

# **Regulation of BCL6: p38 MAPK signalling and CTCF transcriptional regulation converge at exon 1**

by

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**ABSTRACT**

BCL6 is a zinc finger transcriptional repressor, which is highly expressed in germinal centre B-cells, and is essential for germinal centre formation and T-dependent antibody responses. Deregulated BCL6 expression is associated with certain non-Hodgkin's lymphomas. High expression is observed in breast cancer. Tight lineage and temporal regulation of BCL6 is, therefore, required for normal immunity and abnormal regulation occurs in cancer. Regulatory mechanisms have been analysed in two settings. Firstly, BCL6 is strongly induced by the tyrosine kinase inhibitor, Imatinib, in chronic myeloid leukaemia lymphoid blast crisis cell lines, and this effect was used in order to study the effects of phospho-protein signalling on BCL6 expression and a major finding is that p38 MAPK induced BCL6. Also, p38 is, at least in part, responsible for BCL6 expression in basal conditions in the germinal centre representative Burkitt's lymphoma cell lines and that qualitatively different CD40 stimuli can either induce or repress BCL6 expression. Luciferase assays showed that p38 acts at a 300bp sequence immediately 5' of exon 1, and probably also at more distal sequences. Overall it appears that the balance between positive and negative regulatory controls BCL6 expression with inhibitory signalling pathways being predominant in most circumstances. Focusing on BCL6 exon 1, a binding site for the multifunctional regulator CTCF was identified. CTCF interacts in vitro and in vivo with this sequence. Reduced expression of CTCF in germinal centre cells caused a moderate reduction of BCL6 expression. Finally, although no clear differences were observed in the methylation status of the CTCF binding site on exon 1, a significant enrichment of active histone modifications at this site was observed in BCL6 expressing cells, suggesting that CTCF may have a role in the epigenetic regulation of BCL6.



## DECLARATION

This thesis has been done following a Split-site PhD programme. I spent two years working under the co-supervision of Dr. Simon D. Wagner and Dr. Andy Porter at Imperial College of London, UK and one year and a half at the University of Cantabria, Spain supervised by Dr. M. Dolores Delgado. Dr Wagner visited the University of Cantabria whilst I was working there on April 2010.

This thesis is the result of my own work, except where otherwise acknowledged by given complete references.

One journal paper was published in the Molecular Immunology Journal:

\*Batlle A, Papadopoulou V, Gomes AR, Willimott S, Melo JV, Naresh K, Lam EW, Wagner SD. Mol Immunol. "CD40 and B-cell receptor signalling induce MAPK family members that can either induce or repress BCL6 expression". 2009 May;46(8-9):1727-35. Epub 2009 Mar 6

This paper is completely based on the results of my work which is presented in a slightly modified form in Chapters 3 and 4 of this dissertation. I would like to note that the immunocytochemistry presented in the referred paper and in the dissertation were carried out by Pr. Kikkeri Naresh.

Furthermore, the contents of chapters 3 and 4 were presented in two panel communications and in one oral communication at the following conferences:

\*Batlle A; Lam E ; Wagner SD ."BCL6 May Be a Survival Factor in Pre-B Cell Ph+ Blast Crisis Cell Lines". 49th Annual Meeting of the American Society of Hematology. Blood (ASH annual meeting abstracts), Nov 2007, 110: 4536. 15.

\*Batlle A, Papadopoulou V, Gomes A.R, Willimott S, Melo J.V., Neresh K, Lam EW, Wagner S.D. "Membrane and soluble CD40 ligand have different effects on BCL6 expression and produce different proteína/DNA complexes at a site in exon 1 on the

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*BCL6* gene". 14th congress of the European hematology association. Haematologica June 2009;94(s2)

Batlle A. Oral presentation at the "X Jornada de Investigación del Departamento de Biología Molecular de la UC" (Cabezón de la Sal, Viernes 18 de Septiembre 2009) sesión "Transcripción y Respuesta Inmune "con la ponencia: Regulación de BCL6 por CTCF

*To my husband Ismael*



*“There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after.”*

*J.R.R. Tolkien*



## ACKNOWLEDGMENTS

The present work could have never been done without the support of many people, most of whom are from both the Department of Haematology, Imperial College, Hammersmith Hospital and the Molecular Biology Department, Universidad de Cantabria.

