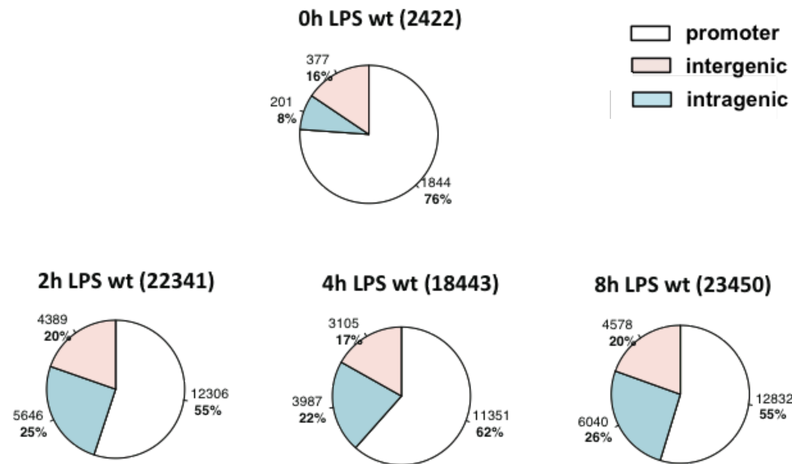



**Figure 40** ***Myc binding sites increase in number after 2h of LPS stimulation and are not restricted to gene promoters.***
Pie charts showing genomic distribution of Myc binding sites. The total number of peaks is reported in brackets and divided in subgroups on the basis of annotation: peaks on promoters, in intergenic region and gene body or intragenic regions.

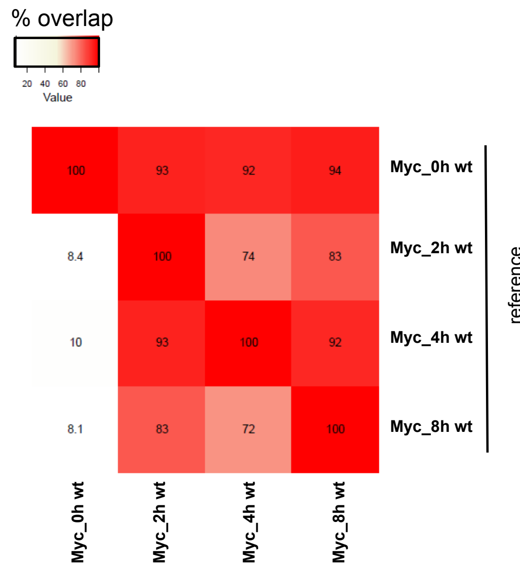



**Figure 41** ***Overlap between Myc ChIP-seq peaks*.**
Percentages of peaks overlapping (at least 1bp) with the reference samples are reported in each column.

In order to better characterize distal sites, we took advantage of chromatin profiles generated in our lab on splenic B cells of either wild type (C) or young Eμ-*myc* mice (P) along with those obtained in Eμ-*myc* lymphomas (T) (Sabò et al., 2014). We reasoned that the wild-type B cells *in vivo* should be very similar to our untreated B-cells *in vitro* (t0 of LPS) in terms of Myc levels and active promoter/enhancers profiles. Indeed, by western blot we verified that Myc protein levels are low in Eμ-*myc* control cells (C) and LPS-untreated wild type cells and increase to a similar extent either in Eμ-*myc* transgenic pre-tumoral cells (P) or upon LPS treatment (**Figure 42**).

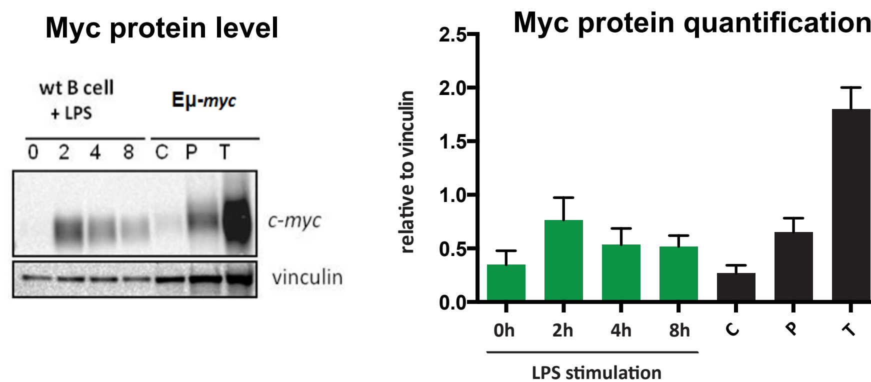



**Figure 42** ***Levels of Myc protein in normal B cells and Eμ-myc transgenic B cells.***
Immunoblot of cell extracts to monitor Myc protein levels in LPS stimulated B cells and *Eμ-myc* control (C), pre-tumoral (P) and tumor (T) samples.

As shown in the qualitative heatmaps (**Figures 43**, **Figure 44**), the distribution of Myc binding sites in resting B cells (0h) is comparable with the Eμ-*myc* C sample and following 2h LPS is very similar to the Eμ-*myc* P sample, reflecting the Myc protein levels in the different conditions (**Figure 42**). According to the distribution of the epigenetic marks, Myc, when induced, binds already active promoters marked by the presence of H3K4me3

and H3K27ac (**Figure 43**). Consistently most of the unbound promoters show no active marks (**Figure 44**). Considering the distal Myc peaks, most of them are found in H3K4me1/H3K27ac positive regions that, when placed outside promoters and/or CpG islands, can be classified as active enhancers (Heintzman et al., 2009).

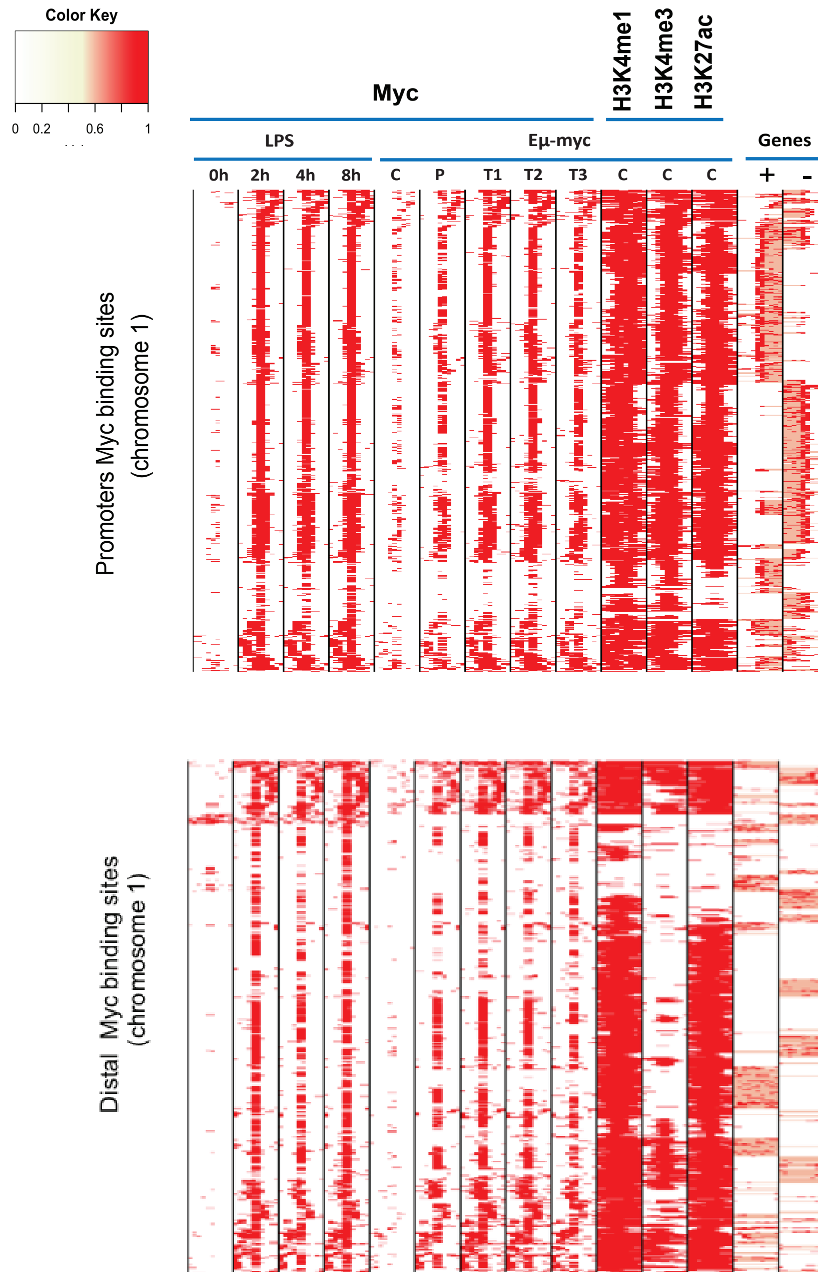



**Figure 43** ***Myc binds open and active chromatin*.**
Heatmaps showing the distribution of Myc peaks at annotated promoters (top panel) and enhancers sites (bottom panel) in LPS-stimulated wt B cells (0, 2, 4, 8h) and Eµ-*myc* (C, P, T1, T2, T3) samples. The distribution of histone marks (H3K4me1, H3K4me3, H3K27ac) in Eµ-*myc* control sample is also shown. Each row represents a different genomic interval (6 kb width centered on Myc peaks). The panels include every annotated promoter and every enhancers at chromosome 1 identified as Myc-associated by ChIP-seq in at least one of the experimental samples. Annotated genes (exons in red, introns in pink; + sense, - antisense strand) are also shown.

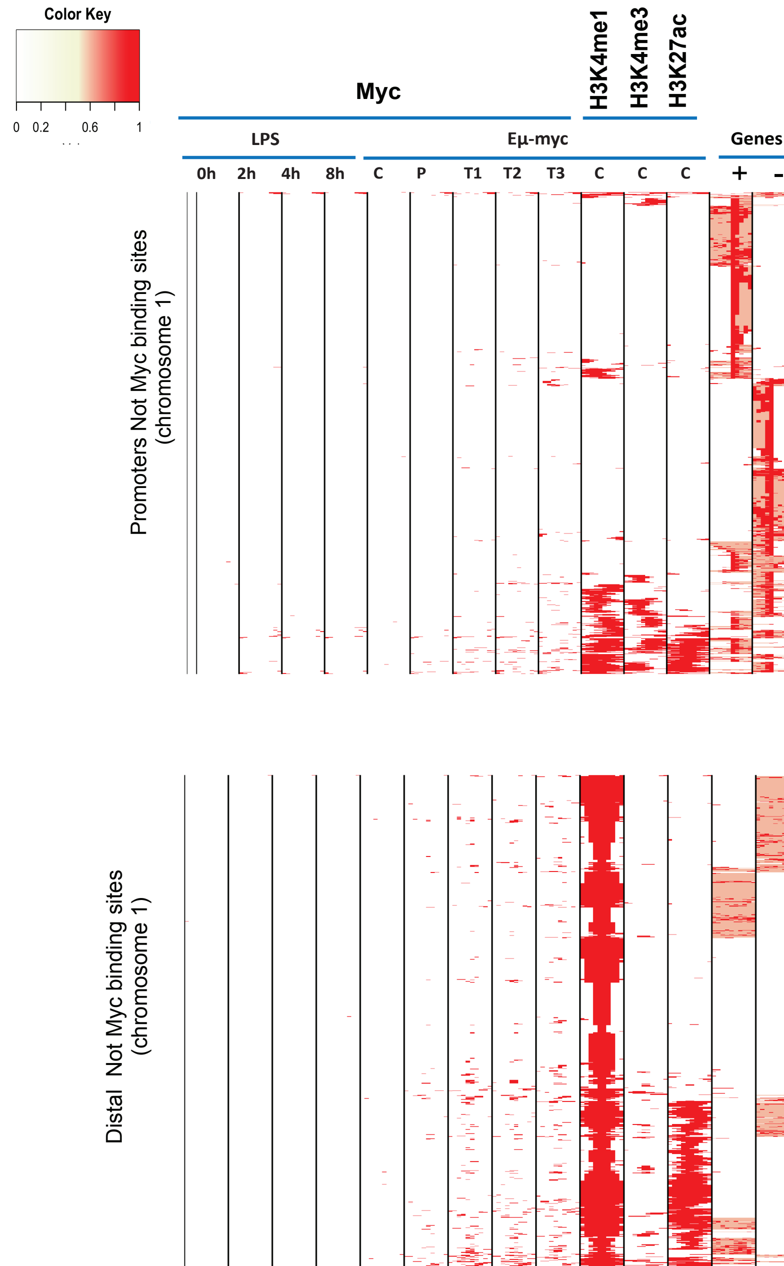



**Figure 44** ***Promoters and enhancers not bound by Myc are mainly inactive.***
Regions not bound by Myc at annotated promoters (top panel) and at enhancers (bottom panel) in LPS-stimulated wt B cells (0, 2, 4, 8h) and Eμ-*myc* samples are shown. The distribution of histone marks (H3K4me1, H3K4me3, H3K27ac) in Eμ-*myc* control sample is also shown. Each row represents a different genomic interval (6 kb width centered on trascriptional start site for the promoters and 6 kb width centered on H3K4me1 peaks for the enhancers). Annotated genes (exons in red, introns in pink; + sense, - antisense strand) are also shown.

In addition, the hierarchy of Myc binding at promoters is conserved at different Myc levels after LPS treatment, as previously shown (Sabò et al., 2014), and is very similar to what we already observed in the Eμ-*myc* C->P->T progression (**Figure 45**).

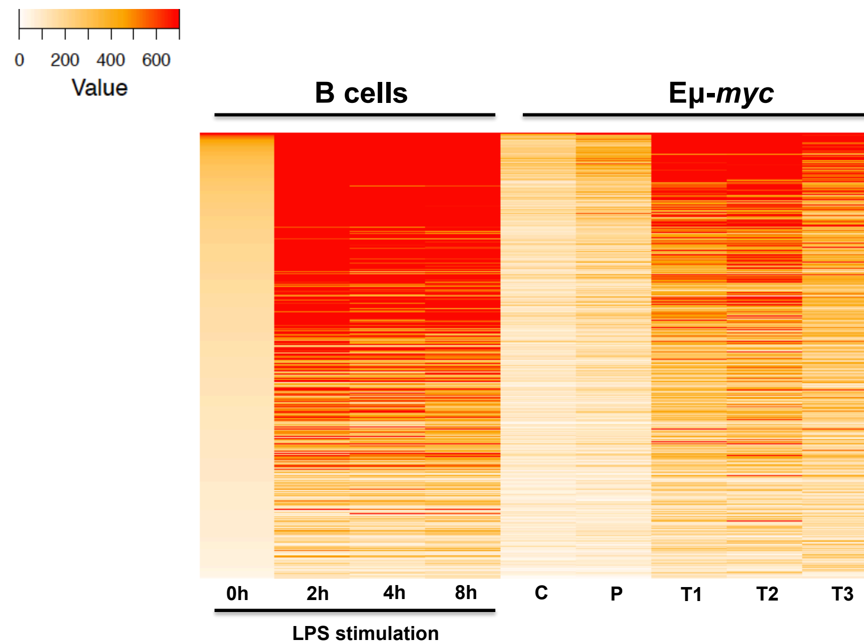



**Figure 45** ***Myc-binding hierarchy on promoters is conserved at different Myc expression levels after LPS stimulation and shows a similar pattern of distribution in Eμ-myc samples.***
Quantitative heatmap showing library size-normalized ChIP-seq read counts at Myc bound promoters in B cells after LPS stimulation (0, 2, 4, 8h) or in Eμ-*myc* samples (Control, Pre-tumoral and three Tumors) as indicated.

Moreover, as reported above, Myc peaks at t0 are mostly positioned at promoters and we found that Myc binding sites at promoters are generally stronger (higher enrichment) compared to the ones at enhancers (**Figure 46**) representing higher affinity binding sites. The fact that the few distal peaks at t0 have a high enrichment suggest that they could actually be not yet annotated promoters.

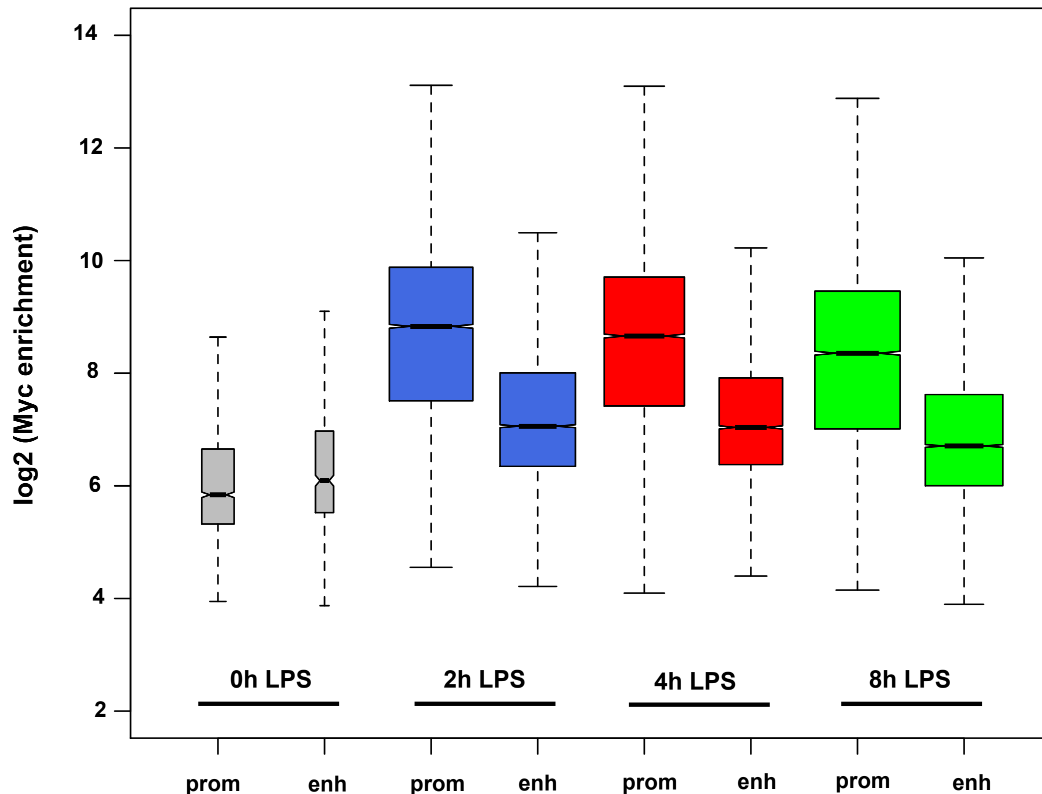



**Figure 46** ***Myc binding intensity at promoters and enhancers peaks.***
Peak enrichment of Myc binding sites in wt B cells without LPS (0h) and after 2h, 4h, 8h of LPS stimulation. The boxes are drawn with widths proportional to the square roots of the number of observations in the groups.

Myc is known to preferentially bind a DNA target sequence, the "E-box" consensus CACGTGA and few variant motifs (Blackwell and Weintraub 1990). We thus performed motif analysis on Myc binding sites and checked for the occurrence of canonical (CACGTG) and non-canonical E-boxes (CACGCG, CATGCG, CACGAG, CATGTG) as defined in the papers of Grandori et al., 1996 and Perna et al. 2012. In stimulated cells, around 60% of Myc binding sites (both at promoters and enhancers) were devoid of any E-box; the remaining 40% of sites contained either the canonical E-box (around 8%) or one of the non-canonical E-boxes (around 30%). In resting conditions, when Myc is lowly expressed, and bound only to its highest affinity targets, the fraction of

bound promoters that contains an E-box was higher reaching around 60% (**Figure 47**). Indeed canonical E-box containing sites have the highest peak enrichment followed by those containing non-canonical E-boxes and those without any E-box (**Figure 48**). This hierarchy between Eboxes and enrichment was detected also for enhancers even if in this case the fractions of bound enhancers that contain Eboxes at time 0h and during stimulation were similar (**Figures 47; Figure 48**) arguing that Myc, once it's induced and invades chromatin, does not discriminate enhancers based on presence or absence of Eboxes containing sites**.**

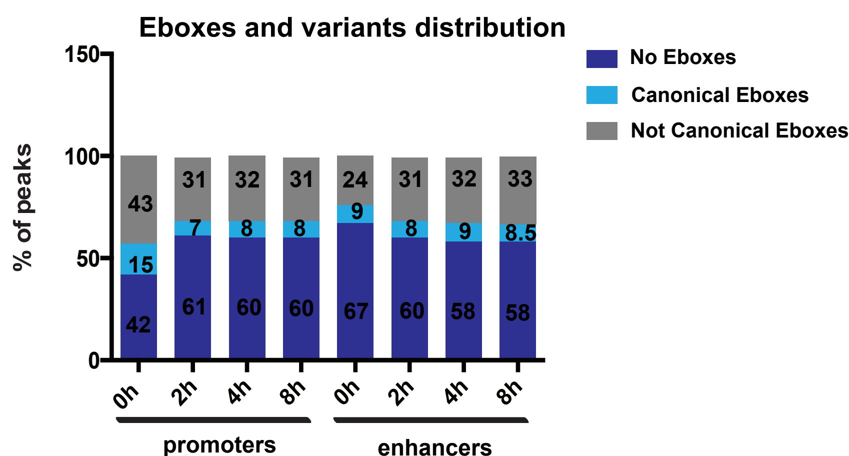



**Figure 47** ***Percentages of Myc binding sites containing canonical or not canonical E-boxes in promoters and distal sites of resting and stimulated wt B cells.***
Percentages of Myc binding sites containing canonical and not canonical E-boxes in the region surrounding the peak summit (+/-75bp) for Myc binding site at promoters and enhancers in wt B cells during LPS stimulation.

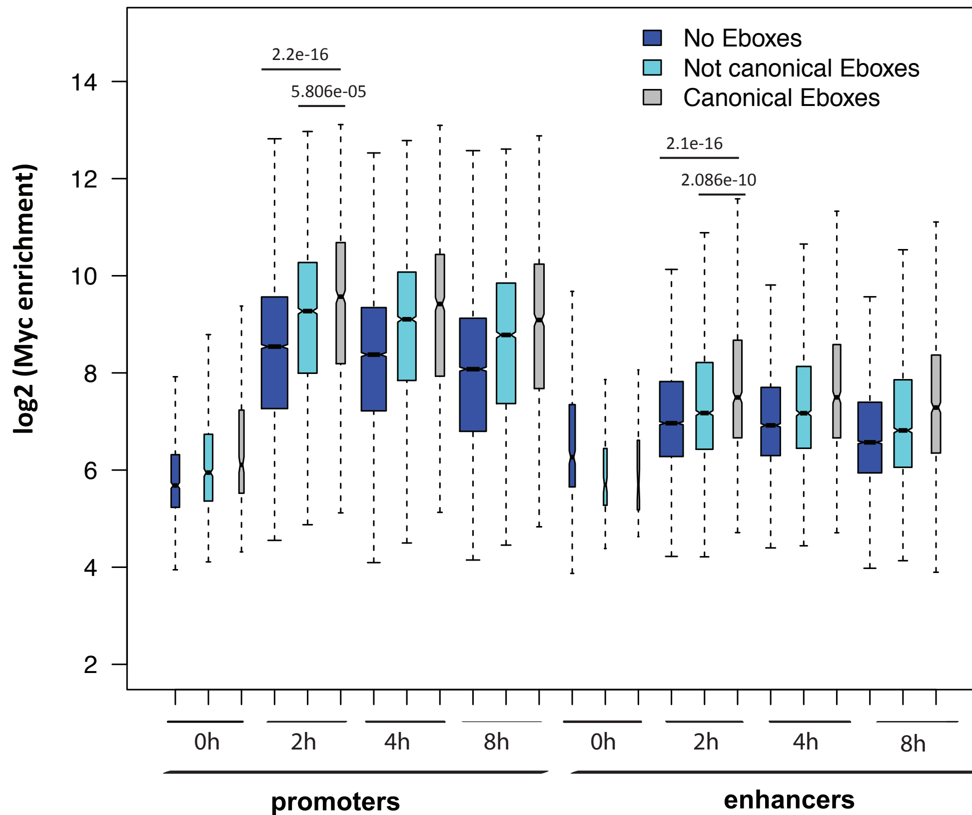



**Figure 48** ***Myc binding intensity correlates with presence of canonical and non-canonical Eboxes.*** Myc enrichment as log2 (ChIP-input) reads is reported for promoters and distal sites at different time points divided on the basis of the presence of canonical and not canonical E-boxes in the region of peak summit +/-75 bp. The boxes are drawn with widths proportional to the square roots of the number of observations in the groups.

We can hypothesize that when Myc is highly expressed such as post LPS-mediated B cell activation, it can bind to the open chromatin not only through specific interaction (canonical E-box recognition) but also through less specific binding to the DNA (variant sites, such as non canonical E-boxes) and/or protein-protein interactions (Sabò A. & Amati B. 2014; Afek et al.2014; Siggers and Gordan 2014). This observation fits with a model proposed in our lab for which there is a temporal order of Myc binding to the DNA and a hierarchy of binding from high to low affinity binding sites. In particular, Myc/Max dimers are first recruited to active promoters and enhancers via protein-protein interactions, and

then they may scan locally DNA sequence and stabilize on medium/high affinity sites (E-boxes and variants). Once Myc is high or overexpressed, the selectivity of binding decrease and it can occupy also variant sites with lower affinity for Myc/Max binding (Lin C et al.2012; Sabò & Amati 2014; Guo et al.,2014). In summary, in resting cells, Myc binds higher fraction of high affinity Ebox containing sites compared to activated cells where high Myc levels increase the probability of a less sequence specific binding to the DNA.

## 4.6 Correlation between Myc genomic occupancy and Myc driven transcription

In order to determine which of the Myc-dependent LPS regulated genes were direct Myc targets, we combined Myc binding and gene expression profiles. Consistent with the phenomenon of 'invasion', from 2h of LPS onward a large majority of all expressed genes were bound by Myc at their promoters, whether regulated by LPS, Myc, or none (**Table 1**). Thus Myc binding to promoters was not predictive of Myc-dependent regulation as also reported for serum-responsive genes in fibroblasts (Perna et al., 2012).

| TOTAL EXPRESSED GENES 11617 | | 2h LPS | | 4h LPS | | 8h LPS | |
|---|---|---|---|---|---|---|---|
| | | N° genes in each categories | % of Myc bound | N° genes in each categories | % of Myc bound | N° genes in each categories | % of Myc bound |
| Myc dependent genes | 1 | 83 | 66% | 687 | 83% | 724 | 90% |
| | 2 | 2 | 100% | 44 | 61% | 77 | 67% |
| | 3 | 7 | 86% | 504 | 63% | 748 | 78% |
| | 4 | 20 | 95% | 79 | 86% | 16 | 87% |
| | TOT | 112 | 87% | 1314 | 75% | 1565 | 84% |
| Myc independent genes | 5 | 579 | 89% | 598 | 86% | 488 | 89% |
| | 6 | 770 | 84% | 811 | 86% | 602 | 83% |
| | TOT | 1349 | 96% | 1409 | 86% | 1090 | 87% |

**Table 1** ***Summary of Myc binding and gene regulation in LPS-stimulated B-cells.***
The table gives the total numbers of genes in each regulatory category and the percentages of promoters bound by Myc at different time points after LPS stimulation. The regulatory categories, as defined in Figure 37, as the following: 1. Myc-dependent LPS induced (LPS+/Myc+); 2. Myc-dependent repressed LPS induced (LPS+/Myc-) 3. Myc-dependent LPS repressed (LPS-/Myc-); 4. Myc-dependent induced LPS repressed (LPS-/Myc+) ; 5. Myc-independent LPS induced; 6. Myc- independent LPS repressed.

The above notwithstanding, Myc-dependent LPS induced genes (regulatory group 1) enriched while Myc-dependent LPS repressed genes (regulatory group 3) depleted for Myc bound genes compared to the whole population, suggesting that Myc mediated repression could occur either via other proteins (regulated by Myc) or via indirect binding of Myc to chromatin (justifying a lower or even lost ChIP-seq signal). We thus restricted our lists of Myc-dependent genes to the genes that are also bound.

Since the mere presence of a Myc peak at promoters was not predictive of gene regulation, we wondered whether the intensity of the peak could be more informative. Compared to non-regulated genes, Myc dependent LPS induced genes have higher Myc binding intensity whereas Myc dependent LPS repressed genes have lower one (**Figure 49**). In particular, among the LPS induced genes, those that were Myc dependent were

more highly bound compared to the Myc independent genes. The same distinction was not apparent for repressed genes.

Consistent with these findings, a quantile stratification of Myc binding strength (low, medium and high intensity) confirmed that Myc dependent LPS Induced genes (regulatory group 1) contained the highest percentage of high affinity Myc binding sites (**Figure 50**).

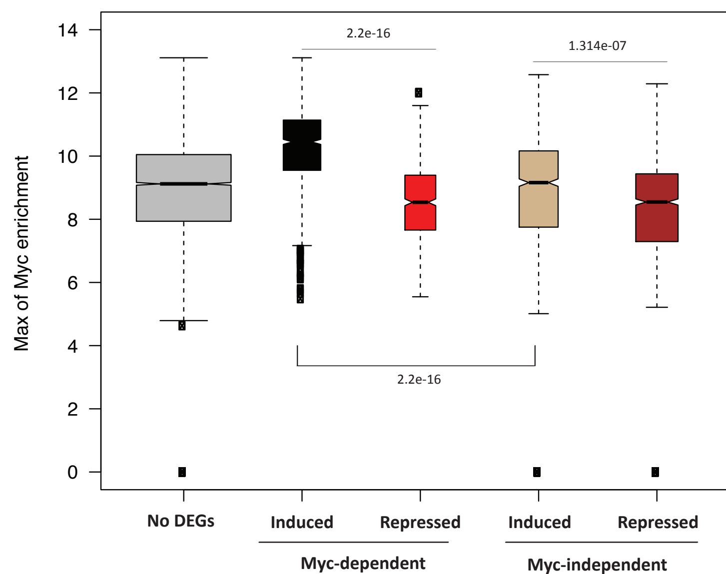



**Figure 49** ***Myc dependent Induced genes have the highest Myc enrichment at promoters.*** Box plots showing the maximum Myc enrichment during the time course of LPS stimulation for the different gene categories: No DEGs, Myc-dependent LPS induced (regulatory group 1) or Myc-dependent LPS repressed (regulatory group 3), Myc-independent LPS induced (regulatory group 5) and Myc-independent LPS repressed. Width of boxplots are proportional to the square-roots of the number of observations in the groups.

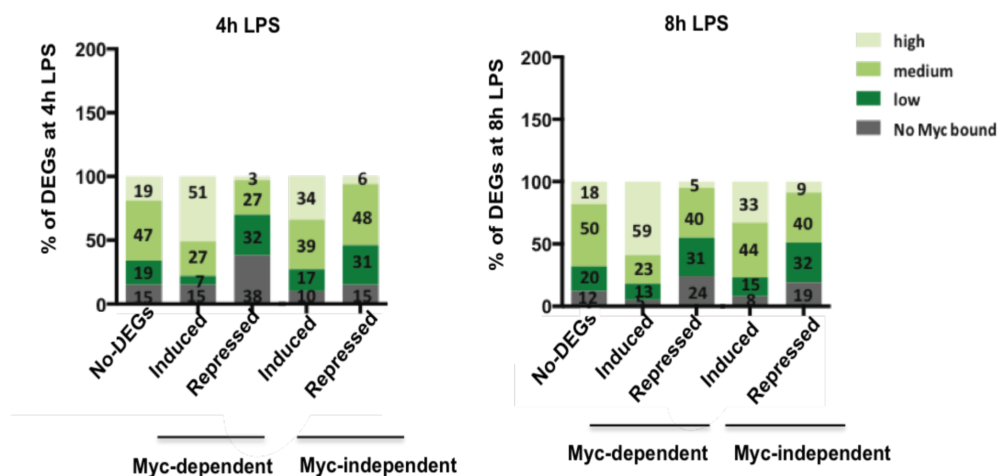



**Figure 50** ***Percentages of peaks belonging to different levels of Myc enrichment in the different categories of DEGs.***
Percentages of genes bound by Myc with high, medium and low affinity intensity in each gene category defined by gene expression (NoDEGs, Myc-dependent LPS induced (regulatory group 1), Myc-dependent LPS repressed (regulatory group 3), Myc independent LPS induced (regulatory group 5) and Myc independent LPS repressed (regulatory group 6) at 4 and 8h after LPS stimulation. At each time point the distribution of Myc enrichment was divided in quantile to define 3 different levels of affinity: low, medium and high (see Materials & Methods for details).

Since Myc preferentially binds the E-box motif CACGTG and a series of variant motifs (Blackwell T.K et al 1993; Perna et al., 2012; Grandori et el., 1996), we calculated the frequency of canonical and non canonical E-boxes under the peak summit of Myc-bound promoters corresponding to the different regulatory categories (**Figure 51**). Myc-dependent induced genes had the highest fraction and Myc-dependent repressed genes the lowest fraction of sites containing an E-box, with the Myc-independent genes in an intermediate situation (**Figure 51**).

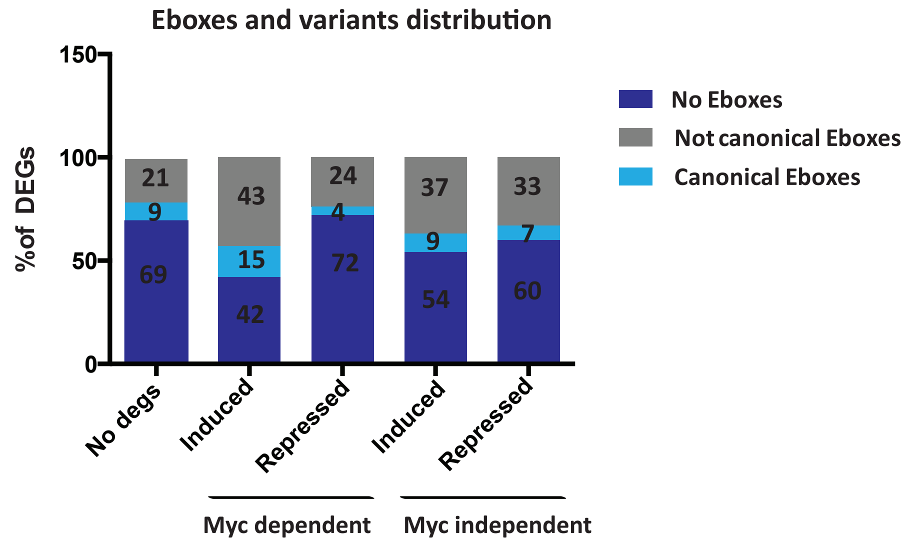



**Figure 51** ***Distribution of Myc binding sites containing canonical and non-canonical E-boxes in differentially regulated promoters*****.**
Percentages of genes in each category (No DEGs, Myc-dependent LPS induced (regulatory group 1), Myc-dependent LPS repressed (regulatory group 3) and Myc-independent LPS induced (regulatory group 5) and Myc-independent LPS repressed (regulatory group 6) in at least one time point of stimulation) having a Myc peak at their promoter without any E-box, or with a canonical or not canonical E-box (identified on the summit of the peaks +/-75 bp).

In conclusion, the analysis of DNA sequence and binding intensity of Myc gave us a coherent picture: Myc dependent LPS induced genes are the highest affinity (presence of E-box and highest Myc enrichment) while Myc dependent repressed are the lowest (absence of E-box and lowest Myc enrichment) suggesting that on repressed genes Myc could be less frequently directly bound to DNA and possibly interact with other TFs, such as Miz1, Sp1 or NF-kb, antagonizing or reversing their activation function (Crescenzi et al., 1994; Tanaka et al.2001; Herkert and Eilers, 2010). Distinct transcription factors may contribute to the Myc transcriptional response and may raise the possibility to discriminate between Myc dependent induced and repressed genes and perhaps between Myc dependent and Myc independent regulation. To address this issue, we performed a position weight matrix (PWM) enrichment analysis on the set of sequences

around the summit of Myc peaks in the promoters of each different category of DEGs. We identified motifs with a significant z score (>3) for each time point (**Table 2**) respect to a genomic background constituted by sequences at the summit of Myc promoter peaks of NoDEGs. Among the motifs enriched in the Myc dependent LPS Induced genes there were the ones for Myc itself, another validation of the higher Myc binding affinity to the DNA of these gene category. Since many motifs are in common among the different categories, we decided to manually curate our table keeping only the motifs that specifically belong to a single category of gene regulation (in at least one time point), discarding the ones that belong to classes found in more than one category and also the ones that are not expressed in our system. The resulting lists of motifs are summarized in Table 2. Among the motifs identified univocally in the Myc dependent LPS Induced gene promoters were for example the binding sites for NFX2, a thyroid specific transcription factor which plays a role in ribosomal gene transcription (Diermeier et al., 2013) or p53. Among Myc dependent repressed genes, we could score binding sites for the SMAD transcription factor family, known to be inhibited by direct interaction with Myc (Xin-Hua Feng et al 2002). The lists of motifs enriched into the Myc independent genes categories could also be useful for identification of different mechanisms of regulation compared to the dependent genes. For example among the Myc independent induced genes we can identify motifs for CREBB, NFKb1 and 2, transcription factors that have role in growth control and cell signaling activation response respectively. The role of any of these TFs in Myc activity will require further investigation.

## Myc-dependent genes bound by Myc

zscore>3

| 2h LPS | | | | | | 4h LPS | | | | | | 8h LPS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Induced (12/76)** | | | **Repressed (1/5)** | | | **Induced (24/248)** | | | **Repressed (22/200)** | | | **Induced (34/300)** | | | **Repressed (44/292)** | | |
| TFs | *zscore* | Class | TFs | *zscore* | Class | TFs | *zscore* | Class | TFs | zscore | Class | TFs | *zscore* | Class | TFs | zscore | Class |
| FOXD2 | Inf | 241 | KAISO | Inf | 270 | APEX1 | inf | 352 | APEX1 | Inf | 352 | ARI3A | Inf | 406 | AR | Inf | 66 |
| HEY2 | 5.37 | 429 | | | | ATF4 | Inf | 404 | ARID5A | Inf | 107 | CEBPG | Inf | 403 | ARID5A | Inf | 66 |
| NF2L2 | 4.87 | 15 | | | | FOXD1 | Inf | 241 | BPTF | Inf | 227 | GMEB1 | Inf | 197 | ATF4 | Inf | 404 |
| NFE2L2 | 4.87 | 45 | | | | FOXD2 | Inf | 241 | FOXC2 | Inf | 99 | HOXA11 | Inf | 172 | FOXB1 | Inf | 99 |
| MYC::MAX | 4.79 | 429 | | | | FOXG1 | Inf | 241 | GFI1B | Inf | 247 | HXB1 | Inf | 265 | GATA5 | Inf | 237 |
| KAISO | 4.34 | 270 | | | | FOXO4 | Inf | 241 | HLTF | Inf | 59 | HXC6 | Inf | 267 | GFI1B | Inf | 247 |
| NFE2 | 4.34 | 112 | | | | HAND1 | Inf | 40 | MYF5 | Inf | 369 | IRF7 | Inf | 424 | HNF6 | Inf | 252 |
| NRF2 | 4.34 | 45 | | | | HNF4G | Inf | 251 | NFATC1 | Inf | 91 | IRF9 | Inf | 427 | IRF9 | Inf | 427 |
| NR2F6 | 3.97 | 9 | | | | MAFK | Inf | 15 | NR4A2 | Inf | 2 | NKX2 | Inf | 3 | LMX1B | Inf | 406 |
| NR1H3 | 3.63 | 371 | | | | MYF5 | Inf | 369 | PO6F1 | Inf | 406 | NR1H4 | Inf | 290 | MEF2A | Inf | 430 |
| SRBP2 | 3.62 | 437 | | | | NF2L2 | Inf | 15 | POU3F3 | Inf | 406 | NR1I2 | Inf | 106 | NKX1-2 | Inf | 406 |
| TF3C2 | 3.21 | 313 | | | | NFAC1 | Inf | 93 | ZFP652 | Inf | 393 | NRI13 | Inf | 106 | NKX2-3 | Inf | 72 |
| | | | | | | NFATC1 | Inf | 91 | ZNF713 | Inf | 418 | NR2E3 | Inf | 104 | NRI13 | inf | 106 |
| | | | | | | NFE2L2 | Inf | 45 | SPI1 | 5.8 | 211 | RORG | Inf | 18 | NR3C2 | Inf | 409 |
| | | | | | | NFIA+NFIB+ | | | SPIB | 4.37 | 211 | TBX21 | Inf | 82 | OG2X | Inf | 406 |
| | | | | | | NFIC+NFIX | Inf | 41 | RUNX2 | 3.84 | 207 | THA | Inf | 331 | PAX6 | Inf | 56 |
| | | | | | | NKXX2 | Inf | 7 | SMAD1 | 3.79 | 314 | TP73 | Inf | 297 | PO4F2 | Inf | 308 |
| | | | | | | NRF2 | Inf | 45 | TF65 | 3.37 | 442 | IRF1 | 9.91 | 424 | PO5F1 | Inf | 26 |
| | | | | | | TCF21 | Inf | 129 | THA | 3.37 | 331 | MYC | 8.53 | 429 | PO6F1 | Inf | 406 |
| | | | | | | ZNF282 | Inf | 83 | AR | 3.16 | 66 | MYCN | 8.39 | 429 | POU3F4 | Inf | 406 |
| | | | | | | BHE41 | 5.36 | 429 | GCM1 | 3.16 | 114 | IRF2 | 7.82 | 424 | POU5F1 | Inf | 26 |
| | | | | | | MYC | 5.05 | 429 | SPIC | 3.07 | 211 | SPI1 | 5.93 | 211 | RAX | Inf | 406 |
| | | | | | | TBX2 | 3.8 | 82 | | | | STAT2 | 5.63 | 425 | SCRT2 | Inf | 128 |
| | | | | | | ENOA | 3.71 | 429 | | | | SPZ1 | 4.51 | 60 | SOX11 | Inf | 23 |
| | | | | | | MAX | 3.02 | 429 | | | | P53 | 4.13 | 295 | TAL1 | Inf | 326 |
| | | | | | | | | | | | | MLXPL | 3.75 | 25 | TAL1:TCF3 | Inf | 36 |
| | | | | | | | | | | | | TATA_BOX | 3.59 | 58 | TFE2 | Inf | 326 |
| | | | | | | | | | | | | RARG | 3.56 | 2 | THA | Inf | 331 |
| | | | | | | | | | | | | STAT3 | 3.28 | 42 | ZFP410 | Inf | 302 |
| | | | | | | | | | | | | NHLH1 | 3.17 | 423 | ZNF238 | Inf | 388 |
| | | | | | | | | | | | | MAX | 3.14 | 429 | KLF1 | 5.61 | 335 |
| | | | | | | | | | | | | EPAS1 | 3.02 | 50 | IRF8 | 5.36 | 426 |
| | | | | | | | | | | | | | | | STAT4 | 5.36 | 323 |
| | | | | | | | | | | | | | | | ESRRA | 5.31 | 12 |
| | | | | | | | | | | | | | | | KLF3 | 5.1 | 335 |
| | | | | | | | | | | | | | | | ATF6A | 4.64 | 225 |
| | | | | | | | | | | | | | | | MAFA | 4.27 | 273 |
| | | | | | | | | | | | | | | | ZNF75A | 4.09 | 94 |
| | | | | | | | | | | | | | | | ZEP2 | 4.04 | 345 |
| | | | | | | | | | | | | | | | ESR2 | 3.85 | 2 |
| | | | | | | | | | | | | | | | TATA_BOX | 3.85 | 58 |
| | | | | | | | | | | | | | | | POU2F1 | 3.59 | 406 |
| | | | | | | | | | | | | | | | POU2F2 | 3.27 | 406 |
| | | | | | | | | | | | | | | | SMAD2 | 3.18 | 315 |