My most sincere thanks go to my supervisors, Dr. Simon Wagner, and Dr. M. Dolores Delgado, for providing me invaluable support, for their guidance, for their ability to solve problems that appear to have no solution and, above all, for introducing me to the fascinating world of research. I would certainly hope to keep working with them in the future

May I also express my most sincere gratitude to Prof. Junia VD Melo, who introduced me to Dr Wagner, and who has helped me with many aspects of this project. I would also want to thank to Prof. Jesus San Miguel, who helped and encouraged me to go to London in order to work with Prof. Junia VD Melo.

I am also very grateful to Dr. Marta Albajar who has always believed in me, has been very supportive and for introducing me to the molecular biology group at the University of Cantabria.

Many thanks to Prof. Javier Leon for having me in the laboratory, for his helpful comments and, for his financial support. I am very grateful to the Foundation Marqués de Valdecilla for its support and for providing most of the financial resources. Thanks also to Amgen for the economical support

My sincere gratitude also goes to everybody from both laboratories, particularly Dr Shaun Willimott, Dr Vasiliki Papadopoulou, Dr. Jennifer Zobel, Julia Hong, Dr. Hetal Patel, Dr. Jamshid S. Korashad, Dr. Georgios Nteliopoulos, Manuel Rosa Garrido, Cristina Abaira, Gabriel Bretones, Pilar Frade and Rosa Blanco for showing me many

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different techniques and for providing a very nice environment for work full of enthusiasm and high professional standards.

I also want to thank Dr Andy Porter, for his help and support. Thanks also to Professor Eric Lam. I am very grateful for the practical advice he has given me for my experiments.

Thanks to Dr. Arturo Iriondo, head of the Department of Haematology of the Hospital Universitario Marqués de Valdecilla, for bringing me the idea and opportunity of doing this four year research period and for his very important support. I am also very grateful to Elida del Cerro for her assistance with the primary cells samples and with the flow cytometry analysis. I also gratefully acknowledge Patricia Burón for her help with the FISH experiments. Thanks as well to Dr. Andres Insunza, Mercedes Colorado, Marisa Arribas and Isabel Mendez for providing me with the primary cells samples.

My biggest thanks go to my husband who gave me a tremendous amount of support. Without it, this work would have never happened. Many thanks also to my parents, who have always believed in me and who have always encouraged me to keep going. In addition, I would like to thank my grandfather, Prof. Antonio López Borrasca, who transmitted me his great enthusiasm for science.

## ABBREVIATIONS

|                |                                                            |
|----------------|------------------------------------------------------------|
| aa             | Amino Acid                                                 |
| ALL Ph+        | Acute Lymphoblastic Leukaemia with Philadelphia Chromosome |
| AML            | Acute Myelogenous Leukemia                                 |
| ATR            | Ataxia-Telangiectasia and Rad3 related                     |
| 5-Aza-dC       | 5-Aza-2'-deoxycytidine                                     |
| BC CML         | Blast Crisis of CML                                        |
| <i>BCL6</i>    | <i>B Cell Lymphoma 6</i>                                   |
| BcoR           | BCL6 Corepressor                                           |
| BSA            | Bovine Serum Albumin                                       |
| BTB/POZ domain | (for BR-C, ttk and bab)/ (for Pox virus and Zinc finger)   |
| bp             | Base Pairs                                                 |
| Chx            | Cicloheximide                                              |
| CDKN1A         | Cyclin-Dependent Kinase Inhibitor 1A                       |
| C/EBP $\beta$  | CCAAT/enhancer-Binding Protein Beta                        |
| CKI            | Cdk Inhibitor Protein                                      |
| CML            | Chronic Myelogenous Leukaemia                              |
| CTCF           | CCCTC binding Factor                                       |
| CTS            | CTCF Target Site                                           |
| DAPI           | 4',6'-diamino-2 phenylindole dihydrochloride               |
| DBD            | DNA Binding Domain                                         |
| DLBCL          | Diffuse Large B cell Lymphoma                              |
| DUSPs          | Dual-Specificity Phosphatases                              |
| DMSO           | Dimethyl Sulphoxide                                        |
| dNTP           | Deoxyribonucleoside Triphosphate                           |
| ELK1           | ETS like gene 1                                            |
| EBV            | Epstein Barr Virus                                         |
| FC             | Follicular center                                          |
| F              | Fetal Calf Serum                                           |
| 5'-FU          | 5'fluorouridine                                            |