**Myc-independent genes bound by Myc**

zscore>3

| 2h LPS | | | | | | 4h LPS | | | | | | 8h LPS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Induced (39/304)** | | | **Repressed (33/300)** | | | **Induced (47/269)** | | | **Repressed (53/309)** | | | **Induced (31/255)** | | | **Repressed (43/278)** | | |
| TFs | *zscore* | Class | TFs | *zscore* | Class | TFs | *zscore* | Class | TFs | zscore | Class | TFs | *zscore* | Class | TFs | zscore | Class |
| ATF5 | Inf | 223 | ARID5A | Inf | 107 | BACH1 | Inf | 15 | ATF5 | Inf | 223 | BARHL2 | Inf | 406 | AIRE | Inf | 216 |
| CEBPE | Inf | 403 | ATF5 | Inf | 223 | CART1 | Inf | 406 | BACH1 | Inf | 15 | BRAC | Inf | 75 | AR | Inf | 66 |
| DBX2 | Inf | 406 | DBX2 | Inf | 406 | CBP | Inf | 355 | CUX1 | Inf | 233 | CPHX | Inf | 175 | ATF4 | Inf | 404 |
| HOXC6 | Inf | 406 | EN2 | Inf | 406 | CUX1 | Inf | 233 | ESX1 | Inf | 406 | FOXA1 | Inf | 411 | CEBPE | Inf | 403 |
| HXA13 | Inf | 217 | FOXA2 | Inf | 411 | DRGX | Inf | 406 | FOXL1 | Inf | 241 | GATA1 | Inf | 229 | FOXD2 | Inf | 241 |
| IRC900814 | Inf | 122 | FOXC2 | Inf | 99 | E2F8 | Inf | 95 | FOXO4 | Inf | 241 | HBP1 | Inf | 118 | GATA5 | Inf | 237 |
| NEUROD2 | Inf | 38 | FOXF1 | Inf | 20 | HES7 | Inf | 429 | GCM2 | Inf | 246 | HMX3 | Inf | 406 | HBP1 | Inf | 118 |
| NFIA | Inf | 41 | FOXK1 | Inf | 241 | ISX | Inf | 406 | GRHL1 | Inf | 395 | IRF9 | Inf | 427 | HES5 | Inf | 429 |
| NFIB | Inf | 41 | FOXQ1 | Inf | 381 | LEF1 | Inf | 87 | GSC | Inf | 180 | PEBB | Inf | 300 | HES7 | Inf | 429 |
| NR2E3 | Inf | 104 | GSC2 | Inf | 407 | LHX2 | Inf | 406 | GSC2 | Inf | 407 | PTF1A | Inf | 309 | HNF6 | Inf | 252 |
| PEBB | Inf | 300 | HMX3 | Inf | 406 | LHX3 | Inf | 406 | HES5 | Inf | 429 | PTX1 | Inf | 180 | HOXC11 | Inf | 173 |
| PITX1 | Inf | 180 | HXA10 | Inf | 255 | LHX5 | Inf | 406 | HES7 | Inf | 429 | TFE2 | Inf | 326 | HOXC12 | Inf | 172 |
| POU3F1 | Inf | 406 | MSX3 | Inf | 406 | LHX9 | Inf | 406 | HOMEZ | Inf | 182 | THA | Inf | 331 | HOXC13 | Inf | 183 |
| PO4F1 | Inf | 406 | NFIA | Inf | 41 | MSX1 | Inf | 406 | HOXD11 | Inf | 172 | ZBTB12 | Inf | 151 | HOXD13 | Inf | 183 |
| TBX4 | Inf | 75 | NFIB | Inf | 41 | MYF5 | Inf | 369 | HXA9 | Inf | 263 | ZBTB49 | Inf | 285 | HSFY2 | Inf | 399 |
| TBX5 | Inf | 75 | PHOX2A | Inf | 406 | NFIA+NFIB | | | IRF6 | Inf | 123 | ZNF238 | Inf | 388 | HXA7 | Inf | 262 |
| ZN238 | Inf | 347 | PITX2 | Inf | 6 | +NFIC+NFIX | Inf | 41 | LEF1 | Inf | 87 | STAT2 | 6.01 | 425 | HXC13 | Inf | 183 |
| TF65 | 11.71 | 442 | PO4F1 | Inf | 406 | NF-KAPPAB | Inf | 442 | MAFF | Inf | 48 | NHLH1 | 5.58 | 423 | IRF7 | Inf | 424 |
| RELA | 6.96 | 442 | PROX1 | Inf | 402 | NF2L2 | Inf | 15 | MAFK | Inf | 15 | TWST1 | 4.63 | 62 | IRF9 | Inf | 427 |
| REL | 6.7 | 442 | SP100 | Inf | 144 | NFATC1 | Inf | 91 | MEF2A | Inf | 430 | NF2L2 | 4.54 | 15 | MSX2 | Inf | 406 |
| NFKB1 | 6.63 | 438 | T | Inf | 75 | NR2E3 | Inf | 404 | MEF2B | Inf | 401 | HSF1 | 4.28 | 254 | MYBB | Inf | 280 |
| POU2F1 | 6.21 | 406 | TCF2 | Inf | 205 | NR4A3 | Inf | 13 | MEF2C | Inf | 212 | STAT1 | 4.09 | 425 | NKX2-3 | Inf | 72 |
| THA | 5.96 | 331 | ZBTB3 | Inf | 153 | PBX1 | Inf | 17 | MEF2D | Inf | 430 | IRF3 | 3.93 | 424 | NR4A2 | Inf | 2 |
| POU3F4 | 5.7 | 406 | ZNF410 | Inf | 390 | PHOX2B | Inf | 406 | MYF5 | Inf | 369 | E2F4 | 3.85 | 157 | ONECUT1 | Inf | 397 |
| POU5F1P1 | 5.28 | 79 | MYF6 | 9.19 | 423 | POU3F3 | Inf | 406 | NFIA | Inf | 41 | STAT3 | 3.84 | 42 | ONECUT2 | Inf | 397 |
| ZN589 | 5.2 | 319 | SPI1 | 5.2 | 211 | POU5F1P1 | Inf | 79 | NFIB | Inf | 41 | BHE41 | 3.63 | 429 | ONECUT3 | Inf | 397 |
| NR4A3 | 5.1 | 13 | SPIB | 4.3 | 211 | PROP1 | Inf | 406 | NFIA+NFIB | | | IRF1 | 3.63 | 424 | PITX2 | Inf | 6 |
| NFKB2 | 4.97 | 443 | SCRT1 | 4.18 | 128 | RELA | Inf | 442 | +NFIC+NFIX | Inf | 41 | IRF2 | 3.63 | 424 | PTF1A | Inf | 309 |
| HSF2 | 4.32 | 152 | STAT3 | 4.04 | 42 | SOX5 | Inf | 241 | NF-KAPPAB | Inf | 442 | HNF4G | 3.45 | 251 | PTX1 | Inf | 180 |
| P73 | 4.05 | 298 | IRF9 | 4.02 | 427 | SOX8 | Inf | 5 | NR1I2 | Inf | 106 | E2F7 | 3.43 | 50 | SP100 | Inf | 144 |
| TFAP4 | 4.02 | 129 | IRF8 | 3.21 | 426 | TBX21 | Inf | 82 | NR2F2 | Inf | 2 | EPAS1 | 3.24 | 50 | STA5A | Inf | 320 |
| BHE41 | 3.78 | 429 | IRF4 | 3.09 | 428 | TWIST1 | Inf | 62 | ONECUT1 | Inf | 397 | | | | TAL1 | Inf | 326 |
| PAX6 | 3.38 | 56 | SPIC | 3.01 | 211 | UNCX | Inf | 406 | ONECUT2 | Inf | 397 | | | | TAL1::TCF3 | Inf | 36 |
| POU3F2 | 3.35 | 406 | | | | ZNF282 | Inf | 83 | ONECUT3 | Inf | 397 | | | | TBX19 | Inf | 75 |
| MYF5 | 3.21 | 369 | | | | ZNF306 | Inf | 389 | PO4F3 | Inf | 197 | | | | TFE2 | Inf | 326 |
| TLX1::NFIC | 3.18 | 67 | | | | FOXO3 | 6.48 | 241 | PO5F1 | Inf | 24 | | | | THA | Inf | 331 |
| EPAS1 | 3.13 | 50 | | | | NFKB1 | 6.37 | 438 | POU5F1 | Inf | 26 | | | | ZBTB49 | Inf | 285 |
| MYC | 3.05 | 429 | | | | ZIC2 | 4.74 | 161 | SOX2 | Inf | 22 | | | | MBD1 | 4.37 | 125 |
| POU3F3 | 3.04 | 406 | | | | REL | 4.54 | 442 | SOX21 | Inf | 406 | | | | IRF4 | 4.35 | 428 |
| | | | | | | RARB | 4.23 | 312 | SOX8 | Inf | 5 | | | | SOX9 | 4.22 | 22 |
| | | | | | | POU2F3 | 4.13 | 406 | SOX9 | Inf | 22 | | | | MLXPL | 3.85 | 25 |
| | | | | | | TBP | 3.94 | 258 | SOX21 | Inf | 406 | | | | MYOG | 3.75 | 433 |
| | | | | | | POU2F1 | 3.65 | 406 | TAL1::GATA1 | Inf | 310 | | | | HNRPK | 3.39 | 365 |
| | | | | | | ZBTB3 | 3.48 | 153 | TEAD3 | Inf | 34 | | | | | | |
| | | | | | | TF5 | 3.38 | 442 | TEAD4 | Inf | 35 | | | | | | |
| | | | | | | ZIC3 | 3.3 | 161 | TWIST1 | Inf | 62 | | | | | | |
| | | | | | | E2F1 | 3.04 | 157 | ZFP652 | Inf | 393 | | | | | | |
| | | | | | | KLF3 | 3.03 | 335 | ZNF282 | Inf | 83 | | | | | | |
| | | | | | | | | | ZNF713 | Inf | 418 | | | | | | |
| | | | | | | | | | NF1 | 7.51 | 41 | | | | | | |
| | | | | | | | | | ESR2 | 4.47 | 2 | | | | | | |
| | | | | | | | | | CAATT_BOX | 4.14 | 441 | | | | | | |
| | | | | | | | | | ZBT7B | 3.97 | 339 | | | | | | |
| | | | | | | | | | DBD_MUTANT | 3.52 | 414 | | | | | | |
| | | | | | | | | | PURA | 3.37 | 311 | | | | | | |

**Table 2** ***Enriched transcription factor binding sites at promoters of Myc- bound transcriptional dependent and independent genes.***

Significant enriched motifs respect to the backgroud (zscore>3) are reported for each time point and each category of genes.'Inf' comes from the total absence of the motif in the background. Since many TFs can be classified inside the same categories of regulation, a column of the corrisponding class of the transcription factor is also shown for each gene category.

| Myc-dependent genes bound by Myc | | | | | |
|---|---|---|---|---|---|
| Induced_at least 1 time point | | | Repressed_at least 1 time point | | |
| TFs | Class | Max_zscore | TFs | Class | Max_zscore |
| GMEB1 | Class197 | Inf | BPTF | Class227 | Inf |
| NKX2 | Class3 | Inf | GFI1B | Class247 | Inf |
| NRF2 | Class45 | Inf | HLTF | Class59 | Inf |
| P53 | Class295 | 4.13 | NR3C2 | Class409 | Inf |
| NR2F6 | Class9 | 3.97 | ZFP410 | Class302 | Inf |
| SRBP2 | Class437 | 3.62 | STAT4 | Class323 | 5.36 |
| TF3C2 | Class313 | 3.21 | ESRRA | Class12 | 5.31 |
| | | | ATF6A | Class225 | 4.64 |
| | | | ZNF75A | Class94 | 4.09 |
| | | | ZEP2 | Class345 | 4.04 |
| | | | RUNX2 | Class207 | 3.84 |
| | | | SMAD1 | Class314 | 3.79 |
| | | | SMAD2 | Class315 | 3.18 |
| | | | GCM1 | Class114 | 3.16 |

| Myc-independent genes bound by Myc | | | | | |
|---|---|---|---|---|---|
| Induced_at least 1 time point | | | Repressed_at least 1 time point | | |
| TFs | Class | Max_zscore | TFs | Class | Max_zscore |
| CBP | Class355 | Inf | GRHL1 | Class395 | Inf |
| NR4A3 | Class13 | Inf | HOMEZ | Class182 | Inf |
| PEBB | Class300 | Inf | MAFF | Class48 | Inf |
| ZBTB12 | Class151 | Inf | MEF2B | Class401 | Inf |
| ZIC2 | Class161 | Inf | MEF2C | Class212 | Inf |
| ZN589 | Class319 | Inf | MYBB | Class280 | Inf |
| ZNF306 | Class389 | Inf | SP100 | Class144 | Inf |
| NFKB1 | Class438 | 6.63 | STA5A | Class320 | Inf |
| TBP | Class258 | 6.01 | TCF2 | Class205 | Inf |
| NFKB2 | Class443 | 4.97 | ZBT7B | Class339 | Inf |
| HSF2 | Class152 | 4.32 | ZNF410 | Class390 | Inf |
| HSF1 | Class254 | 4.28 | MBD1 | Class125 | 4.37 |
| E2F4 | Class157 | 3.85 | IRF4 | Class428 | 4.35 |
| ZN238 | Class347 | 3.3 | HNRPK | Class365 | 3.39 |
| E2F1 | Class157 | 3.04 | PURA | Class311 | 3.37 |

**Table 3** ***Summary of TFs identified at promoters of Myc-bound transcriptional dependent and independent genes.***
Lists of TF PWMs enriched in at least one time point with associated class and highest zscore presented during the time course are shown. Common PWMs among categories and PWMs of the same class were excluded.

## 4.7 Mechanism of transcriptional regulation: RNAPol2 recruitment versus elongation

Even though Myc has been reported to enhance the loading of RNA polymerase II (RNAPol2) on target promoters (Martinato et al. 2008), it was suggested that it mainly modulates transcriptional pause release and elongation, in particular by recruiting the P-

TEFb complex (cyclin T1 and Cdk9), which phosphorylates the carboxy-terminal domain of RNAPol2 on Ser2 favouring transcriptional elongation (Rahl et al. 2010; Bouchard et al., 2004; Eberhardy et al., 2001).

ChIP-qPCR data on the promoters of selected Myc-dependent LPS induced gene indicate that RNAPol2 is already present before LPS treatment and increases after stimulation in a Myc-dependent manner (**Figure 52**). These data would be consistent with a role for Myc in RNAPol2 recruitment at its target genes, without excluding a role for elongation.

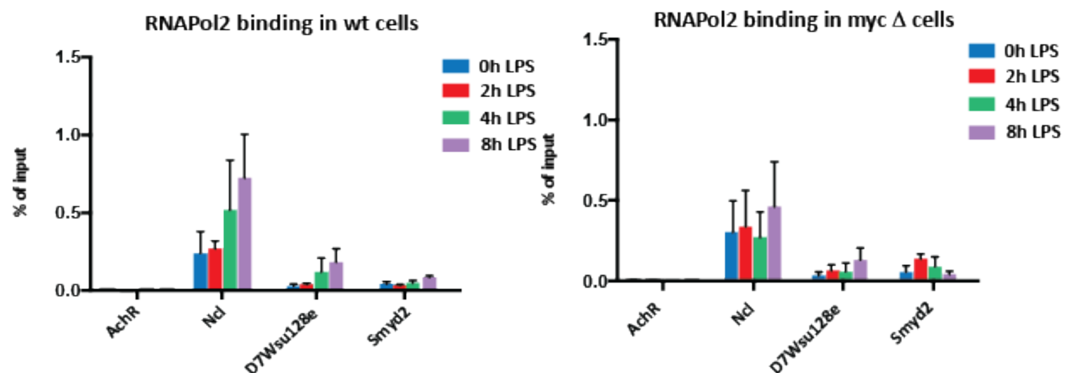



**Figure 52** ***RNAPol2 binding to the promoters of Myc target genes increases upon LPS stimulation.***

ChIP-qPCR for RNAPol2 on the TSS of Myc-regulated genes in wt and c-myc$^{\Delta/\Delta}$ cells, at 0, 2, or 4h after LPS stimulation. The average ± s.d. of 3 independent experiments is shown.

In order to clarify the role of Myc in RNA polymerase recruitment, promoter clearance and/or elongation we performed RNAPol2 ChIP-seq experiments in wt and *c-myc*$^{\Delta/\Delta}$ cells upon LPS treatment. In wt cells, the numbers of RNApol2 peaks increased during LPS stimulation, which did not occur in the absence of Myc, albeit we could identify more peaks in *c-myc*$^{\Delta/\Delta}$ than in wt cells at the earliest time-points (0 and 2h, **Figure 53a**). Almost all the peaks identified at 0h LPS were retrieved also in LPS stimulated samples (**Figure 53b**) both in wt and *c-myc*$^{\Delta/\Delta}$ cells. In addition, we observed a large overlap between wt and *c-myc*$^{\Delta/\Delta}$ cells, with 68 to 97% of the peaks in wt cells also contained in *c-myc*$^{\Delta/\Delta}$ cells and, reciprocally, 60 to 97% of peaks in *c-myc*$^{\Delta/\Delta}$ cells included in wt cells.

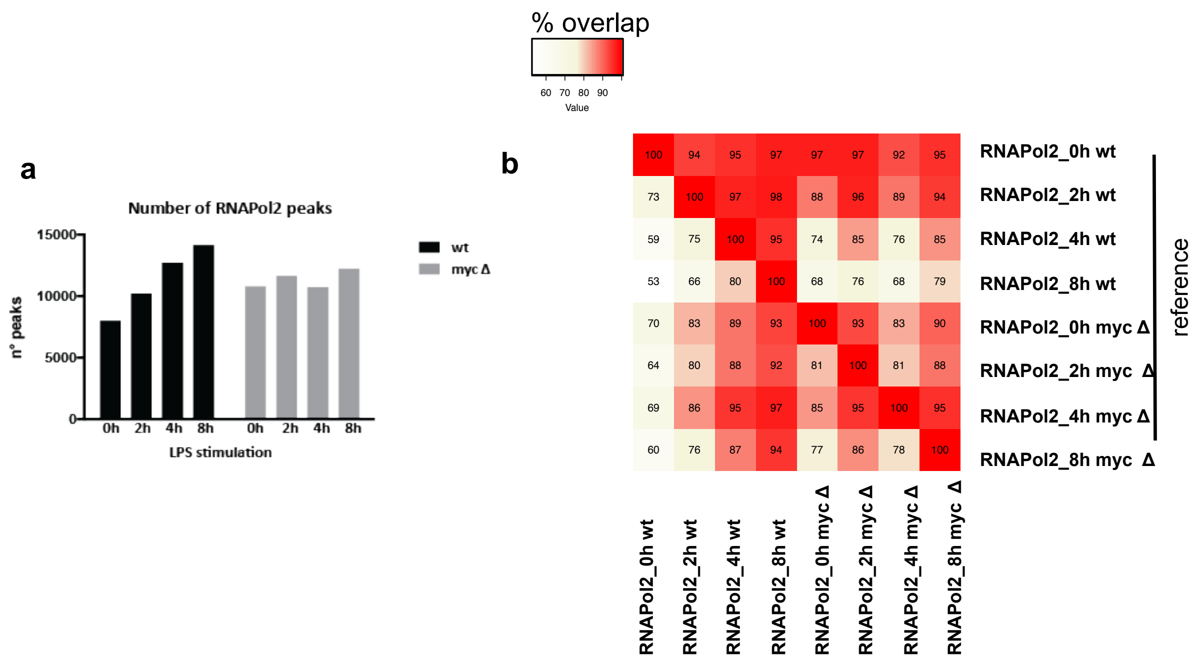



**Figure 53** ***RNAPol2 peaks numbers and overlap between RNAPol2 peaks in different samples.*** **a**) Number of peaks in wt and *c-myc*$^{\Delta/\Delta}$ cells upon LPS treatment. **b)** Percentages of RNAPol2 peaks overlapping (by least 1bp) with the reference samples are reported in each column.

As already discussed, we considered the epigenetic profiles of control B cells *in* vivo (the C sample of our Eμ-*myc* study, Sabò et al. 2014) as surrogates for unstimulated B-cells in our experiments: based on this analysis, we deduced that almost all the Myc bound promoters are already pre-loaded with RNAPol2 and marked by H3K4me3 and H3K27ac in resting conditions (**Figure 54a**). RNAPol2 is pre-engaged not only on promoters of Myc binding sites but also on enhancers. Indeed, distal RNAPol2 binding sites show H3K4me1, H3K27ac marks and to a lesser extent also a Myc peak, arguing that most of the active enhancers are Pol2 and Myc loaded (**Figure 54b**). Unlike Myc-bound elements, those that were not bound showed no RNAPol2, as observed at either promoters (**Figure 55a**) or enhancers (**Figure 55b**), the latter selected as distal H3K4me1 sites with no Myc. In most cases, lack of Myc and Pol2 also correlated with lack of the corresponding activation marks (H3K4me3 in promoters, and H3K27ac in either promoters or enhancers).

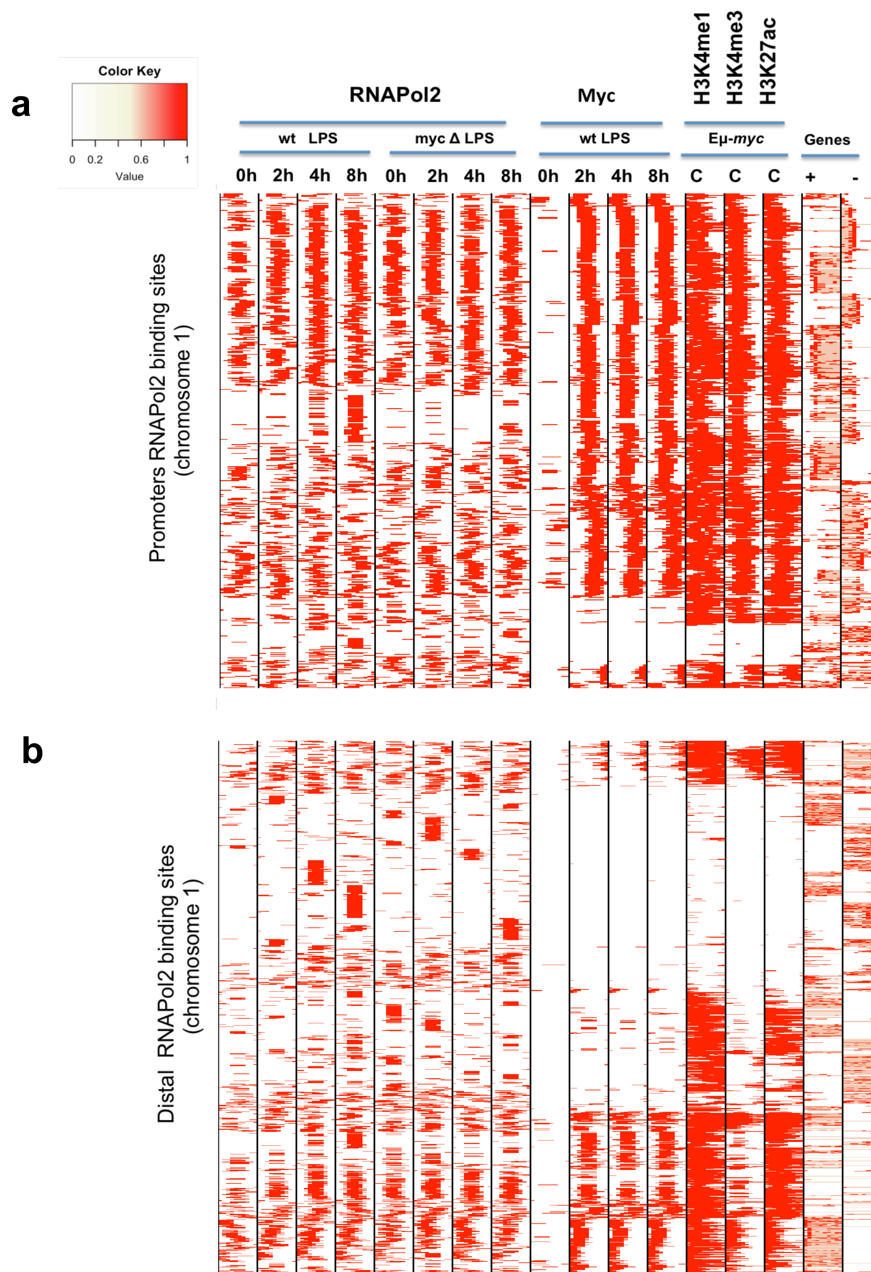



**Figure 54** ***RNAPol2 is pre-loaded on active promoters and distal sites.***
Heatmaps showing the distribution of RNAPol2 and Myc peaks at annotated promoters (top panel) and at distal H3K4me1 positive binding sites (botton panel) upon LPS stimulation of wt and *c-myc*$^{\Delta/\Delta}$ B cells (0, 2, 4h) at chromosome 1. The distribution of histone marks (H3K4me1, H3K4me3, H3K27ac) in Eμ-*myc* control sample is also shown. Each row represents a different genomic interval (6 kb width centered on the midpoint of the RNAPol2 peaks). Annotated genes (exons in red, introns in pink; + sense, - antisense strand) are also shown.

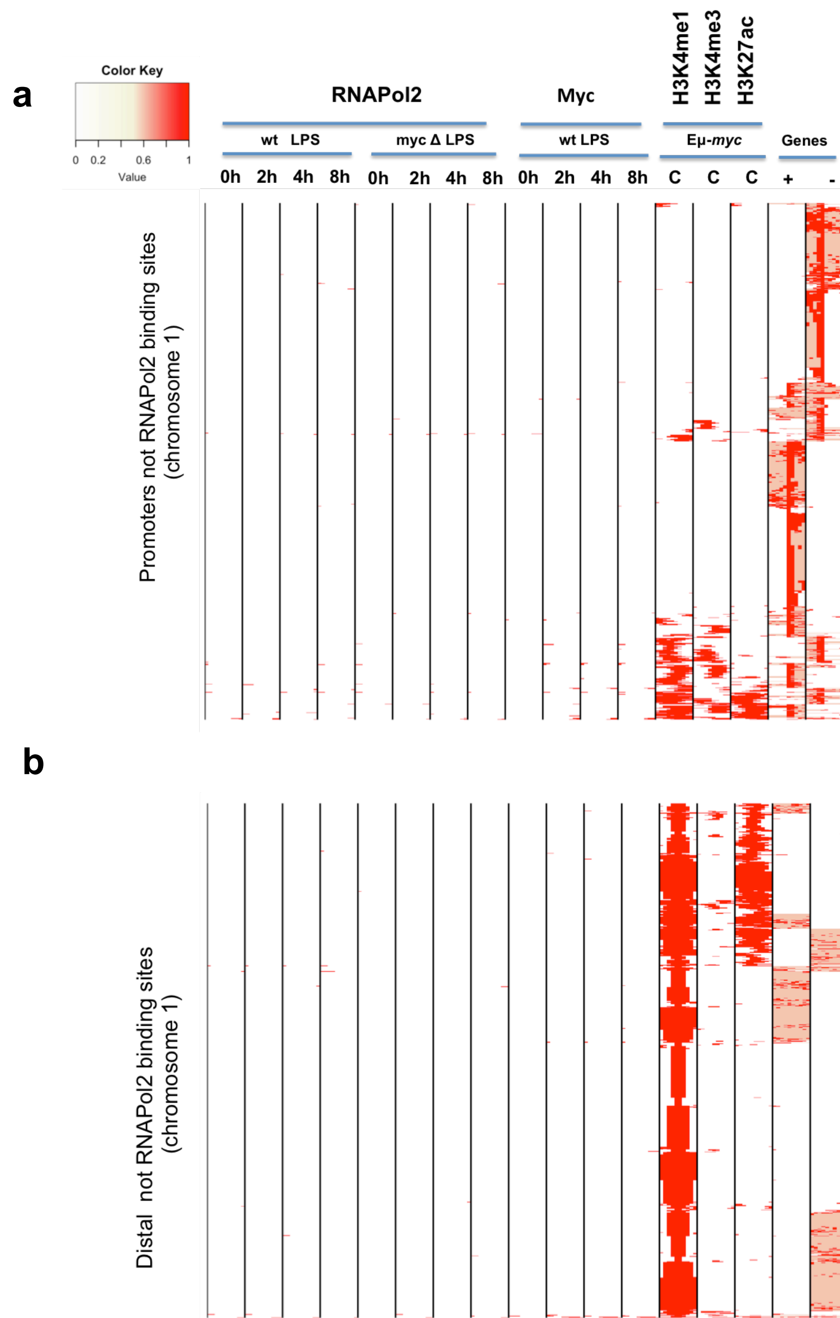



**Figure 55** ***RNAPol2 unbound regions are mainly inactive.***
Regions not bound by RNAPol2 and Myc at annotated promoters (top panel) and at enahncers (distal H3K4me1 positive sites) (botton panel) upon LPS stimulation of wt and *c-myc*$^{\Delta/\Delta}$ B cells (0, 2, 4, 8h) at chromosome 1. The distribution of histone marks (H3K4me1, H3K4me3, H3K27ac) in Eμ-*myc* control sample is also shown. Each row represents a different genomic interval. In the top heatmap no Myc and RNApol2 bound promoters (6 kb width centered on the transcription start site); in the bottom heatmap no Myc and RNAPol2 bound enhancers (6 kb width centered on H3K4me1 peaks) are shown. Annotated genes (exons in red, introns in pink; + sense, - antisense strand) are also shown.

It is noteworthy here that the above heatmaps, which represent a qualitative yes/no score, did not reveal any obvious differences between wt and c-myc$^{\Delta/\Delta}$ cells in terms of RNAPol2 distribution at promoters and distal sites (**Figure 54; Figure 55**). A more quantitative analysis was thus needed to evaluate if and to which extent Myc could affect RNAPol2recruitment and/or elongation. To this aim, we plotted the distribution of RNAPol2 reads in the TSS region and the gene body of the different regulatory groups identified by RNA-seq and we also examined the stalling index, based on the ratio of RNAPol2 reads in the promoter and gene body (Rahl et al.2010; Zeitlinger et al. 2007) (**Figure 56**; for the sake of simplicity, only the results of the 4h LPS time point is shown here). Different effects of RNApol2 occupancy were observed in the groups of DEGs. Most remarkably, Myc-dependent induced genes showed higher level of RNAPol2 both on the TSS and gene body in wt cells, which were essentially lost in *c-myc*$^{\Delta/\Delta}$cells (**Figure 56a**). Unexpectedly, Myc-independent induced genes still showed some dependency upon Myc in RNAPol2 recruitment at either promoters or gene bodies (**Figure 56b**), a feature shared also with non-regulated genes and, on promoters, with repressed genes (**Figure 56a; Figure 56b**). While these observations remain to be validated, they point to possible non-specific and/or indirect effects of Myc on Pol2 loading.

The above considerations limit the significance of the stalling index (see also Sabò et al., 2014). We note, however, that in no instance did activated genes show the *decrease* in stalling index that would be expected from a selective augmentation of elongation, as was proposed to occur upon activation of all expressed genes by Myc (Rahl et al., 2009; Lin C. et al., 2012). These data do not allow us to assign any exclusive role to either loading or elongation in Myc-regulated transcription. Tentatively, we speculate that both steps may be involved.

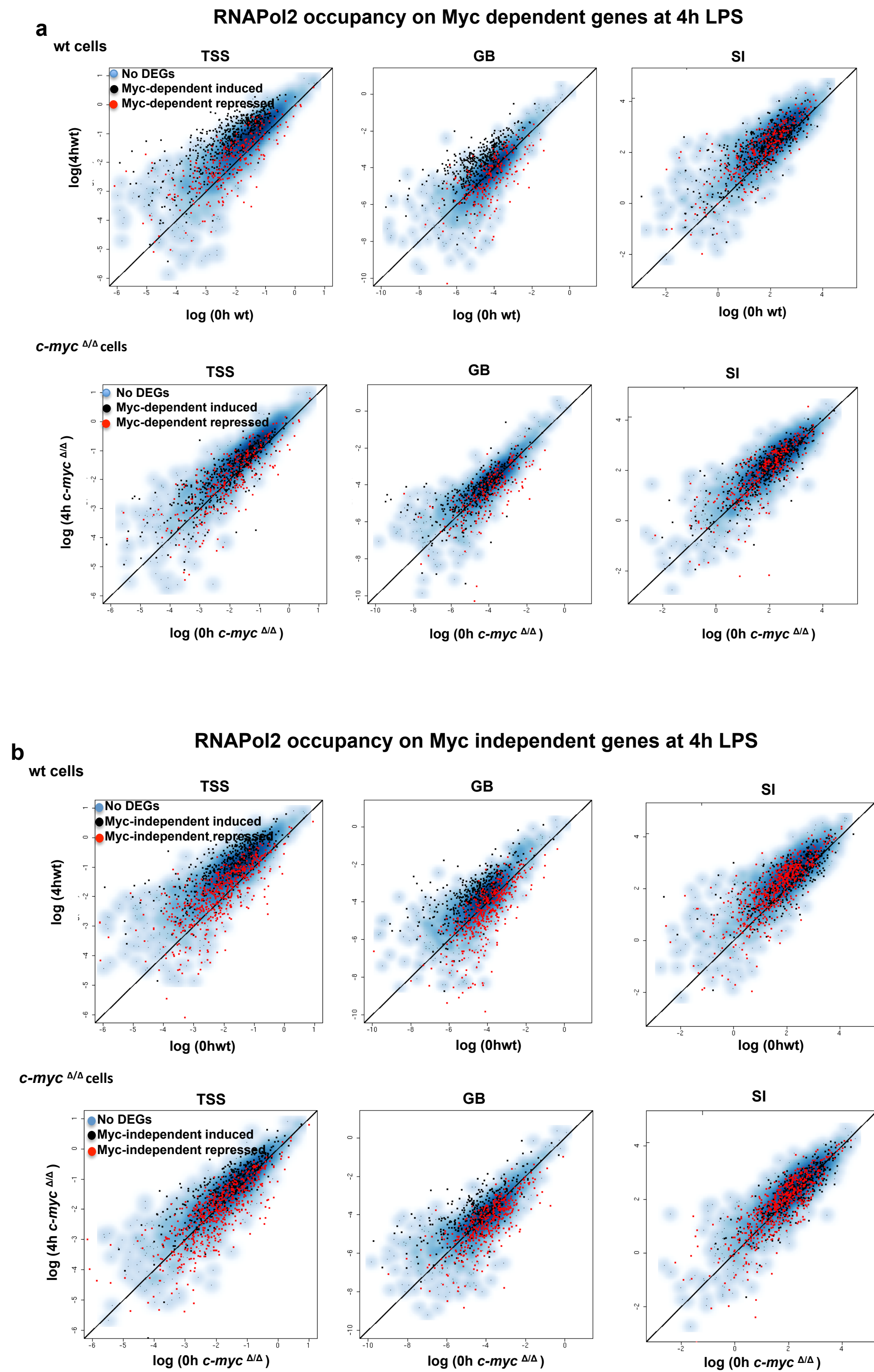



**Figure 56** ***Stalling index analysis on the different regulatory groups of genes***
Stalling index and Log10 reads at 4h LPS in wt and *c-myc*$^{\Delta/\Delta}$ cells on TSS and gene body are reported on No-DEGs, Myc-dependent induced and repressed genes (**a**) and on Myc-independent induced and repressed genes (**b**).

## 4.8 Investigating biological functions of Myc dependent genes

To investigate the biological processes regulated by Myc in B-cells, we applied gene ontology (GO) and Gene set enrichment analysis (GSEA) to our RNA-seq data. Myc-dependent LPS induced and repressed genes affect biological processes consistently regulated by Myc in many other systems (Kress et al., 2015). In particular, we concentrate on Myc-dependent induced and Myc bound genes (called "Myc-dependent induced bound genes") and Myc-dependent repressed and Myc bound genes (called "Myc-dependent repressed bound genes"). The few Myc–dependent induced bound genes at 2h of LPS were manually checked and, as expected, they were involved in mitogenic/signal response. At 4h and 8h after LPS Myc-dependent induced genes were mainly involved, among others, in RNA metabolic processes, energy metabolism, translation and ribosome assembly (**Table 4**). Given specificity to this finding, we performed a pre-ranked analysis with GSEA for the Myc-dependent induced and repressed genes and we observed at 8h after LPS a significant enrichment of gene sets involved in ribosome biogenesis, translation and mitochondrial biosynthesis (**Table 5**), as confirmed by GO. In addition, ingenuity pathway analysis (IPA) also underlines a significant enrichment of signaling pathways involved in initial translation (eIF2 and eIF4), mTOR and mitochondrial metabolic pathways (**Table 6**). It has already been demonstrated that Myc promotes the RNAPolII mediated transcription of many translation initiation factors for example through E-box's binding at the promoter of eIF4 (Jones et al., 1996).

We thus confirmed that Myc induction causes a re-organization of cell's physiology, activating genes involved in the whole protein biosynthetic apparatus, growth and energy metabolism.

Among the Myc-dependent repressed genes, the overall picture is less clear (**Table 4**). At 4h and 8h post LPS, this category enriched for genes implicated in negative regulation of cellular growth, cell cycle and in signal transduction. Other down-regulated genes belong to GADD45 signaling and it was already shown that *Gadd45a (*mainly involved in growth arrest and DNA-damage response) is a dowregulated Myc target gene (Yap et al., 2011).

In conclusion, RNA-seq profiling indicates that Myc can selective up and down regulate genes in response to LPS. These genes control processes such as translation, energy metabolism, nucleotide biosynthesis consistent with the role of Myc in driving cellular activation in response to extracellular cues (de Alboran et al., 2001).

# Gene ontology of Myc dependent bound genes

**4h LPS**

**Myc-dependent induced bound genes**

| Gene Set Name | # Genes in Gene Set (K) | # Genes in Overlap (k) | k/K | p-value | FDR q-value |
|---|---|---|---|---|---|
| BIOPOLYMER_METABOLIC_PROCESS | 1684 | 84 | 0.0499 | 1.87E-29 | 1.54E-26 |
| NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS | 1244 | 70 | 0.0563 | 1.3E-27 | 5.36E-25 |
| RNA_METABOLIC_PROCESS | 841 | 52 | 0.0618 | 1.72E-22 | 4.73E-20 |
| TRANSCRIPTION_DNA_DEPENDENT | 636 | 33 | 0.0519 | 1.42E-12 | 2.12E-10 |
| TRANSCRIPTION | 753 | 36 | 0.0478 | 1.44E-12 | 2.12E-10 |
| RNA_BIOSYNTHETIC_PROCESS | 638 | 33 | 0.0517 | 1.55E-12 | 2.12E-10 |
| RNA_PROCESSING | 173 | 17 | 0.0983 | 2.69E-11 | 3.17E-9 |
| RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS_AND_ASSEMBLY | 86 | 12 | 0.1395 | 3.84E-10 | 3.96E-8 |
| REGULATION_OF_RNA_METABOLIC_PROCESS | 471 | 25 | 0.0531 | 4.77E-10 | 4.37E-8 |
| RIBOSOME_BIOGENESIS_AND_ASSEMBLY | 18 | 7 | 0.3889 | 7.94E-10 | 6.55E-8 |
| REGULATION_OF_NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METAB OLIC_PROCESS | 618 | 27 | 0.0437 | 6.44E-9 | 4.43E-7 |
| TRANSCRIPTION_FROM_RNA_POLYMERASE_II_PROMOTER | 457 | 23 | 0.0503 | 6.45E-9 | 4.43E-7 |
| REGULATION_OF_TRANSCRIPTIONDNA_DEPENDENT | 461 | 23 | 0.0499 | 7.59E-9 | 4.81E-7 |
| RRNA_PROCESSING | 15 | 6 | 0.4000 | 1.11E-8 | 6.53E-7 |
| ORGANELLE_ORGANIZATION_AND_BIOGENESIS | 473 | 23 | 0.0486 | 1.22E-8 | 6.72E-7 |
| RRNA_METABOLIC_PROCESS | 16 | 6 | 0.3750 | 1.76E-8 | 9.05E-7 |
| REGULATION_OF_TRANSCRIPTION | 566 | 25 | 0.0442 | 1.9E-8 | 9.21E-7 |
| PROTEIN_METABOLIC_PROCESS | 1231 | 39 | 0.0317 | 2.44E-8 | 1.12E-6 |
| REGULATION_OF_GENE_EXPRESSION | 673 | 27 | 0.0401 | 3.74E-8 | 1.62E-6 |
| REGULATION_OF_TRANSCRIPTION_FROM_RNA_POLYMERASE_II_PROMOTER | 289 | 17 | 0.0588 | 6.7E-8 | 2.76E-6 |
| REGULATION_OF_CELLULAR_METABOLIC_PROCESS | 787 | 29 | 0.0368 | 7.23E-8 | 2.84E-6 |
| REGULATION_OF_METABOLIC_PROCESS | 799 | 29 | 0.0363 | 9.93E-8 | 3.72E-6 |
| CELLULAR_PROTEIN_METABOLIC_PROCESS | 1117 | 35 | 0.0313 | 1.68E-7 | 6.04E-6 |
| CELLULAR_MACROMOLECULE_METABOLIC_PROCESS | 1131 | 35 | 0.0309 | 2.26E-7 | 7.75E-6 |
| BIOPOLYMER_MODIFICATION | 650 | 24 | 0.0369 | 9.35E-7 | 3.09E-5 |
| PROTEIN_MODIFICATION_PROCESS | 631 | 23 | 0.0365 | 1.95E-6 | 6.19E-5 |
| CELL_CYCLE_GO_0007049 | 315 | 15 | 0.0476 | 5.4E-6 | 1.65E-4 |
| BIOPOLYMER_CATABOLIC_PROCESS | 117 | 9 | 0.0769 | 9.97E-6 | 2.94E-4 |
| RNA_SPLICING | 91 | 8 | 0.0879 | 1.17E-5 | 3.32E-4 |
| CELLULAR_MACROMOLECULE_CATABOLIC_PROCESS | 104 | 8 | 0.0769 | 3.1E-5 | 8.53E-4 |
| MACROMOLECULE_CATABOLIC_PROCESS | 137 | 9 | 0.0657 | 3.52E-5 | 9.38E-4 |
| POSITIVE_REGULATION_OF_BIOLOGICAL_PROCESS | 709 | 21 | 0.0296 | 1.1E-4 | 2.83E-3 |
| MACROMOLECULAR_COMPLEX_ASSEMBLY | 280 | 12 | 0.0429 | 1.26E-4 | 3.14E-3 |
| RNA_CATABOLIC_PROCESS | 23 | 4 | 0.1739 | 1.35E-4 | 3.25E-3 |
| POSITIVE_REGULATION_OF_CELLULAR_PROCESS | 668 | 20 | 0.0299 | 1.38E-4 | 3.25E-3 |
| CHROMATIN_REMODELING | 25 | 4 | 0.1600 | 1.89E-4 | 4.34E-3 |
| CELLULAR_CATABOLIC_PROCESS | 212 | 10 | 0.0472 | 2.11E-4 | 4.71E-3 |
| CELLULAR_COMPONENT_ASSEMBLY | 298 | 12 | 0.0403 | 2.24E-4 | 4.85E-3 |
| DNA_METABOLIC_PROCESS | 257 | 11 | 0.0428 | 2.41E-4 | 5.09E-3 |
| ESTABLISHMENT_AND_OR_MAINTENANCE_OF_CHROMATIN_ARCHITECTURE | 77 | 6 | 0.0779 | 2.85E-4 | 5.88E-3 |
| AMINO_ACID_METABOLIC_PROCESS | 78 | 6 | 0.0769 | 3.06E-4 | 6.03E-3 |
| REGULATION_OF_CELL_CYCLE | 182 | 9 | 0.0495 | 3.07E-4 | 6.03E-3 |
| CATABOLIC_PROCESS | 225 | 10 | 0.0444 | 3.4E-4 | 6.52E-3 |
| CHROMATIN_MODIFICATION | 55 | 5 | 0.0909 | 4.52E-4 | 8.47E-3 |
| POSITIVE_REGULATION_OF_RNA_METABOLIC_PROCESS | 120 | 7 | 0.0583 | 5.34E-4 | 9.75E-3 |
| POST_TRANSLATIONAL_PROTEIN_MODIFICATION | 476 | 15 | 0.0315 | 5.44E-4 | 9.75E-3 |
| CHROMOSOME_ORGANIZATION_AND_BIOGENESIS | 124 | 7 | 0.0565 | 6.5E-4 | 1.14E-2 |
| AMINO_ACID_AND_DERIVATIVE_METABOLIC_PROCESS | 101 | 6 | 0.0594 | 1.21E-3 | 2.05E-2 |
| TRANSPORT | 795 | 20 | 0.0252 | 1.22E-3 | 2.05E-2 |
| TRANSCRIPTION_FROM_RNA_POLYMERASE_III_PROMOTER | 19 | 3 | 0.1579 | 1.32E-3 | 2.18E-2 |