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|                 |                                                                                                      |
|-----------------|------------------------------------------------------------------------------------------------------|
| GC              | Germinal Center                                                                                      |
| GFP             | Green Fluorescent Protein                                                                            |
| GCK             | germinal centre kinase                                                                               |
| hTERT           | Human Telomerase Reverse Transcriptase                                                               |
| ICR             | Imprinting Control Region                                                                            |
| Ig              | immunoglobulin                                                                                       |
| IL              | Interleukin                                                                                          |
| INF             | Interferon                                                                                           |
| IRF4            | Interferon Regulatory Factor 4                                                                       |
| kb              | Kilobase                                                                                             |
| kDa             | Kilodalton                                                                                           |
| LB              | Luria Broth                                                                                          |
| LCR             | Locus Control Region                                                                                 |
| MAPK            | Mitogen-Activated Protein Kinases                                                                    |
| MAPKKK or MAP3K | Mitogen-Activated Protein Kinase Kinase Kinase                                                       |
| MAPKK or MAP2K  | Mitogen-Activated Protein Kinase Kinase                                                              |
| MAPKAP-KSs      | MAPK-activated Protein Kinases                                                                       |
| MEF2            | Myocyte-Specific Enhancer Factor 2                                                                   |
| MKK             | Mitogen-activated protein kinase kinase                                                              |
| MKP             | MAPK phosphatases                                                                                    |
| MNK             | MAP Kinase-Interacting Serine/Threonine Kinase                                                       |
| MTA3            | Metastasis-Associated Protein 3                                                                      |
| NCoR            | Nuclear Receptor Corepressor                                                                         |
| NF-kB           | Nuclear Factor kappa-light-chain-enhancer of<br>activated B cells                                    |
| NHL             | Non Hodgkin Lymphoma                                                                                 |
| PAMPs           | Pathogen-Associated Molecular Patterns                                                               |
| PCR             | Polymerase Chain Reaction                                                                            |
| PBS             | Phosphate Buffer Saline                                                                              |
| PEST            | a peptide sequence which is rich in Proline (P),<br>glutamic acid (E), Serine (S), and Treonine (T). |
| PI              | Propidium Iodide                                                                                     |
| PPM1D           | p38 Inhibitor Protein Phosphatase 1D                                                                 |
| PRAK            | p38-Regulated/Activated Kinase                                                                       |

|              |                                                                        |
|--------------|------------------------------------------------------------------------|
| <i>PRDM1</i> | Positive Regulatory Domain 1                                           |
| PRR          | Pattern Recognition Receptors                                          |
| RAG1/2       | Recombination Activating Genes                                         |
| R10F         | RPMI-10 %-FCS                                                          |
| rpm          | Revolutions per minute                                                 |
| RPMI         | Culture medium Roswell Park Memorial Institute                         |
| RT           | Room Temperature                                                       |
| rRNA         | Ribosomic Ribonucleic Acid                                             |
| rDNA         | Ribosomic Deoxyribonucleic Acid                                        |
| SIRS         | Systemic Inflammatory Response                                         |
| STAT         | Signal Transducers and Activators of Transcription<br>protein          |
| SDS          | Sodium Dodecyl Sulfate                                                 |
| SMRT         | Silencing Mediator of the Retinoid and Thyroid<br>hormone receptors    |
| SHM          | Somatic Hypermutation                                                  |
| TAB1         | Transforming growth factor beta-Activated kinase-<br>Binding protein 1 |
| Th           | T helper                                                               |
| TNF          | Tumor Necrosis Factor                                                  |
| TPA          | 12-O-tetradecanylphorbol 13-acetate                                    |
| ZAP70        | Zeta-chain-Associated Protein kinase 70                                |
| ZF           | Zinc Finger                                                            |