**4h LPS**

**Myc-dependent repressed bound genes**

| Gene Set Name | # Genes in Gene Set (K) | # Genes in Overlap (k) | k/K | p-value | FDR q-value |
|---|---|---|---|---|---|
| BIOPOLYMER_MODIFICATION | 650 | 24 | 0.0369 | 1.45E-12 | 1.19E-9 |
| PROTEIN_MODIFICATION_PROCESS | 631 | 23 | 0.0365 | 5.59E-12 | 2.3E-9 |
| PROTEIN_METABOLIC_PROCESS | 1231 | 30 | 0.0244 | 7.06E-11 | 1.94E-8 |
| CELLULAR_PROTEIN_METABOLIC_PROCESS | 1117 | 28 | 0.0251 | 1.68E-10 | 3.47E-8 |
| CELLULAR_MACROMOLECULE_METABOLIC_PROCESS | 1131 | 28 | 0.0248 | 2.23E-10 | 3.67E-8 |
| BIOPOLYMER_METABOLIC_PROCESS | 1684 | 34 | 0.0202 | 4.98E-10 | 6.85E-8 |
| POST_TRANSLATIONAL_PROTEIN_MODIFICATION | 476 | 16 | 0.0336 | 3.15E-8 | 3.72E-6 |
| SIGNAL_TRANSDUCTION | 1634 | 27 | 0.0165 | 1.71E-6 | 1.77E-4 |
| RESPONSE_TO_STRESS | 508 | 13 | 0.0256 | 1.22E-5 | 1.12E-3 |
| NEGATIVE_REGULATION_OF_GROWTH | 40 | 4 | 0.1000 | 9.15E-5 | 7.2E-3 |
| GROWTH | 77 | 5 | 0.0649 | 9.6E-5 | 7.2E-3 |
| REGULATION_OF_CELL_CYCLE | 182 | 7 | 0.0385 | 1.11E-4 | 7.5E-3 |
| NEGATIVE_REGULATION_OF_CELLULAR_PROCESS | 646 | 13 | 0.0201 | 1.4E-4 | 8.24E-3 |
| REGULATION_OF_CELL_GROWTH | 46 | 4 | 0.0870 | 1.59E-4 | 8.74E-3 |
| GLYCOPROTEIN_METABOLIC_PROCESS | 90 | 5 | 0.0556 | 2.01E-4 | 1.01E-2 |
| REGULATION_OF_BIOLOGICAL_QUALITY | 419 | 10 | 0.0239 | 2.16E-4 | 1.01E-2 |
| NEGATIVE_REGULATION_OF_BIOLOGICAL_PROCESS | 677 | 13 | 0.0192 | 2.2E-4 | 1.01E-2 |
| REGULATION_OF_CELLULAR_METABOLIC_PROCESS | 787 | 14 | 0.0178 | 2.77E-4 | 1.2E-2 |
| REGULATION_OF_METABOLIC_PROCESS | 799 | 14 | 0.0175 | 3.22E-4 | 1.33E-2 |
| CELL_CYCLE_ARREST_GO_0007050 | 57 | 4 | 0.0702 | 3.65E-4 | 1.44E-2 |
| REGULATION_OF_GROWTH | 58 | 4 | 0.0690 | 3.91E-4 | 1.47E-2 |
| ONE_CARBON_COMPOUND_METABOLIC_PROCESS | 26 | 3 | 0.1154 | 4.77E-4 | 1.65E-2 |
| CELL_SURFACE_RECEPTOR_LINKED_SIGNAL_TRANSDUCTION_GO_0007166 | 641 | 12 | 0.0187 | 4.79E-4 | 1.65E-2 |
| BIOSYNTHETIC_PROCESS | 470 | 10 | 0.0213 | 5.32E-4 | 1.7E-2 |
| PROTEIN_AMINO_ACID_DEPHOSPHORYLATION | 63 | 4 | 0.0635 | 5.36E-4 | 1.7E-2 |
| PHOSPHORYLATION | 313 | 8 | 0.0256 | 5.88E-4 | 1.8E-2 |
| REGULATION_OF_GENE_EXPRESSION | 673 | 12 | 0.0178 | 7.32E-4 | 2.16E-2 |
| DEPHOSPHORYLATION | 70 | 4 | 0.0571 | 7.99E-4 | 2.27E-2 |
| CENTRAL_NERVOUS_SYSTEM_DEVELOPMENT | 123 | 5 | 0.0407 | 8.43E-4 | 2.32E-2 |
| NEGATIVE_REGULATION_OF_CELLULAR_METABOLIC_PROCESS | 259 | 7 | 0.0270 | 9.31E-4 | 2.48E-2 |
| GLYCOPROTEIN_BIOSYNTHETIC_PROCESS | 74 | 4 | 0.0541 | 9.84E-4 | 2.49E-2 |
| NEGATIVE_REGULATION_OF_METABOLIC_PROCESS | 262 | 7 | 0.0267 | 9.95E-4 | 2.49E-2 |
| G_PROTEIN_COUPLED_RECEPTOR_PROTEIN_SIGNALING_PATHWAY | 342 | 8 | 0.0234 | 1.04E-3 | 2.53E-2 |
| NEGATIVE_REGULATION_OF_CELL_CYCLE | 79 | 4 | 0.0506 | 1.26E-3 | 2.91E-2 |
| NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS | 1244 | 17 | 0.0137 | 1.27E-3 | 2.91E-2 |
| HEART_DEVELOPMENT | 37 | 3 | 0.0811 | 1.36E-3 | 3.03E-2 |

**8h LPS**
**Myc-dependent induced bound genes**

| Gene Set Name | # Genes in Gene Set (K) | # Genes in Overlap (k) | k/K | p-value | FDR q-value |
|---|---|---|---|---|---|
| CELLULAR_BIOSYNTHETIC_PROCESS | 321 | 67 | 0.2087 | 9.47E-58 | 6.58E-55 |
| TRANSLATION | 180 | 55 | 0.3056 | 1.6E-57 | 6.58E-55 |
| BIOSYNTHETIC_PROCESS | 470 | 73 | 0.1553 | 3.12E-53 | 8.57E-51 |
| MACROMOLECULE_BIOSYNTHETIC_PROCESS | 321 | 61 | 0.1900 | 5.25E-50 | 1.08E-47 |
| NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS | 1244 | 102 | 0.0820 | 1.34E-47 | 2.21E-45 |
| PROTEIN_METABOLIC_PROCESS | 1231 | 98 | 0.0796 | 1.41E-44 | 1.94E-42 |
| CELLULAR_PROTEIN_METABOLIC_PROCESS | 1117 | 92 | 0.0824 | 5.07E-43 | 5.97E-41 |
| CELLULAR_MACROMOLECULE_METABOLIC_PROCESS | 1131 | 92 | 0.0813 | 1.41E-42 | 1.46E-40 |
| RNA_METABOLIC_PROCESS | 841 | 68 | 0.0809 | 2.42E-31 | 2.22E-29 |
| BIOPOLYMER_METABOLIC_PROCESS | 1684 | 95 | 0.0564 | 4.38E-31 | 3.62E-29 |
| RNA_PROCESSING | 173 | 27 | 0.1561 | 2.08E-20 | 1.56E-18 |
| TRANSCRIPTION | 753 | 49 | 0.0651 | 7.55E-19 | 5.19E-17 |
| REGULATION_OF_METABOLIC_PROCESS | 799 | 49 | 0.0613 | 8.38E-18 | 5.32E-16 |
| RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS_AND_ASSEMBLY | 86 | 19 | 0.2209 | 1.01E-17 | 5.94E-16 |
| REGULATION_OF_CELLULAR_METABOLIC_PROCESS | 787 | 48 | 0.0610 | 2.28E-17 | 1.25E-15 |
| REGULATION_OF_GENE_EXPRESSION | 673 | 41 | 0.0609 | 5.42E-15 | 2.79E-13 |
| TRANSCRIPTION_DNA_DEPENDENT | 636 | 39 | 0.0613 | 2.06E-14 | 9.88E-13 |
| PROTEIN_RNA_COMPLEX_ASSEMBLY | 67 | 15 | 0.2239 | 2.27E-14 | 9.88E-13 |
| RNA_BIOSYNTHETIC_PROCESS | 638 | 39 | 0.0611 | 2.28E-14 | 9.88E-13 |
| MACROMOLECULAR_COMPLEX_ASSEMBLY | 280 | 26 | 0.0929 | 4.18E-14 | 1.73E-12 |
| RNA_SPLICING | 91 | 16 | 0.1758 | 1.73E-13 | 6.76E-12 |
| CELLULAR_COMPONENT_ASSEMBLY | 298 | 26 | 0.0872 | 1.8E-13 | 6.76E-12 |
| POSITIVE_REGULATION_OF_CELLULAR_PROCESS | 668 | 37 | 0.0554 | 2.02E-12 | 7.25E-11 |
| POSITIVE_REGULATION_OF_BIOLOGICAL_PROCESS | 709 | 38 | 0.0536 | 2.71E-12 | 9.32E-11 |
| REGULATION_OF_NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS | 618 | 34 | 0.0550 | 1.93E-11 | 6.37E-10 |
| ORGANELLE_ORGANIZATION_AND_BIOGENESIS | 473 | 29 | 0.0613 | 4.71E-11 | 1.49E-9 |
| CELL_PROLIFERATION_GO_0008283 | 513 | 29 | 0.0565 | 3.16E-10 | 9.66E-9 |
| PROTEIN_FOLDING | 58 | 11 | 0.1897 | 4.56E-10 | 1.34E-8 |
| REGULATION_OF_TRANSCRIPTION | 566 | 30 | 0.0530 | 7.19E-10 | 2.04E-8 |
| TRANSCRIPTION_FROM_RNA_POLYMERASE_II_PROMOTER | 457 | 26 | 0.0569 | 2.29E-9 | 6.29E-8 |
| NUCLEOBASENUCLEOSIDE_AND_NUCLEOTIDE_METABOLIC_PROCESS | 52 | 10 | 0.1923 | 2.47E-9 | 6.56E-8 |
| TRANSLATIONAL_INITIATION | 39 | 9 | 0.2308 | 2.8E-9 | 7.21E-8 |
| REGULATION_OF_RNA_METABOLIC_PROCESS | 471 | 26 | 0.0552 | 4.27E-9 | 1.07E-7 |
| REGULATION_OF_TRANSLATIONAL_INITIATION | 31 | 8 | 0.2581 | 8.25E-9 | 2,00E-07 |
| MITOCHONDRION_ORGANIZATION_AND_BIOGENESIS | 48 | 9 | 0.1875 | 1.98E-8 | 4.66E-7 |
| REGULATION_OF_TRANSCRIPTIONDNA_DEPENDENT | 461 | 24 | 0.0521 | 5.06E-8 | 1.16E-6 |
| MRNA_PROCESSING_GO_0006397 | 73 | 10 | 0.1370 | 7.44E-8 | 1.66E-6 |
| RIBOSOME_BIOGENESIS_AND_ASSEMBLY | 18 | 6 | 0.3333 | 1.17E-7 | 2.54E-6 |
| MRNA_METABOLIC_PROCESS | 84 | 10 | 0.1190 | 2.88E-7 | 6.1E-6 |
| REGULATION_OF_APOPTOSIS | 341 | 19 | 0.0557 | 4.47E-7 | 9.22E-6 |
| REGULATION_OF_PROGRAMMED_CELL_DEATH | 342 | 19 | 0.0556 | 4.67E-7 | 9.4E-6 |
| CELL_DEVELOPMENT | 577 | 25 | 0.0433 | 8.36E-7 | 1.64E-5 |
| POSITIVE_REGULATION_OF_CELLULAR_METABOLIC_PROCESS | 229 | 15 | 0.0655 | 9.83E-7 | 1.88E-5 |
| APOPTOSIS_GO | 431 | 21 | 0.0487 | 1,00E-06 | 1.88E-5 |
| PROGRAMMED_CELL_DEATH | 432 | 21 | 0.0486 | 1.04E-6 | 1.91E-5 |
| REGULATION_OF_DEVELOPMENTAL_PROCESS | 440 | 21 | 0.0477 | 1.39E-6 | 2.46E-5 |
| RRNA_PROCESSING | 15 | 5 | 0.3333 | 1.4E-6 | 2.46E-5 |
| POSITIVE_REGULATION_OF_METABOLIC_PROCESS | 236 | 15 | 0.0636 | 1.43E-6 | 2.46E-5 |
| REGULATION_OF_CELLULAR_COMPONENT_ORGANIZATION_AND_BIOGENESIS | 125 | 11 | 0.0880 | 1.6E-6 | 2.69E-5 |
| NEGATIVE_REGULATION_OF_CELLULAR_PROCESS | 646 | 26 | 0.0402 | 1.98E-6 | 3.26E-5 |
| RRNA_METABOLIC_PROCESS | 16 | 5 | 0.3125 | 2.01E-6 | 3.26E-5 |
| RESPONSE_TO_STRESS | 508 | 22 | 0.0433 | 3.8E-6 | 6.03E-5 |
| ALCOHOL_METABOLIC_PROCESS | 88 | 9 | 0.1023 | 4.11E-6 | 6.39E-5 |
| NEGATIVE_REGULATION_OF_BIOLOGICAL_PROCESS | 677 | 26 | 0.0384 | 4.58E-6 | 6.99E-5 |
| REGULATION_OF_TRANSLATION | 93 | 9 | 0.0968 | 6.5E-6 | 9.7E-5 |
| REGULATION_OF_PROTEIN_METABOLIC_PROCESS | 173 | 12 | 0.0694 | 6.59E-6 | 9.7E-5 |
| MITOCHONDRIAL_TRANSPORT | 21 | 5 | 0.2381 | 8.86E-6 | 1.28E-4 |
| NITROGEN_COMPOUND_METABOLIC_PROCESS | 155 | 11 | 0.0710 | 1.28E-5 | 1.82E-4 |
| DNA_METABOLIC_PROCESS | 257 | 14 | 0.0545 | 1.85E-5 | 2.58E-4 |
| REGULATION_OF_CELLULAR_PROTEIN_METABOLIC_PROCESS | 162 | 11 | 0.0679 | 1.94E-5 | 2.66E-4 |
| NUCLEOTIDE_METABOLIC_PROCESS | 42 | 6 | 0.1429 | 2.48E-5 | 3.36E-4 |
| POSITIVE_REGULATION_OF_TRANSCRIPTION | 144 | 10 | 0.0694 | 3.79E-5 | 5.05E-4 |
| CELL_CYCLE_GO_0007049 | 315 | 15 | 0.0476 | 4.47E-5 | 5.86E-4 |
| NEGATIVE_REGULATION_OF_APOPTOSIS | 150 | 10 | 0.0667 | 5.37E-5 | 6.92E-4 |
| NEGATIVE_REGULATION_OF_PROGRAMMED_CELL_DEATH | 151 | 10 | 0.0662 | 5.68E-5 | 7.21E-4 |
| NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_TRANSPORT | 31 | 5 | 0.1613 | 6.59E-5 | 8.24E-4 |
| POSITIVE_REGULATION_OF_NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_P ROCESS | 154 | 10 | 0.0649 | 6.7E-5 | 8.25E-4 |
| AMINO_ACID_AND_DERIVATIVE_METABOLIC_PROCESS | 101 | 8 | 0.0792 | 8.98E-5 | 1.09E-3 |
| CHROMATIN_MODIFICATION | 55 | 6 | 0.1091 | 1.18E-4 | 1.41E-3 |
| RNA_SPLICINGVIA_TRANSESTERIFICATION_REACTIONS | 35 | 5 | 0.1429 | 1.2E-4 | 1.42E-3 |
| CELLULAR_RESPIRATION | 19 | 4 | 0.2105 | 1.24E-4 | 1.42E-3 |
| TRNA_METABOLIC_PROCESS | 19 | 4 | 0.2105 | 1.24E-4 | 1.42E-3 |
| REGULATION_OF_CELL_PROLIFERATION | 308 | 14 | 0.0455 | 1.3E-4 | 1.46E-3 |
| PROTEIN_COMPLEX_ASSEMBLY | 167 | 10 | 0.0599 | 1.31E-4 | 1.46E-3 |
| NUCLEOTIDE_BIOSYNTHETIC_PROCESS | 20 | 4 | 0.2000 | 1.53E-4 | 1.68E-3 |
| TRANSPORT | 795 | 25 | 0.0314 | 1.74E-4 | 1.87E-3 |
| AMINE_METABOLIC_PROCESS | 141 | 9 | 0.0638 | 1.75E-4 | 1.87E-3 |
| SPLICEOSOME_ASSEMBLY | 21 | 4 | 0.1905 | 1.87E-4 | 1.98E-3 |
| CELLULAR_CATABOLIC_PROCESS | 212 | 11 | 0.0519 | 2.17E-4 | 2.27E-3 |
| POSITIVE_REGULATION_OF_I_KAPPAB_KINASE_NF_KAPPAB_CASCADE | 87 | 7 | 0.0805 | 2.24E-4 | 2.31E-3 |
| POSITIVE_REGULATION_OF_TRANSCRIPTIONDNA_DEPENDENT | 118 | 8 | 0.0678 | 2.64E-4 | 2.66E-3 |
| POSITIVE_REGULATION_OF_CELL_PROLIFERATION | 149 | 9 | 0.0604 | 2.64E-4 | 2.66E-3 |
| POSITIVE_REGULATION_OF_RNA_METABOLIC_PROCESS | 120 | 8 | 0.0667 | 2.96E-4 | 2.95E-3 |
| REGULATION_OF_I_KAPPAB_KINASE_NF_KAPPAB_CASCADE | 93 | 7 | 0.0753 | 3.37E-4 | 3.31E-3 |
| CATABOLIC_PROCESS | 225 | 11 | 0.0489 | 3.61E-4 | 3.51E-3 |
| POSITIVE_REGULATION_OF_SIGNAL_TRANSDUCTION | 126 | 8 | 0.0635 | 4.12E-4 | 3.95E-3 |
| NEGATIVE_REGULATION_OF_DEVELOPMENTAL_PROCESS | 197 | 10 | 0.0508 | 4.94E-4 | 4.68E-3 |
| CELLULAR_RESPONSE_TO_EXTRACELLULAR_STIMULUS | 12 | 3 | 0.2500 | 5.43E-4 | 5.04E-3 |
| PURINE_NUCLEOTIDE_METABOLIC_PROCESS | 12 | 3 | 0.2500 | 5.43E-4 | 5.04E-3 |
| GLUCOSE_METABOLIC_PROCESS | 28 | 4 | 0.1429 | 5.92E-4 | 5.42E-3 |
| INTRACELLULAR_TRANSPORT | 280 | 12 | 0.0429 | 6.47E-4 | 5.84E-3 |
| ESTABLISHMENT_OF_LOCALIZATION | 870 | 25 | 0.0287 | 6.52E-4 | 5.84E-3 |
| MACROMOLECULE_CATABOLIC_PROCESS | 137 | 8 | 0.0584 | 7.18E-4 | 6.37E-3 |
| ESTABLISHMENT_AND_OR_MAINTENANCE_OF_CHROMATIN_ARCHITECTURE | 77 | 6 | 0.0779 | 7.43E-4 | 6.52E-3 |
| AMINO_ACID_METABOLIC_PROCESS | 78 | 6 | 0.0769 | 7.96E-4 | 6.91E-3 |
| REGULATION_OF_TRANSCRIPTION_FROM_RNA_POLYMERASE_II_PROMOTER | 289 | 12 | 0.0415 | 8.52E-4 | 7.32E-3 |
| REGULATION_OF_BIOLOGICAL_QUALITY | 419 | 15 | 0.0358 | 9.42E-4 | 8.01E-3 |
| AEROBIC_RESPIRATION | 15 | 3 | 0.2000 | 1.09E-3 | 9.14E-3 |
| CELLULAR_COMPONENT_DISASSEMBLY | 33 | 4 | 0.1212 | 1.12E-3 | 9.14E-3 |
| NUCLEAR_EXPORT | 33 | 4 | 0.1212 | 1.12E-3 | 9.14E-3 |

**8h LPS**

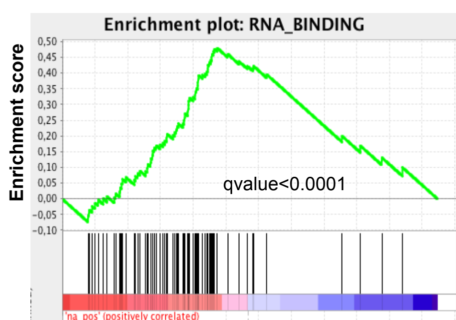
**Myc-dependent repressed bound genes**

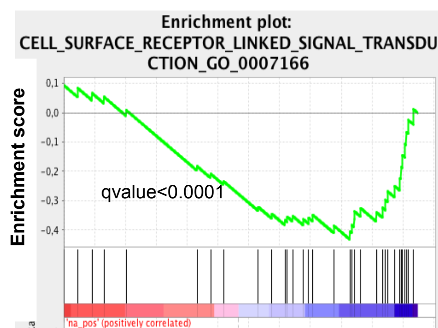
| Gene Set Name | # Genes in Gene Set (K) | # Genes in Overlap (k) | k/K | p-value | FDR q-value |
|---|---|---|---|---|---|
| SIGNAL_TRANSDUCTION | 1634 | 67 | 0.0410 | 1.57E-19 | 1.3E-16 |
| BIOPOLYMER_METABOLIC_PROCESS | 1684 | 60 | 0.0356 | 8.25E-15 | 3.41E-12 |
| REGULATION_OF_TRANSCRIPTION | 566 | 30 | 0.0530 | 5.07E-12 | 9.54E-10 |
| NEGATIVE_REGULATION_OF_CELLULAR_PROCESS | 646 | 32 | 0.0495 | 5.78E-12 | 9.54E-10 |
| NEGATIVE_REGULATION_OF_BIOLOGICAL_PROCESS | 677 | 32 | 0.0473 | 1.94E-11 | 2.67E-9 |
| REGULATION_OF_TRANSCRIPTIONDNA_DEPENDENT | 461 | 26 | 0.0564 | 3.52E-11 | 4.15E-9 |
| REGULATION_OF_NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS | 618 | 30 | 0.0485 | 4.39E-11 | 4.53E-9 |
| REGULATION_OF_RNA_METABOLIC_PROCESS | 471 | 26 | 0.0552 | 5.62E-11 | 5.16E-9 |
| TRANSCRIPTION | 753 | 33 | 0.0438 | 6.75E-11 | 5.25E-9 |
| REGULATION_OF_METABOLIC_PROCESS | 799 | 34 | 0.0426 | 7.52E-11 | 5.25E-9 |
| REGULATION_OF_GENE_EXPRESSION | 673 | 31 | 0.0461 | 7.63E-11 | 5.25E-9 |
| REGULATION_OF_CELLULAR_METABOLIC_PROCESS | 787 | 33 | 0.0419 | 2.09E-10 | 1.33E-8 |
| ESTABLISHMENT_OF_LOCALIZATION | 870 | 33 | 0.0379 | 2.56E-9 | 1.51E-7 |
| INTRACELLULAR_SIGNALING_CASCADE | 667 | 28 | 0.0420 | 4.89E-9 | 2.69E-7 |
| TRANSCRIPTION_DNA_DEPENDENT | 636 | 27 | 0.0425 | 7.3E-9 | 3.76E-7 |
| RNA_BIOSYNTHETIC_PROCESS | 638 | 27 | 0.0423 | 7.79E-9 | 3.78E-7 |
| NEGATIVE_REGULATION_OF_CELLULAR_METABOLIC_PROCESS | 259 | 17 | 0.0656 | 9.7E-9 | 4.45E-7 |
| NEGATIVE_REGULATION_OF_METABOLIC_PROCESS | 262 | 17 | 0.0649 | 1.15E-8 | 5,00E-07 |
| TRANSPORT | 795 | 30 | 0.0377 | 1.54E-8 | 6.35E-7 |
| RESPONSE_TO_STRESS | 508 | 23 | 0.0453 | 2.99E-8 | 1.17E-6 |
| NEGATIVE_REGULATION_OF_TRANSCRIPTION | 188 | 14 | 0.0745 | 4.07E-8 | 1.53E-6 |
| BIOPOLYMER_MODIFICATION | 650 | 26 | 0.0400 | 4.54E-8 | 1.63E-6 |
| RNA_METABOLIC_PROCESS | 841 | 30 | 0.0357 | 5.3E-8 | 1.82E-6 |
| PROTEIN_MODIFICATION_PROCESS | 631 | 25 | 0.0396 | 9.87E-8 | 3.26E-6 |
| PROTEIN_METABOLIC_PROCESS | 1231 | 37 | 0.0301 | 1.18E-7 | 3.74E-6 |
| NEGATIVE_REGULATION_OF_NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLI C_PROCESS | 211 | 14 | 0.0664 | 1.71E-7 | 5.22E-6 |
| POST_TRANSLATIONAL_PROTEIN_MODIFICATION | 476 | 21 | 0.0441 | 1.83E-7 | 5.38E-6 |
| ANATOMICAL_STRUCTURE_DEVELOPMENT | 1013 | 32 | 0.0316 | 2.92E-7 | 8.32E-6 |
| CELLULAR_PROTEIN_METABOLIC_PROCESS | 1117 | 33 | 0.0295 | 8.4E-7 | 2.31E-5 |
| CELLULAR_MACROMOLECULE_METABOLIC_PROCESS | 1131 | 33 | 0.0292 | 1.1E-6 | 2.93E-5 |
| NEGATIVE_REGULATION_OF_TRANSCRIPTION_DNA_DEPENDENT | 130 | 10 | 0.0769 | 2.63E-6 | 6.79E-5 |
| ORGANELLE_ORGANIZATION_AND_BIOGENESIS | 473 | 19 | 0.0402 | 2.77E-6 | 6.92E-5 |
| NEGATIVE_REGULATION_OF_RNA_METABOLIC_PROCESS | 132 | 10 | 0.0758 | 3.02E-6 | 7.34E-5 |
| TRANSCRIPTION_FROM_RNA_POLYMERASE_II_PROMOTER | 457 | 18 | 0.0394 | 6.59E-6 | 1.55E-4 |
| REGULATION_OF_TRANSCRIPTION_FROM_RNA_POLYMERASE_II_PROMOTER | 289 | 14 | 0.0484 | 7.05E-6 | 1.62E-4 |
| SYSTEM_DEVELOPMENT | 861 | 26 | 0.0302 | 8.42E-6 | 1.88E-4 |
| MULTICELLULAR_ORGANISMAL_DEVELOPMENT | 1049 | 29 | 0.0276 | 1.35E-5 | 2.94E-4 |
| REGULATION_OF_CATALYTIC_ACTIVITY | 276 | 13 | 0.0471 | 2,00E-05 | 4.23E-4 |
| CYTOSKELETON_ORGANIZATION_AND_BIOGENESIS | 208 | 11 | 0.0529 | 3.02E-5 | 6.22E-4 |
| CATION_TRANSPORT | 147 | 9 | 0.0612 | 5.14E-5 | 1.03E-3 |
| ION_TRANSPORT | 185 | 10 | 0.0541 | 5.73E-5 | 1.13E-3 |
| METAL_ION_TRANSPORT | 117 | 8 | 0.0684 | 6.15E-5 | 1.18E-3 |
| PROTEIN_AMINO_ACID_DEPHOSPHORYLATION | 63 | 6 | 0.0952 | 8.28E-5 | 1.55E-3 |
| NEGATIVE_REGULATION_OF_GROWTH | 40 | 5 | 0.1250 | 8.87E-5 | 1.63E-3 |
| REGULATION_OF_MOLECULAR_FUNCTION | 324 | 13 | 0.0401 | 1.03E-4 | 1.78E-3 |
| MESODERM_DEVELOPMENT | 22 | 4 | 0.1818 | 1.03E-4 | 1.78E-3 |
| REGULATION_OF_TRANSFERASE_ACTIVITY | 161 | 9 | 0.0559 | 1.04E-4 | 1.78E-3 |
| LIPID_METABOLIC_PROCESS | 325 | 13 | 0.0400 | 1.06E-4 | 1.78E-3 |
| CELLULAR_LOCALIZATION | 371 | 14 | 0.0377 | 1.08E-4 | 1.78E-3 |
| ORGAN_DEVELOPMENT | 571 | 18 | 0.0315 | 1.19E-4 | 1.93E-3 |
| INFLAMMATORY_RESPONSE | 129 | 8 | 0.0620 | 1.22E-4 | 1.93E-3 |
| ANATOMICAL_STRUCTURE_MORPHOGENESIS | 376 | 14 | 0.0372 | 1.24E-4 | 1.93E-3 |
| DEPHOSPHORYLATION | 70 | 6 | 0.0857 | 1.49E-4 | 2.28E-3 |
| PROTEIN_KINASE_CASCADE | 293 | 12 | 0.0410 | 1.56E-4 | 2.33E-3 |
| NERVOUS_SYSTEM_DEVELOPMENT | 385 | 14 | 0.0364 | 1.58E-4 | 2.33E-3 |
| CELL_SURFACE_RECEPTOR_LINKED_SIGNAL_TRANSDUCTION_GO_0007166 | 641 | 19 | 0.0296 | 1.71E-4 | 2.44E-3 |
| CELL_PROJECTION_BIOGENESIS | 25 | 4 | 0.1600 | 1.74E-4 | 2.44E-3 |
| REGULATION_OF_CELL_GROWTH | 46 | 5 | 0.1087 | 1.75E-4 | 2.44E-3 |
| ESTABLISHMENT_OF_CELLULAR_LOCALIZATION | 353 | 13 | 0.0368 | 2.38E-4 | 3.27E-3 |
| GROWTH | 77 | 6 | 0.0779 | 2.53E-4 | 3.42E-3 |
| PHOSPHORYLATION | 313 | 12 | 0.0383 | 2.85E-4 | 3.79E-3 |
| RESPONSE_TO_WOUNDING | 190 | 9 | 0.0474 | 3.57E-4 | 4.67E-3 |
| INTRACELLULAR_TRANSPORT | 280 | 11 | 0.0393 | 4.11E-4 | 5.3E-3 |
| REGULATION_OF_PROTEIN_KINASE_ACTIVITY | 155 | 8 | 0.0516 | 4.26E-4 | 5.41E-3 |
| REGULATION_OF_KINASE_ACTIVITY | 157 | 8 | 0.0510 | 4.64E-4 | 5.8E-3 |
| REGULATION_OF_GROWTH | 58 | 5 | 0.0862 | 5.22E-4 | 6.43E-3 |
| SMALL_GTPASE_MEDIATED_SIGNAL_TRANSDUCTION | 89 | 6 | 0.0674 | 5.53E-4 | 6.7E-3 |
| TISSUE_DEVELOPMENT | 138 | 7 | 0.0507 | 1.07E-3 | 1.26E-2 |
| CELL_DEVELOPMENT | 577 | 16 | 0.0277 | 1.1E-3 | 1.26E-2 |
| REGULATION_OF_BIOLOGICAL_QUALITY | 419 | 13 | 0.0310 | 1.17E-3 | 1.32E-2 |
| PROTEIN_POLYMERIZATION | 19 | 3 | 0.1579 | 1.24E-3 | 1.38E-2 |
| PROTEIN_AMINO_ACID_PHOSPHORYLATION | 279 | 10 | 0.0358 | 1.48E-3 | 1.63E-2 |
| APOPTOSIS_GO | 431 | 13 | 0.0302 | 1.5E-3 | 1.63E-2 |
| PROGRAMMED_CELL_DEATH | 432 | 13 | 0.0301 | 1.53E-3 | 1.64E-2 |
| CELL_CYCLE_PROCESS | 193 | 8 | 0.0415 | 1.76E-3 | 1.86E-2 |
| REGULATION_OF_DEVELOPMENTAL_PROCESS | 440 | 13 | 0.0295 | 1.8E-3 | 1.88E-2 |
| REGULATION_OF_APOPTOSIS | 341 | 11 | 0.0323 | 2.02E-3 | 2.06E-2 |
| REGULATION_OF_PROGRAMMED_CELL_DEATH | 342 | 11 | 0.0322 | 2.06E-3 | 2.08E-2 |
| NEGATIVE_REGULATION_OF_CELL_CYCLE | 79 | 5 | 0.0633 | 2.11E-3 | 2.1E-2 |
| NEGATIVE_REGULATION_OF_CELL_PROLIFERATION | 156 | 7 | 0.0449 | 2.16E-3 | 2.12E-2 |
| REGULATION_OF_G_PROTEIN_COUPLED_RECEPTOR_PROTEIN_SIGNALING_PATHWAY | 23 | 3 | 0.1304 | 2.19E-3 | 2.13E-2 |
| MICROTUBULE_BASED_PROCESS | 82 | 5 | 0.0610 | 2.49E-3 | 2.39E-2 |
| BRAIN_DEVELOPMENT | 51 | 4 | 0.0784 | 2.72E-3 | 2.57E-2 |
| CELLULAR_LIPID_METABOLIC_PROCESS | 255 | 9 | 0.0353 | 2.78E-3 | 2.61E-2 |
| NUCLEOCYTOPLASMIC_TRANSPORT | 87 | 5 | 0.0575 | 3.22E-3 | 2.98E-2 |
| RESPONSE_TO_EXTERNAL_STIMULUS | 312 | 10 | 0.0321 | 3.32E-3 | 3.04E-2 |
| NUCLEAR_TRANSPORT | 88 | 5 | 0.0568 | 3.38E-3 | 3.07E-2 |
| CALCIUM_ION_TRANSPORT | 27 | 3 | 0.1111 | 3.5E-3 | 3.1E-2 |
| POSITIVE_REGULATION_OF_BIOLOGICAL_PROCESS | 709 | 17 | 0.0240 | 3.55E-3 | 3.11E-2 |
| ANATOMICAL_STRUCTURE_FORMATION | 56 | 4 | 0.0714 | 3.82E-3 | 3.31E-2 |
| CELL_CYCLE_ARREST_GO_0007050 | 57 | 4 | 0.0702 | 4.07E-3 | 3.5E-2 |
| POSITIVE_REGULATION_OF_CELLULAR_PROCESS | 668 | 16 | 0.0240 | 4.64E-3 | 3.94E-2 |
| SUPEROXIDE_METABOLIC_PROCESS | 10 | 2 | 0.2000 | 5.48E-3 | 4.61E-2 |
| DI___TRI_VALENT_INORGANIC_CATION_TRANSPORT | 32 | 3 | 0.0938 | 5.69E-3 | 4.74E-2 |
| MACROMOLECULE_LOCALIZATION | 235 | 8 | 0.0340 | 5.8E-3 | 4.79E-2 |

**Table 4.** ***Gene Ontology for Myc-dependent induced and repressed bound genes*.**

GO functional analysis was performed for Myc-dependent induced bound genes and Myc-dependent repressed bound genes at 4 and 8h LPS. All genes set with significant FDR (<0.05) are shown for all the regulatory groups of genes. Numbers of genes overlapping with the indicated dataset (k), total genes in the dataset (K), ratio (k/K), pvalue and FDR are repo

## Gene set enrichment analysis




| 8h LPS Myc-dependent genes | | | |
|---|---|---|---|
| **Gene set name** | **SIZE** | **NES** | **FDR q-val** |
| STRUCTURAL_CONSTITUENT_OF_RIBOSOME | 64 | 3.939.561 | <0.0001 |
| RNA_BINDING | 76 | 34.074.485 | <0.0001 |
| TRANSLATION | 48 | 30.823.843 | <0.0001 |
| MITOCHONDRIAL_PART | 34 | 2.964.111 | <0.0001 |
| | | | |
| G_PROTEIN_COUPLED_RECEPTOR_PROTEIN_SIGNALING_PATHWAY | 17 | -21.026.623 | 0.0028776978 |
| CELL_SURFACE_RECEPTOR_LINKED_SIGNAL_TRANSDUCTION_GO_0007166 | 34 | -18.834.964 | 0.005194805 |
| HYDROLASE_ACTIVITY_ACTING_ON_ESTER_BONDS | 28 | -18.667.593 | 0.0013869626 |
| SIGNAL_TRANSDUCTION | 114 | -18.325.193 | <0.0001 |

**Table 5.** ***Myc–dependent bound genes are mainly involved in metabolic, RNA and signal transduction processes.***

Table of top scoring gene sets through GSEA pre-ranked in Myc-dependent induced bound and repressed bound genes after 8h of LPS stimulation. The number of genes (size) in each set, the normalized enrichment score (NES) and the test of statistical significance (FDR q-value) are indicated.

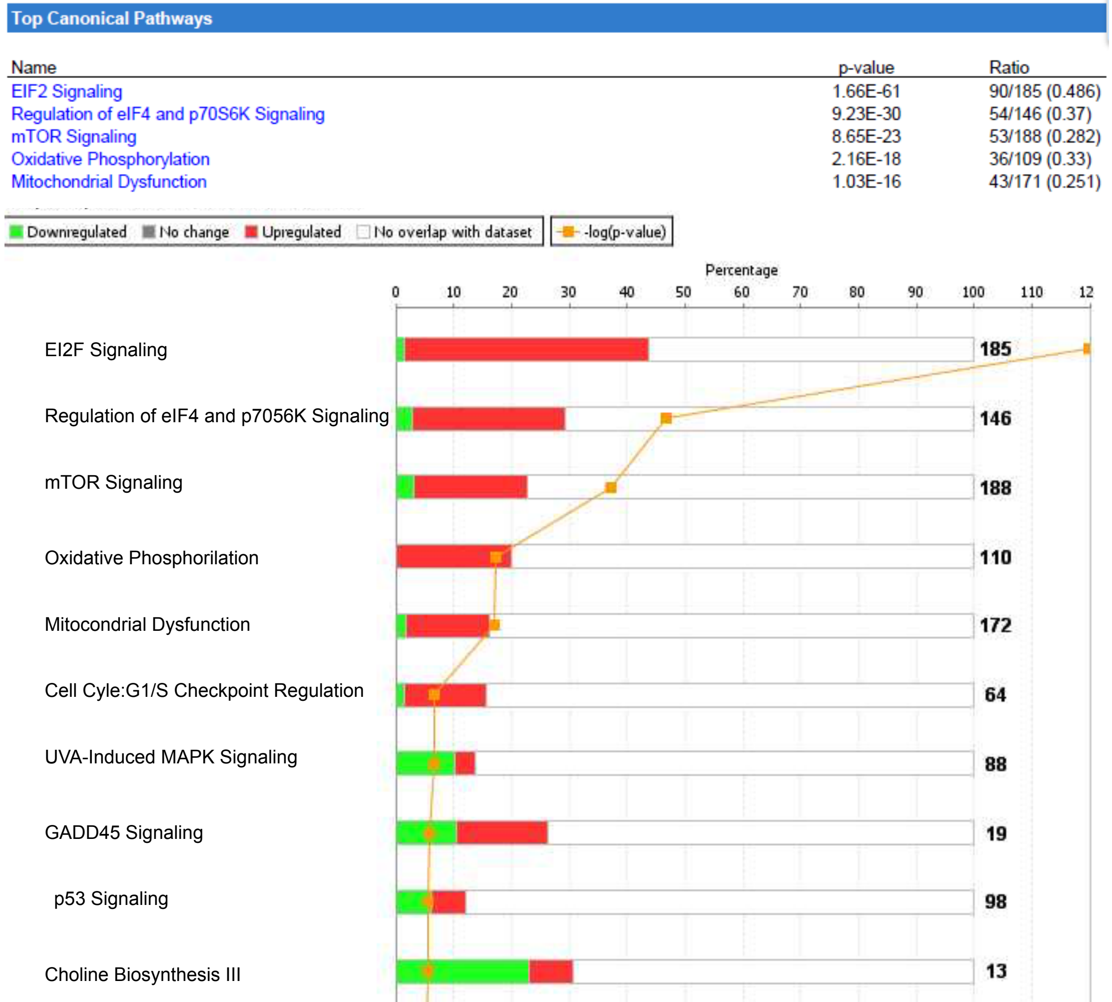



**Table 6** ***Ingenuity Pathway analysis of Myc dependent bound genes at 8h LPS.***

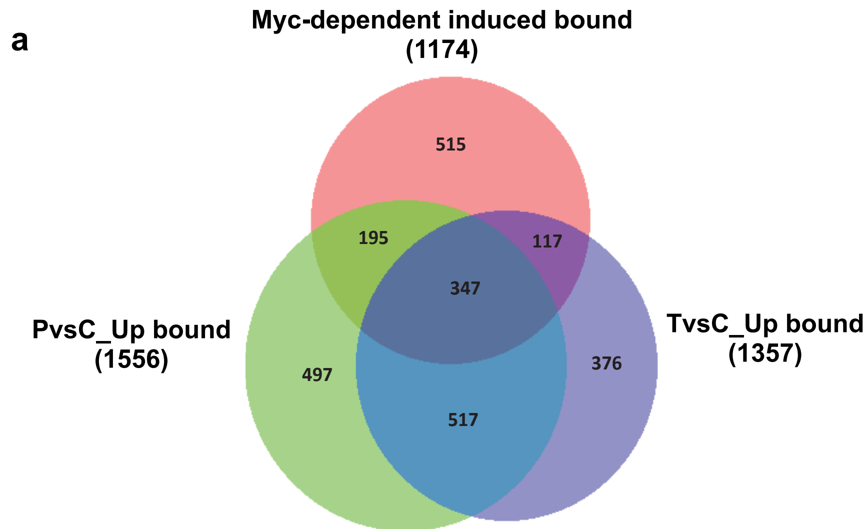
Table of top canonical pathways identified for Myc dependent genes at 8h LPS and barplots with percentages of the corrisponding Myc-dependent induced bound (red) and Myc-dependent repressed bound (green) genes inside each pathways are reported below.

## 4.9 Comparison of Myc dependent genes in physiology and pathology

To further characterize Myc target genes in a physiological versus pathological context, we compared the core of genes identified in mitogen-activated B cells with the genes regulated by Myc during lymphomagenesis. Specifically, we compared Myc-dependent bound genes in primary B cells with differentially regulated and Myc-bound genes at the pre-tumoral and tumoral stages in Eμ-*myc* mouse B cells *in vivo* relative to their wild type counterpart (Sabò et al. 2014). This analysis showed that around 56% (659/1174) of the Myc-dependent induced bound genes at either 2h, 4h or 8h after LPS treatment in wild type B cells were also up regulated during lymphomagenesis in Eμ-*myc* B cells (**Figure 57a).** Among all these common genes, half of them (347/659) were specifically shared with pretumoral and tumoral lymphomas. The biological processes in which these genes are implicated concern nucleotide biosynthesis and metabolic processes necessary for normal cell growth (**Figure 57b**).

The overlap between Myc-dependent repressed genes in LPS-treated B-cells and tumor progression was lower (**Figure 58a).** In total 28% of Myc dependent bound repressed genes were shared with Eμ-*myc* B cells. These common genes enriched for a lower number of terms with lower statistical significances and less precise biological meanings, such as signal transduction, general regulation of transcription and post-translational events (**Figure 58b**).