| <b>INDEX</b>                                                                                               | <b>Page</b> |
|------------------------------------------------------------------------------------------------------------|-------------|
| <b>ABSTRACT</b>                                                                                            | 3           |
| <b>DECLARATION</b>                                                                                         | 5           |
| <b>ACKNOWLEDGMENTS</b>                                                                                     | 7           |
| <b>ABBREVIATIONS</b>                                                                                       | 13          |
| <b>INDEX</b>                                                                                               | 17          |
| <b>FIGURES INDEX</b>                                                                                       | 23          |
| <b>TABLES INDEX</b>                                                                                        | 25          |
| <b>CHAPTER 1. -GENERAL INTRODUCTION</b>                                                                    | 27          |
| <b>1.1. – Outline</b>                                                                                      | 29          |
| <b>1.2. – Immune response and germinal centers</b>                                                         | 30          |
| <b>1.3. – B cell development</b>                                                                           | 31          |
| <b>1.4. – BCL6 is a master transcription factor in germinal centre development and the immune response</b> | 35          |
| 1.4.1. Discovery of BCL6                                                                                   | 35          |
| 1.4.2. BCL6 expression                                                                                     | 35          |
| 1.4.3. Characteristics of BCL6 protein                                                                     | 36          |
| 1.4.4. Mechanism of action                                                                                 | 37          |
| 1.4.5. Transcriptional and post-transcriptional regulation of BCL6                                         | 40          |
| 1.4.6. BCL6 deregulation in lymphomas                                                                      | 42          |
| <b>1.5.-BCR-ABL involvement in BCL6 regulation</b>                                                         | 44          |
| <b>1.6- MAPK involvement in BCL6 regulation</b>                                                            | 46          |
| 1.6.1. MAPK family                                                                                         | 47          |
| 1.6.2. p38 functions in normal and cancer cells                                                            | 47          |
| 1.6.3. p38 and the immune system                                                                           | 49          |
| 1.6.4. p38 and cancer                                                                                      | 51          |

---

|                                                                         |    |
|-------------------------------------------------------------------------|----|
| 1.6.5. p38 and haematological malignancies                              | 52 |
| <b>1.7.CTCF as a potential regulator of BCL6</b>                        | 53 |
| 1.7.1. CTCF structural and functional domains                           | 54 |
| 1.7.2 CTCF postranslational modifications and interacting partners      | 55 |
| 1.7.3. CTCF intracellular localization and expression                   | 56 |
| 1.7.4. CTCF molecular functions                                         | 57 |
| a. Transcriptional repression or activation                             | 57 |
| b. Chromatin insulation                                                 | 58 |
| 1.7.5. CTCF role in cell proliferation, differentiation and apoptosis   | 62 |
| 1.7.6. Genome wide analysis of CTCF binding sites                       | 63 |
| 1.7.7. CTCF in human cancer                                             | 65 |
| <b>1.8. - Aims</b>                                                      | 68 |
| <b>CHAPTER 2. - MATERIAL AND METHODS</b>                                | 71 |
| <b>2.1. - Cell culture methods</b>                                      | 73 |
| 2.1.1. - Cell lines culture                                             | 73 |
| 2.1.2. - Primary cells                                                  | 73 |
| 2.1.3. - Cell counting, cell cycle and viability                        | 73 |
| <b>2.2. - Purification of primary lymphoma cells using a FAC-Sorter</b> | 74 |
| <b>2.3. - Fluorescence In Situ Hybridization (FISH)</b>                 | 76 |
| <b>2.4. Drug treatments</b>                                             | 76 |
| 2.4.1. Signaling pathways inhibitors                                    | 76 |
| 2.4.2. CD40 and BCR stimulation                                         | 78 |
| 2.4.3. Anisomycin and Cycloheximide                                     | 78 |
| 2.4.4. Demethylation treatment                                          | 78 |
| <b>2.5. Construction of plasmid vectors</b>                             | 79 |
| 2.5.1. Dominant negative STAT5                                          | 79 |
| 2.5.2. Luciferase Reporter Vectors                                      | 79 |
| 2.5.3. CTCF vectors                                                     | 81 |