**a**




**b**

**Gene ontology of all common Myc Induced genes**

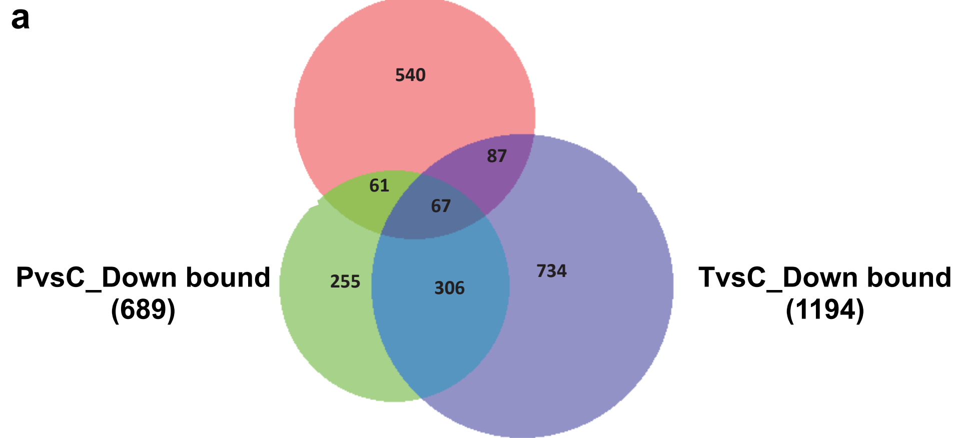
| Gene Set Name | # Genes in Gene Set (K) | # Genes in Overlap (k) | k/K | p-value | FDR q-value |
|---|---|---|---|---|---|
| NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS | 1244 | 150 | 0.1206 | 2.73E-72 | 2.25E-69 |
| BIOPOLYMER_METABOLIC_PROCESS | 1684 | 154 | 0.0914 | 9.21E-58 | 3.8E-55 |
| RNA_METABOLIC_PROCESS | 841 | 89 | 0.1058 | 4.62E-38 | 1.27E-35 |
| CELL_CYCLE_GO_0007049 | 315 | 54 | 0.1714 | 5.18E-34 | 1.07E-31 |
| CELL_CYCLE_PROCESS | 193 | 41 | 0.2124 | 5.69E-30 | 9.39E-28 |
| ORGANELLE_ORGANIZATION_AND_BIOGENESIS | 473 | 54 | 0.1142 | 5.02E-25 | 6.05E-23 |
| RNA_PROCESSING | 173 | 35 | 0.2023 | 5.13E-25 | 6.05E-23 |
| MITOTIC_CELL_CYCLE | 153 | 33 | 0.2157 | 1.32E-24 | 1.36E-22 |
| CELL_CYCLE_PHASE | 170 | 33 | 0.1941 | 4.62E-23 | 4.23E-21 |
| DNA_METABOLIC_PROCESS | 257 | 37 | 0.1440 | 5.46E-21 | 4.5E-19 |
| M_PHASE | 114 | 26 | 0.2281 | 2.45E-20 | 1.84E-18 |
| PROTEIN_METABOLIC_PROCESS | 1231 | 78 | 0.0634 | 3.05E-19 | 2.1E-17 |
| CELLULAR_PROTEIN_METABOLIC_PROCESS | 1117 | 72 | 0.0645 | 3.1E-18 | 1.96E-16 |
| NUCLEOBASENUCLEOSIDE_AND_NUCLEOTIDE_METABOLIC_PROCESS | 52 | 18 | 0.3462 | 4.3E-18 | 2.53E-16 |
| BIOSYNTHETIC_PROCESS | 470 | 45 | 0.0957 | 4.76E-18 | 2.62E-16 |
| CELLULAR_MACROMOLECULE_METABOLIC_PROCESS | 1131 | 72 | 0.0637 | 6.03E-18 | 3.11E-16 |
| TRANSCRIPTION | 753 | 56 | 0.0744 | 4.22E-17 | 2.05E-15 |
| TRANSCRIPTION_DNA_DEPENDENT | 636 | 50 | 0.0786 | 2.32E-16 | 1.06E-14 |
| RNA_BIOSYNTHETIC_PROCESS | 638 | 50 | 0.0784 | 2.63E-16 | 1.14E-14 |
| REGULATION_OF_CELLULAR_METABOLIC_PROCESS | 787 | 56 | 0.0712 | 2.91E-16 | 1.17E-14 |
| M_PHASE_OF_MITOTIC_CELL_CYCLE | 85 | 20 | 0.2353 | 2.99E-16 | 1.17E-14 |
| RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS_AND_ASSEMBLY | 86 | 20 | 0.2326 | 3.82E-16 | 1.43E-14 |
| REGULATION_OF_METABOLIC_PROCESS | 799 | 56 | 0.0701 | 5.6E-16 | 2.01E-14 |
| MITOSIS | 82 | 19 | 0.2317 | 2.25E-15 | 7.72E-14 |
| CELLULAR_BIOSYNTHETIC_PROCESS | 321 | 34 | 0.1059 | 2.93E-15 | 9.68E-14 |
| CELL_PROLIFERATION_GO_0008283 | 513 | 43 | 0.0838 | 3.17E-15 | 1.01E-13 |
| REGULATION_OF_CELL_CYCLE | 182 | 26 | 0.1429 | 4.43E-15 | 1.35E-13 |
| REGULATION_OF_NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS | 618 | 47 | 0.0761 | 6.45E-15 | 1.9E-13 |
| CHROMOSOME_ORGANIZATION_AND_BIOGENESIS | 124 | 21 | 0.1694 | 6.05E-14 | 1.72E-12 |
| RNA_SPLICING | 91 | 18 | 0.1978 | 2.31E-13 | 6.34E-12 |
| RESPONSE_TO_ENDOGENOUS_STIMULUS | 200 | 25 | 0.1250 | 3.32E-13 | 8.84E-12 |
| NUCLEOTIDE_METABOLIC_PROCESS | 42 | 13 | 0.3095 | 1.01E-12 | 2.6E-11 |
| RESPONSE_TO_DNA_DAMAGE_STIMULUS | 162 | 22 | 0.1358 | 1.6E-12 | 4,00E-11 |
| DNA_REPLICATION | 102 | 18 | 0.1765 | 1.8E-12 | 4.38E-11 |
| RESPONSE_TO_STRESS | 508 | 38 | 0.0748 | 4.22E-12 | 9.94E-11 |
| RIBOSOME_BIOGENESIS_AND_ASSEMBLY | 18 | 9 | 0.5000 | 1.88E-11 | 4.2E-10 |
| MACROMOLECULAR_COMPLEX_ASSEMBLY | 280 | 27 | 0.0964 | 1.88E-11 | 4.2E-10 |
| ESTABLISHMENT_OF_LOCALIZATION | 870 | 50 | 0.0575 | 3.18E-11 | 6.9E-10 |
| TRANSCRIPTION_FROM_RNA_POLYMERASE_II_PROMOTER | 457 | 34 | 0.0744 | 6.55E-11 | 1.39E-9 |
| CELLULAR_COMPONENT_ASSEMBLY | 298 | 27 | 0.0906 | 7.84E-11 | 1.62E-9 |
| RIBONUCLEOTIDE_METABOLIC_PROCESS | 16 | 8 | 0.5000 | 2.59E-10 | 5.2E-9 |
| MACROMOLECULE_BIOSYNTHETIC_PROCESS | 321 | 27 | 0.0841 | 4.15E-10 | 8.16E-9 |
| TRANSPORT | 795 | 45 | 0.0566 | 5.01E-10 | 9.62E-9 |
| DNA_REPAIR | 125 | 17 | 0.1360 | 5.27E-10 | 9.87E-9 |
| REGULATION_OF_RNA_METABOLIC_PROCESS | 471 | 33 | 0.0701 | 5.82E-10 | 1.07E-8 |

| | | | | | |
|---|---|---|---|---|---|
| TRANSLATION | 180 | 20 | 0.1111 | 6.38E-10 | 1.14E-8 |
| INTERPHASE | 68 | 13 | 0.1912 | 7.7E-10 | 1.35E-8 |
| POSITIVE_REGULATION_OF_CELLULAR_PROCESS | 668 | 40 | 0.0599 | 9.39E-10 | 1.61E-8 |
| REGULATION_OF_GENE_EXPRESSION | 673 | 40 | 0.0594 | 1.16E-9 | 1.96E-8 |
| POSITIVE_REGULATION_OF_BIOLOGICAL_PROCESS | 709 | 41 | 0.0578 | 1.6E-9 | 2.64E-8 |
| CELLULAR_CATABOLIC_PROCESS | 212 | 21 | 0.0991 | 1.98E-9 | 3.2E-8 |
| INTERPHASE_OF_MITOTIC_CELL_CYCLE | 62 | 12 | 0.1935 | 2.98E-9 | 4.73E-8 |
| AMINO_ACID_METABOLIC_PROCESS | 78 | 13 | 0.1667 | 4.51E-9 | 7.02E-8 |
| REGULATION_OF_TRANSCRIPTION | 566 | 35 | 0.0618 | 4.64E-9 | 7.08E-8 |
| BIOPOLYMER_MODIFICATION | 650 | 38 | 0.0585 | 4.72E-9 | 7.08E-8 |
| REGULATION_OF_MITOSIS | 41 | 10 | 0.2439 | 5.74E-9 | 8.35E-8 |
| CATABOLIC_PROCESS | 225 | 21 | 0.0933 | 5.77E-9 | 8.35E-8 |
| RRNA_PROCESSING | 15 | 7 | 0.4667 | 6.59E-9 | 9.37E-8 |
| CELLULAR_LOCALIZATION | 371 | 27 | 0.0728 | 9.5E-9 | 1.33E-7 |
| RRNA_METABOLIC_PROCESS | 16 | 7 | 0.4375 | 1.15E-8 | 1.58E-7 |
| MITOTIC_SPINDLE_ORGANIZATION_AND_BIOGENESIS | 10 | 6 | 0.6000 | 1.17E-8 | 1.58E-7 |
| MACROMOLECULE_LOCALIZATION | 235 | 21 | 0.0894 | 1.25E-8 | 1.66E-7 |
| AMINO_ACID_AND_DERIVATIVE_METABOLIC_PROCESS | 101 | 14 | 0.1386 | 1.36E-8 | 1.77E-7 |
| ESTABLISHMENT_OF_CELLULAR_LOCALIZATION | 353 | 26 | 0.0737 | 1.39E-8 | 1.77E-7 |
| NEGATIVE_REGULATION_OF_BIOLOGICAL_PROCESS | 677 | 38 | 0.0561 | 1.4E-8 | 1.77E-7 |
| NUCLEOCYTOPLASMIC_TRANSPORT | 87 | 13 | 0.1494 | 1.78E-8 | 2.22E-7 |
| REGULATION_OF_TRANSCRIPTIONDNA_DEPENDENT | 461 | 30 | 0.0651 | 1.87E-8 | 2.31E-7 |
| NUCLEAR_TRANSPORT | 88 | 13 | 0.1477 | 2.05E-8 | 2.48E-7 |
| SPINDLE_ORGANIZATION_AND_BIOGENESIS | 11 | 6 | 0.5455 | 2.53E-8 | 3.03E-7 |
| MITOCHONDRION_ORGANIZATION_AND_BIOGENESIS | 48 | 10 | 0.2083 | 2.95E-8 | 3.48E-7 |
| PROTEIN_IMPORT | 62 | 11 | 0.1774 | 3.54E-8 | 4.11E-7 |
| PROTEIN_TARGETING | 109 | 14 | 0.1284 | 3.68E-8 | 4.22E-7 |
| NEGATIVE_REGULATION_OF_CELLULAR_PROCESS | 646 | 36 | 0.0557 | 4.01E-8 | 4.53E-7 |
| APOPTOSIS_GO | 431 | 28 | 0.0650 | 5.74E-8 | 6.33E-7 |
| INTRACELLULAR_TRANSPORT | 280 | 22 | 0.0786 | 5.75E-8 | 6.33E-7 |
| PROGRAMMED_CELL_DEATH | 432 | 28 | 0.0648 | 6.03E-8 | 6.54E-7 |
| CELL_DEVELOPMENT | 577 | 33 | 0.0572 | 8.05E-8 | 8.63E-7 |
| PROTEIN_RNA_COMPLEX_ASSEMBLY | 67 | 11 | 0.1642 | 8.18E-8 | 8.65E-7 |
| NITROGEN_COMPOUND_METABOLIC_PROCESS | 155 | 16 | 0.1032 | 9.16E-8 | 9.56E-7 |
| MRNA_METABOLIC_PROCESS | 84 | 12 | 0.1429 | 1.05E-7 | 1.08E-6 |
| PROTEIN_TRANSPORT | 157 | 16 | 0.1019 | 1.1E-7 | 1.12E-6 |
| AMINE_METABOLIC_PROCESS | 141 | 15 | 0.1064 | 1.55E-7 | 1.55E-6 |
| MRNA_PROCESSING_GO_0006397 | 73 | 11 | 0.1507 | 2.03E-7 | 2.02E-6 |
| PROTEIN_MODIFICATION_PROCESS | 631 | 34 | 0.0539 | 2.08E-7 | 2.05E-6 |
| REGULATION_OF_DNA_METABOLIC_PROCESS | 45 | 9 | 0.2000 | 2.12E-7 | 2.06E-6 |
| INTRACELLULAR_PROTEIN_TRANSPORT | 145 | 15 | 0.1034 | 2.23E-7 | 2.14E-6 |
| ESTABLISHMENT_OF_PROTEIN_LOCALIZATION | 190 | 17 | 0.0895 | 2.93E-7 | 2.78E-6 |
| PROTEIN_LOCALIZATION | 214 | 18 | 0.0841 | 3.33E-7 | 3.12E-6 |
| MICROTUBULE_CYTOSKELETON_ORGANIZATION_AND_BIOGENESIS | 35 | 8 | 0.2286 | 3.39E-7 | 3.14E-6 |
| ESTABLISHMENT_AND_OR_MAINTENANCE_OF_CHROMATIN_ARCHITECTURE | 77 | 11 | 0.1429 | 3.55E-7 | 3.26E-6 |
| CELL_CYCLE_CHECKPOINT_GO_0000075 | 48 | 9 | 0.1875 | 3.81E-7 | 3.46E-6 |
| REGULATION_OF_APOPTOSIS | 341 | 23 | 0.0674 | 4.49E-7 | 4.02E-6 |
| REGULATION_OF_PROGRAMMED_CELL_DEATH | 342 | 23 | 0.0673 | 4.72E-7 | 4.19E-6 |
| CARBOXYLIC_ACID_METABOLIC_PROCESS | 178 | 16 | 0.0899 | 6.11E-7 | 5.37E-6 |
| MICROTUBULE_BASED_PROCESS | 82 | 11 | 0.1341 | 6.81E-7 | 5.91E-6 |
| PURINE_RIBONUCLEOTIDE_METABOLIC_PROCESS | 10 | 5 | 0.5000 | 7.03E-7 | 6.04E-6 |
| ORGANIC_ACID_METABOLIC_PROCESS | 180 | 16 | 0.0889 | 7.1E-7 | 6.04E-6 |
| CHROMATIN_MODIFICATION | 55 | 9 | 0.1636 | 1.28E-6 | 1.08E-5 |
| TRNA_METABOLIC_PROCESS | 19 | 6 | 0.3158 | 1.3E-6 | 1.08E-5 |
| DNA_DEPENDENT_DNA_REPLICATION | 56 | 9 | 0.1607 | 1.5E-6 | 1.23E-5 |

**Figure 57** ***Overlap between Myc dependent Induced bound genes in LPS activated B cells and upregulated bound genes in Eμ-myc B cells.***

**a.** Venn diagrams representing the overlap between Myc dependent induced bound genes in at least one time point after LPS stimulation (2 ,4, 8h) with upregulated Myc bound genes in Eμ-*myc* pretumoral (P) *or* tumor (T) compared to control (C) (as described in the paper Sabo et al.2014: qvalue<0.05 and log2(T/C) > or <0). **b.** Gene ontology analysis for all the common genes (659 genes) is shown. All gene sets with significant FDR (<0.05) are reported. Separate analysis on 117, 347 and 195 genes gave very similar results (data not shown).

**Myc-dependent repressed bound genes**
**(755)**




**b**

**Gene ontology of all common Myc repressed genes**

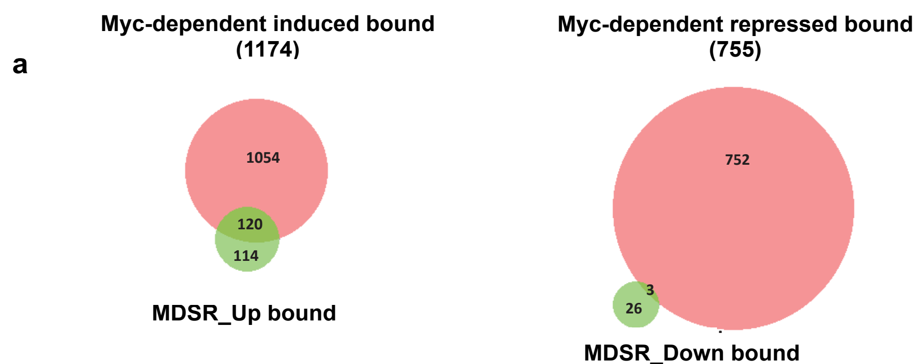
| Gene Set Name | # Genes in Gene Set (K) | # Genes in Overlap (k) | k/K | p-value | FDR q-value |
|---|---|---|---|---|---|
| SIGNAL_TRANSDUCTION | 1634 | 35 | 0.0214 | 4.01E-15 | 3.31E-12 |
| BIOPOLYMER_METABOLIC_PROCESS | 1684 | 24 | 0.0143 | 2.92E-7 | 1.18E-4 |
| REGULATION_OF_METABOLIC_PROCESS | 799 | 16 | 0.0200 | 4.28E-7 | 1.18E-4 |
| INTRACELLULAR_SIGNALING_CASCADE | 667 | 14 | 0.0210 | 1.34E-6 | 2.76E-4 |
| REGULATION_OF_CELLULAR_METABOLIC_PROCESS | 787 | 15 | 0.0191 | 1.82E-6 | 3.01E-4 |
| TRANSCRIPTION | 753 | 14 | 0.0186 | 5.38E-6 | 6.54E-4 |
| REGULATION_OF_TRANSCRIPTION | 566 | 12 | 0.0212 | 7,00E-06 | 6.54E-4 |
| CELLULAR_PROTEIN_METABOLIC_PROCESS | 1117 | 17 | 0.0152 | 7.47E-6 | 6.54E-4 |
| REGULATION_OF_GENE_EXPRESSION | 673 | 13 | 0.0193 | 7.84E-6 | 6.54E-4 |
| NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS | 1244 | 18 | 0.0145 | 7.93E-6 | 6.54E-4 |
| CELLULAR_MACROMOLECULE_METABOLIC_PROCESS | 1131 | 17 | 0.0150 | 8.78E-6 | 6.58E-4 |
| REGULATION_OF_NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS | 618 | 12 | 0.0194 | 1.67E-5 | 1.15E-3 |
| PROTEIN_MODIFICATION_PROCESS | 631 | 12 | 0.0190 | 2.05E-5 | 1.3E-3 |
| PROTEIN_METABOLIC_PROCESS | 1231 | 17 | 0.0138 | 2.58E-5 | 1.51E-3 |
| BIOPOLYMER_MODIFICATION | 650 | 12 | 0.0185 | 2.74E-5 | 1.51E-3 |
| REGULATION_OF_TRANSCRIPTIONDNA_DEPENDENT | 461 | 10 | 0.0217 | 3.4E-5 | 1.75E-3 |
| BIOSYNTHETIC_PROCESS | 470 | 10 | 0.0213 | 4,00E-05 | 1.87E-3 |
| REGULATION_OF_RNA_METABOLIC_PROCESS | 471 | 10 | 0.0212 | 4.07E-5 | 1.87E-3 |
| NEGATIVE_REGULATION_OF_CELLULAR_METABOLIC_PROCESS | 259 | 7 | 0.0270 | 1.38E-4 | 5.98E-3 |
| NEGATIVE_REGULATION_OF_METABOLIC_PROCESS | 262 | 7 | 0.0267 | 1.48E-4 | 6.1E-3 |
| REGULATION_OF_CATALYTIC_ACTIVITY | 276 | 7 | 0.0254 | 2.03E-4 | 7.98E-3 |
| LEUKOCYTE_ACTIVATION | 69 | 4 | 0.0580 | 2.28E-4 | 8.54E-3 |
| PROTEIN_KINASE_CASCADE | 293 | 7 | 0.0239 | 2.92E-4 | 1.05E-2 |
| CELL_ACTIVATION | 77 | 4 | 0.0519 | 3.47E-4 | 1.19E-2 |
| TRANSCRIPTION_DNA_DEPENDENT | 636 | 10 | 0.0157 | 4.59E-4 | 1.49E-2 |
| RNA_BIOSYNTHETIC_PROCESS | 638 | 10 | 0.0157 | 4.7E-4 | 1.49E-2 |
| MACROMOLECULE_BIOSYNTHETIC_PROCESS | 321 | 7 | 0.0218 | 5.03E-4 | 1.51E-2 |
| NEGATIVE_REGULATION_OF_CELLULAR_PROCESS | 646 | 10 | 0.0155 | 5.18E-4 | 1.51E-2 |
| REGULATION_OF_MOLECULAR_FUNCTION | 324 | 7 | 0.0216 | 5.32E-4 | 1.51E-2 |
| REGULATION_OF_PROTEIN_KINASE_ACTIVITY | 155 | 5 | 0.0323 | 5.71E-4 | 1.57E-2 |
| REGULATION_OF_KINASE_ACTIVITY | 157 | 5 | 0.0318 | 6.06E-4 | 1.61E-2 |
| REGULATION_OF_TRANSFERASE_ACTIVITY | 161 | 5 | 0.0311 | 6.78E-4 | 1.75E-2 |
| NEGATIVE_REGULATION_OF_BIOLOGICAL_PROCESS | 677 | 10 | 0.0148 | 7.41E-4 | 1.85E-2 |
| INTERLEUKIN_8_BIOSYNTHETIC_PROCESS | 10 | 2 | 0.2000 | 8.04E-4 | 1.95E-2 |
| RNA_METABOLIC_PROCESS | 841 | 11 | 0.0131 | 1.09E-3 | 2.54E-2 |
| POST_TRANSLATIONAL_PROTEIN_MODIFICATION | 476 | 8 | 0.0168 | 1.11E-3 | 2.54E-2 |
| INTERLEUKIN_8_PRODUCTION | 12 | 2 | 0.1667 | 1.17E-3 | 2.55E-2 |
| PERIPHERAL_NERVOUS_SYSTEM_DEVELOPMENT | 12 | 2 | 0.1667 | 1.17E-3 | 2.55E-2 |
| NEGATIVE_REGULATION_OF_TRANSCRIPTION | 188 | 5 | 0.0266 | 1.35E-3 | 2.87E-2 |

**Figure 58** ***Overlap between Myc dependent repressed bound genes in LPS activated B cells and downregulated bound genes in Eμ-myc B cells.***

**a**. Venn diagrams representing the overlap between Myc-dependent repressed bound genes in at least one time point after LPS stimulation (2 ,4, 8h) with downregulated Myc bound genes in Eμ-*myc* tumor compared to control (as described in the paper Sabo et al.2014: qvalue<0.05 and log2(T/C) > or <0). **b.** Gene ontology analysis for all the common genes (215 genes) is shown. All gene sets with significant FDR (<0.05) are reported. Separate analysis on 87, 67 and 61 genes gave very similar results (data not shown).

In order to find the core of Myc dependent genes during mitogenic stimulation we compared our dataset with Myc dependent serum-response (MDSR) genes in serum-stimulated fibroblasts (Perna et al 2012). Around half of the previously described MDSR genes bound by Myc were retrieved as Myc dependent bound also upon LPS stimulation, albeit many more Myc-dependent genes were identified in the B cells (**Figure 59a**). Once again, the common group included genes involved in nucleotide biosynthesis and RNA processing (**Figure 59b**). On the other hand, only 3 genes repressed and bound by Myc (*Lipa, Cryz, Didt3*) were shared between the two datasets (**Figure 59a**). This is consistent with the lesser relevance of Myc-dependent repression in the stimulated fibroblasts (Perna et al. 2012), possibly due to the lower levels of Myc expression in those cells, compared to LPS-stimulated B-cells. *Lipa* encodes for a lipase involved in hydrolysis of cholesteryl esters and triglycerides reinforced the notion that the lipid pathway is mainly dowregulated by Myc as we shown through the IPA analysis. Instead, *Cryz* encodes for a quinone reductases and Didt3 is a negative regulator of several C/EBP transcription factors mainly involved in apoptosis in response to cell stress.

In conclusion, the multiple analyses performed through the intersection of different datasets will shed light into specific genes and pathways regulated by Myc in B cells under physiological circumstances. These Myc dependent pathways are mainly involved in metabolism activation, growth and translation.




**b**

**Gene ontology of common Myc induced genes**

| Gene Set Name | # Genes in Gene Set (K) | # Genes in Overlap (k) | k/K | p-value | FDR q-value |
|---|---|---|---|---|---|
| NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC_PROCESS | 1244 | 26 | 0.0209 | 9.42E-17 | 7.77E-14 |
| RNA_METABOLIC_PROCESS | 841 | 20 | 0.0238 | 4.56E-14 | 1.88E-11 |
| BIOPOLYMER_METABOLIC_PROCESS | 1684 | 26 | 0.0154 | 1.12E-13 | 3.08E-11 |
| ORGANELLE_ORGANIZATION_AND_BIOGENESIS | 473 | 13 | 0.0275 | 2.9E-10 | 5.97E-8 |
| RNA_PROCESSING | 173 | 8 | 0.0462 | 1.65E-8 | 2.72E-6 |
| RRNA_PROCESSING | 15 | 4 | 0.2667 | 5.33E-8 | 7.33E-6 |
| RRNA_METABOLIC_PROCESS | 16 | 4 | 0.2500 | 7.09E-8 | 8.36E-6 |
| PROTEIN_METABOLIC_PROCESS | 1231 | 16 | 0.0130 | 1.02E-7 | 1.05E-5 |
| RIBOSOME_BIOGENESIS_AND_ASSEMBLY | 18 | 4 | 0.2222 | 1.19E-7 | 1.09E-5 |
| TRANSCRIPTION | 753 | 12 | 0.0159 | 5.48E-7 | 4.28E-5 |
| REGULATION_OF_NUCLEOBASENUCLEOSIDENUCLEOTIDE_AND_NUCLEIC_ACID_METABOLIC _PROCESS | 618 | 11 | 0.0178 | 5.71E-7 | 4.28E-5 |
| CELLULAR_PROTEIN_METABOLIC_PROCESS | 1117 | 14 | 0.0125 | 1.04E-6 | 7.18E-5 |
| CELLULAR_MACROMOLECULE_METABOLIC_PROCESS | 1131 | 14 | 0.0124 | 1.21E-6 | 7.67E-5 |
| RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS_AND_ASSEMBLY | 86 | 5 | 0.0581 | 2.9E-6 | 1.71E-4 |
| TRANSCRIPTION_DNA_DEPENDENT | 636 | 10 | 0.0157 | 5.68E-6 | 2.84E-4 |
| REGULATION_OF_CELLULAR_METABOLIC_PROCESS | 787 | 11 | 0.0140 | 5.8E-6 | 2.84E-4 |
| RNA_BIOSYNTHETIC_PROCESS | 638 | 10 | 0.0157 | 5.84E-6 | 2.84E-4 |
| REGULATION_OF_METABOLIC_PROCESS | 799 | 11 | 0.0138 | 6.69E-6 | 2.99E-4 |
| MITOCHONDRION_ORGANIZATION_AND_BIOGENESIS | 48 | 4 | 0.0833 | 7.12E-6 | 2.99E-4 |
| TRANSLATION | 180 | 6 | 0.0333 | 7.25E-6 | 2.99E-4 |
| CHROMATIN_MODIFICATION | 55 | 4 | 0.0727 | 1.23E-5 | 4.84E-4 |
| CHROMOSOME_ORGANIZATION_AND_BIOGENESIS | 124 | 5 | 0.0403 | 1.74E-5 | 6.51E-4 |
| REGULATION_OF_RNA_METABOLIC_PROCESS | 471 | 8 | 0.0170 | 2.95E-5 | 1.06E-3 |
| G1_S_TRANSITION_OF_MITOTIC_CELL_CYCLE | 27 | 3 | 0.1111 | 4.5E-5 | 1.54E-3 |
| ESTABLISHMENT_AND_OR_MAINTENANCE_OF_CHROMATIN_ARCHITECTURE | 77 | 4 | 0.0519 | 4.68E-5 | 1.54E-3 |
| DNA_METABOLIC_PROCESS | 257 | 6 | 0.0233 | 5.37E-5 | 1.7E-3 |
| REGULATION_OF_GENE_EXPRESSION | 673 | 9 | 0.0134 | 5.96E-5 | 1.82E-3 |
| REGULATION_OF_TRANSCRIPTION | 566 | 8 | 0.0141 | 1.06E-4 | 3.13E-3 |
| CELL_CYCLE_GO_0007049 | 315 | 6 | 0.0190 | 1.63E-4 | 4.65E-3 |
| CELLULAR_BIOSYNTHETIC_PROCESS | 321 | 6 | 0.0187 | 1.81E-4 | 4.82E-3 |
| MACROMOLECULE_BIOSYNTHETIC_PROCESS | 321 | 6 | 0.0187 | 1.81E-4 | 4.82E-3 |
| BIOSYNTHETIC_PROCESS | 470 | 7 | 0.0149 | 2.11E-4 | 5.44E-3 |
| BIOPOLYMER_MODIFICATION | 650 | 8 | 0.0123 | 2.71E-4 | 6.78E-3 |
| CELL_PROLIFERATION_GO_0008283 | 513 | 7 | 0.0136 | 3.58E-4 | 8.68E-3 |
| INTERPHASE_OF_MITOTIC_CELL_CYCLE | 62 | 3 | 0.0484 | 5.45E-4 | 1.28E-2 |
| MITOTIC_CELL_CYCLE | 153 | 4 | 0.0261 | 6.54E-4 | 1.5E-2 |
| INTERPHASE | 68 | 3 | 0.0441 | 7.14E-4 | 1.59E-2 |
| BASE_EXCISION_REPAIR | 17 | 2 | 0.1176 | 8.52E-4 | 1.85E-2 |
| CELL_CYCLE_PHASE | 170 | 4 | 0.0235 | 9.68E-4 | 2.05E-2 |
| CELLULAR_RESPONSE_TO_STIMULUS | 19 | 2 | 0.1053 | 1.07E-3 | 2.15E-2 |
| TRNA_METABOLIC_PROCESS | 19 | 2 | 0.1053 | 1.07E-3 | 2.15E-2 |
| REGULATION_OF_TRANSCRIPTIONDNA_DEPENDENT | 461 | 6 | 0.0130 | 1.21E-3 | 2.37E-2 |

**Figure 59** ***Overlap between Myc dependent bound genes in LPS activated B cells and serum stimulated fibroblasts***

**a.** Venn diagram representing the overlap between Myc dependent induced bound genes in at least one time point (2,4,8h after LPS stimulation) with MDSR, Myc dependent serum response genes bound by Myc upregulated (MDSR_Up bound) and dowregulated (MDSR_Down bound) (as described in material and methods of Perna et al.2012 paper). **b.** Gene ontology analysis for all the common induced genes (120 genes) is shown. All gene sets with significant FDR (<0.05) are reported.

# 5. Discussion

Myc is required for the response of B-lymphocytes upon exposure to activating stimuli (Kelly et al 1983; de Alboran et al., 2001), but a clear picture of the transcriptional programs regulated by Myc in this setting is still missing. In the present study, we provide integrated maps of genome occupancy by Myc and gene expression profiles upon LPS mediated activation of primary B-cells. The dependency of transcriptional responses upon Myc was determined by comparing wild type cells with cells in which conditional deletion of *c-myc* was induced prior to LPS stimulation. Our data clearly pinpoint the requirement for Myc in the up- and down-regulation of specific groups of genes at early time-points following stimulation, in contrast with the concept that Myc acts to generally amplify expression of all active genes. This work represents an accurate description of transcriptional regulation by Myc in a physiological setting, and constitutes a starting point for studies delineating of how select Myc target genes may affect different cellular outcomes.

## 5.1 Myc is required for B cell activation, proliferation and cell growth

For decades, induction of *c-myc* expression has been associated with cell activation in diverse cell types, including normal fibroblasts and lymphocytes (Kelly et al. 1983; Rabitts et al. 1985): starting from a very low baseline during G0-G1 phase, Myc is then rapidly induced by mitogenic stimulation. In line with this concept, we and others (Lin C et al., 2012; de Alboran et al., 2001) have observed that, Myc is rapidly induced at the mRNA and protein levels upon LPS stimulation in B cells, allowing their clonal expansion and

metabolic changes such as increase in size, protein synthesis and global RNA production, which may prepare their differentiation in plasma cells. Also in T cells, Myc expression is required for activation-induced cell growth, proliferation and metabolic reprogramming (Wang et al., 2011). In addition, the strength of TCR activation signalling pathway dictates Myc mRNA expression levels in T cells while other external stimuli such as IL2 maintain high Myc protein levels, since they can ensure a high rate of amino acid uptake and so a high rate of protein synthesis (Preston et al., 2015). Thus, TCR activation alone cannot sustain expression of Myc and the post-transcriptional control of Myc protein is ensured by IL2, generating possible discordances between Myc protein and mRNA levels. These observations in activated T cells seem to be in line with our observations in B cells, in which the c-*myc* mRNA is maximally induced at 2h of LPS activation and declines thereafter, whereas Myc protein levels reach a maximum at the same point, but remain elevated at 4h and 8h (**Figure 21; Figure 42**). At these later time points, we also started to observe a slight increase in cell size consistent with the notion that all the protein biosynthetic apparatus of the cell and thus its mass expand when Myc is highly expressed, both in normal and tumoral lymphocytes (Wang et al. 2011; B. Iritani & R. Eisenman 1999).

This effect of Myc on growth is preceded by a direct and well-described effect of Myc on cell cycle progression. Indeed our data showed that deletion of myc in quiescent B cells impaired S-phase entry, as also observed in a previous study (de Alboran et al., 2001). The decreased rate of proliferation of Myc deleted cells was due to a cell cycle defect, and not

to an increase in apoptosis. On the contrary, Myc deleted cells were more resistant to spontaneous cell death as was also previously published (de Alboran et al., 2003; Murn et al., 2009). Myc deletion has also been shown to promote plasma cell differentiation (Murn et al 2009) but analysis of cell surface markers and terminal differentiation-associated genes did not confirm this in our system. We speculate that Myc may exert its transcriptional activity principally during the initial phases of B cell activation in order to modulate proliferation and growth, and may then be down regulated by transcriptional regulators that control the plasma cell differentation factor network, such as Blimp1 (Lin Y. et al. 1997). Inducing *c-myc* deletion later, after 24-48h of LPS stimulation, could be also useful to clarify its role in the last phases of plasma cell differentation.

## 5.2 Widespread Myc binding to chromatin occurs soon after mitogenic stimulation

We observed that the distribution of Myc binding sites in wild type splenic B cells *in vitro* without stimulation with LPS was comparable to that observed *in vivo* in our previous study (Sabò et al. 2014). Low Myc levels justify the lower numbers of Myc peaks observed in those conditions, and their predominant location at active/poised promoters (Sabò & Amati 2014; Kress et al., 2015). Following 2h of LPS stimulation *in vitro*, Myc protein levels and its genomic distribution were very similar to those seen in Eμ-*myc* transgenic mice at the pre-tumoral stage: the number of Myc binding sites increased on active promoters as well as on distal sites with pre-existing H3K4me1/H3K27ac marks in control cells, which can be equated to active enhancers (Sabò et al., 2014; Nie et al., 2012). Thus, as soon as Myc levels rise upon LPS stimulation, the protein starts binding

open and active chromatin at promoters and distal sites in a widespread manner (the "invasion" phenomenon), in very similar to what occurs in Myc overexpressing B cells.

As outlined in other studies (Walz et al.2014; Sabò et 2014) a sizeable portion of active promoters (which are not only RNAPol2 pre-loaded but also H3K4me3 marked) are already bound at low Myc levels. These sites are still bound in LPS stimulated samples where they represent the most enriched peaks. Thus, relative binding affinities to the different genomic loci are maintained regardless of Myc expression levels in the cell (Sabò et al 2014; Sabò & Amati 2014; Kress et al., 2015). This raise the possibility to distinguish Myc high affinity, highly enriched, E-boxes driven binding sites from low affinity, low enriched and less specific sites.

Altogether, our ChIP-seq data in activated B cells showed that Myc was initially bound to promoters with high affinity sites, characterized by a higher presence of E-boxes, and then concomitant with its increasing levels, bound also low affinity sites, both at promoters and distal enhancers.

## 5.3 Selective transcriptional response precedes RNA amplification

By taking advantage of conditional c-myc deletion, we produced what is to our knowledge the first description of the Myc-dependent transcriptional program in normal B cells. We have shown that upon activation of quiescent B cells with LPS, c-*myc* is rapidly induced and is required for the subsequent induction and repression distinct sets of ca. 1280 and 1040 genes, respectively, within the first 8h upon LPS stimulation. This occurs before the global increase in RNA content, which we could observe only starting from 12h after LPS

stimulation. In summary, as shown in other physiological or pathological settings (Sabò et al.2014; Walz et al 2014;  Ji et al., 2011) we observed a selective transcriptional regulation by Myc which does not fit with the general transcriptional activation by Myc described in the amplifier model (Nie et al.2012; Lin C et al.2012). These results are also in line with what observed during serum stimulation of fibroblasts, in which selective Myc dependent gene activation occurs in the early-mid G1 phase of the cell cycle (Perna et al.2011), RNA amplification occurring only at a later stage (Sabò et al.2014). Altogether, these data imply that the primary action of Myc lies in the transcriptional control of specific groups of genes, RNA amplification occurring as a secondary consequence of cellular activation (Kress et al., 2015).

## 5.4 Myc and the global RNA increase

A bit in contrast with the claim that Myc may induce concomitant increase in both cell size and global RNA amplification (Nie et al. 2012), we noticed a more gradual increase in size (in part Myc dependent) and only a later abrupt increase in global RNA production. The general increase in the transcriptional activity of a cell undergoing metabolic activation and cell size expansion, as happens during plasma cell differentiation, is a highly conserved process, which actually pre-dates Myc in evolution (Marguerat and Bähler, 2012;  das Neves et al., 2010). Thus growth in cell size correlates with RNA amplification and cell cycle but the cause-to effect relationship between these changes is still unclear. Therefore, it will be fundamental to understand how Myc and its target genes can impact on these processes considering that Myc is one of the regulators of cell activation and growth. Recently, one possible mechanism of indirect RNA amplification

promoted by Myc was highlighted by the finding that a single rate limiting enzyme in *de* novo Purine biosynthesis (PRPS2), indirectly induced by Myc through enhanced translation of its mRNA, is required for increased RNA production in Eμ-*myc* B cells (Cunningham JT et al. 2014). In conclusion, the present work is a starting point to delineate and test the pathways that mediate the effects of Myc on cellular physiology and metabolism.

## 5.5 Myc occupancy is not predictive of gene regulation

Combining Myc binding with gene expression profiles, we have shown here and in published work (Sabò et al 2014) that (i.) not all the genes bound by Myc were differentially regulated, (ii.) around 80 % of induced genes (whether Myc dependent or not) and 70% of the repressed genes were Myc bound. For this reason, we concluded that widespread Myc binding to active chromatin (or "invasion") couldn't be equated to productive engagement of Myc at all loci. Upon closer examination of the correlation between Myc binding and transcriptional response, we found that Myc-dependent LPS Induced genes were bound at their promoters with higher frequency and higher intensity compared to Myc-repressed genes, a common feature observed in several other systems in our lab (unpublished data). Conceptually, these observations are consistent with the notion that the mechanisms of Myc repression are likely to be more indirect e.g. via Miz1, AP-2, Sp-1, or other tethering factors with inhibitory activities (Peukert et al., 1997; Gaubatz et al., 1995; Gartel et al 2001).

The above notwithstanding, additional TFs may contribute to either repression or activation by Myc. Motif analysis showed significant enrichment of many TF binding sites

within the Myc peaks in the promoters of induced and repressed genes. For example, the binding motif of Nfatc (nuclear factor of activated T cells) a factor that has a pivotal role in activation of the immune response in T and B cells (Serfling et al., 2000; Bhattacharyya et al. 2011), was enriched at the promoters of Myc-dependent and independent LPS induced genes. Among the motifs enriched in the Myc-dependent LPS repressed genes, we also found zinc finger proteins (such as, Zfp410) and Smad proteins. Smad2 and 3 are known to form a stable complex with Myc on the p15 and p21 promoters, blocking Sp1 dependent transcriptional activation of these genes (Feng X. et al 2002). The distribution of cofactors and TFs in the Myc-regulatory network remains to be completely clarified and a deeper analysis of the motifs listed in this work will be needed in order to validate candidate co-regulators.

## 5.6 Myc binding to enhancers

Whereas the role of Myc at promoters has been extensively studied, Myc binding to enhancers, which was observed in different systems and cell lines (Shi et al., 2013; Lin et al.2012; Sabò et al 2014), still lacks clear functional consequences. We found that in activated B cells a vast portion of Myc peaks falls in distal regions with the characteristic features of enhancers, in particular high H3K4me1 and low H3K4me3 content. These sites showed a lower Myc binding intensity compared to the ones on promoters but showed similar E-box frequencies. By associating enhancers and genes based on linear proximity in the genome, we concluded that Myc binding to enhancers in Eμ-myc B cells strengthens the regulation of the associated genes, whether induced or repressed

(Pelizzola M. and Sabò A., unpublished). A more extensive picture of the enhancer repertoire in B cells will be possible through the analysis of long-range conformation capture experiments already published in B cells (Lin YC. et al., 2012) and/or ongoing in the lab in the Eμ-*myc* model, allowing a more precise assignment of enhancers to specific genes.

## 5.7 Myc-regulated transcription is associated with changes in both RNAPol2 recruitment and elongation

In order to understand if there is a relationship between Myc binding and RNAPol2 recruitment and/or elongation, we profiled RNAPol2 by ChIP-seq in wt and *c-myc*$^{\Delta/\Delta}$ *B* cells before and after LPS treatment. We observed that most of the Myc bound promoters are not only actively marked by H3K4me3 and H3K27ac but are also pre-loaded with RNAPol2, a common behavior observed in several systems (Guccione et al., 2006; Lin C et al., 2012; Nie et al., 2012; Sabò et al.2014; Walz et al., 2014). Besides H3K4me1, RNApol2 was also found at distal Myc-binding sites. The absence of RNApol2 peaks on promoters and enhancers that were not targeted by Myc, together with the absence of active histone marks, further consolidated the concept that Myc does not access inactive chromatin.

With our knockout model, we were in principle in the best situation to investigate how Myc affects the transcription via RNAPol2. In this regard, quantitative analysis of ChIP-seq reads on TSS and gene bodies and their ratios (also known as the Stalling Index) before and after LPS stimulation in wt and *c-myc*$^{\Delta/\Delta}$ cells showed different effects on RNAPol2 distribution in Myc dependent and independent genes. However, our data pointed to a

general increase in Pol2 loading on promoters in LPS-stimulated cells, this effect being partially Myc-dependent. The nature and validity of this phenomenon remain to be addressed. The roles of RNAPol2 loading or elongation in Myc-regulated transcription remain a matter of debate. In human B cells for example, activation of a tet-Myc transgene was shown to induce Pol2 loading at Myc-induced genes (Martinato F at al. 2008). c-Myc inhibition with drug instead was described to affect indiscriminately transcription at the pause-release step (Rahl et al., 2009; Lin C. et al., 2012; Nie et al.,2012; Rahl PB & Young R. 2014). On this basis Myc was described as a universal potentiator of pause-release at all actively transcribed promoters via recruitment of the P-TEFb complex (cyclin T1 and Cdk9). However, our data and the already published one (Sabò et al., 2014), clearly show that no such global role could be attributed to Myc. Given the binding of Myc to multiple types of co-regulators, including histone modifiers and remodelers (Hann SR., 2014), we surmise that diverse mechanisms are likely to account for its transcriptional activity, most likely in a gene- and context-dependent manner.

## 5.8 General vs specific mode of transcriptional regulation by Myc

The genome of naïve B cells is poised for rapid activation. In particular, 90% of promoters from genes in GO lymphocytes are polymerase loaded but un-melted with low levels of TFIIH supporting only basal transcription (Kouzine et al 2013). Myc, trough its transactivation domain, may promote unwinding by recruiting or activating TFHII (Cowling et al., 2007). One effect may be a global increase in global phosphorylation of RNAPol2, with widespread effects on transcription activation and mRNA metabolism. This, together with the observation that Myc, in recruiting p-TEFb, affects the pause release of all

activated genes (Rahl et al., 2010) depicts the generic mechanism of transcriptional amplification by Myc (Nie et al., 2012; Lin C. et al., 2012). Our data support an opposing view, pointing to Myc-specific mechanism of transcriptional regulation (Sabò et al., 2014; Walz et al., 2014) with RNA amplification occurring as a secondary event. Therefore, at the moment, the only unifying model that consistent with all available data is the differential gene regulation by Myc; direct transcriptional amplification remains to be formally proven and is not closely required to explain any of the existing experimental observations. In conclusion, our evidences show indeed that Myc regulates selective gene expression programs in B cells affecting mainly RNAPol2 recruitment of these specific genes and leading to a cellular metabolic remodelling.

## 5.9 A core of Myc target genes involved in metabolism and RNA biogenesis

As already discussed, Myc regulates specific gene programs which are mainly involved in growth, cell cycle, energy metabolism, mitochondrial biogenesis, nucleotide biosynthesis and RNA biogenesis, and maybe feed back on global RNA production.  We thus proposed that Myc can induce RNA amplification indirectly (Sabò et al., 2014; Kress et al., 2015) but how Myc target genes may lead to these physiological changes in activated B cells remains to be addressed. Through comparison of our data in LPS-stimulated B cells with the Eμ-*myc* transcriptional dataset (Sabò et al 2014) and serum activated fibroblasts (Perna et al., 2012) we could identify a core of Myc dependent genes, which are probably required for metabolic activation and growth. For testing the indirect model of RNA amplification, therefore, we could functionally characterize these putative target genes or pathways using RNA interference or chemical drugs in order to un-couple Myc binding to

the genome from increase in cell growth and proliferation. Recently, the importance of these Myc dependent pathways involved in metabolism and translation was underlined in a successful pharmaceutical treatment of Eμ-*myc* lymphomas. The combined targeting of ribosomal DNA biogenesis and mTOR protein translation has provided remarkably therapeutical benefits in the treatment of Myc-driven cancer (Devlin et al 2015). In this regard, the strategy of targeting downstream regulators of Myc both in physiological and pathological contexts may still represent a useful way to truly understand Myc physiological mechanisms of action and overcome Myc-driven tumors.

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