|                                                                                                                                                |     |
|------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 2.5.4. pGMT- vector                                                                                                                            | 82  |
| <b>2.6. Transfections</b>                                                                                                                      | 83  |
| 2.6.1. DG75, MUTU III, BV173 and Z119 cells transfections                                                                                      | 83  |
| 2.6.2. K562 cells transfections                                                                                                                | 84  |
| 2.6.3. 293T cells transfections                                                                                                                | 84  |
| <b>2.7. Luciferase reporter assays</b>                                                                                                         | 85  |
| 2.7.1. In DG75, Z119 and BV173 cells                                                                                                           | 85  |
| 2.7.2. In K562 cells                                                                                                                           | 85  |
| 2.7.3. In 293T cells                                                                                                                           | 85  |
| <b>2.8. Protein analysis</b>                                                                                                                   | 86  |
| 2.8.1. Western blot analysis                                                                                                                   | 86  |
| 2.8.2. Immunofluorescence analysis                                                                                                             | 87  |
| <b>2.9. RNA analysis by reverse transcription (RT) and real-time PCR</b>                                                                       | 89  |
| <b>2.10. Chromatin Immunoprecipitation assay (ChIP)</b>                                                                                        | 92  |
| <b>2.11. Electrophoretic mobility shift assay (EMSA)</b>                                                                                       | 93  |
| 2.11.1. Preparation of radiolabelled probes                                                                                                    | 93  |
| 2.11.2 Nuclear extracts                                                                                                                        | 94  |
| 2.11.3. EMSA reaction                                                                                                                          | 95  |
| 2.11.4. Preparation of methylated probes                                                                                                       | 95  |
| <b>2.12. Bisulfite Genomic Sequencing</b>                                                                                                      | 96  |
| <b>CHAPTER 3. CML lymphoid blast crisis cell lines as model systems to define the effects of BCR-ABL mediated signaling on BCL6 expression</b> | 99  |
| <b>3.1. Introduction</b>                                                                                                                       | 101 |
| <b>3.2. Results</b>                                                                                                                            | 103 |
| 3.2.1. Imatinib induces BCL6 expression in CML lymphoid blast crisis                                                                           | 103 |
| 3.2.2. BCR-ABL downstream pathways as potential regulators of BCL6                                                                             | 104 |
| 3.2.3. STAT 5 drives BCL6 in CML lymphoid blast crisis cell lines                                                                              | 105 |
| 3.2.4. m-Tor represses BCL6 expression in Z119 cells.                                                                                          | 107 |

---

|                                                                                                                                    |     |
|------------------------------------------------------------------------------------------------------------------------------------|-----|
| 3.2.5. p38 MAPK induces BCL6 in CML lymphoid blast crisis cell lines                                                               | 109 |
| 3.2.6. Ectopic expression of BCR-ABL leads to BCL6 down-regulation                                                                 | 110 |
| 3.2.7. STAT5 also regulates BCL6 in germinal centre cells                                                                          | 111 |
| 3.2.8. p38 is important for BCL6 regulation in germinal centre cells                                                               | 112 |
| <b>3.3. Discussion</b>                                                                                                             | 113 |
| <b>CHAPTER 4. CD40 and B-CELL receptor signalling induce MAPK family members that can either induce or repress BCL6 expression</b> | 115 |
| 4.1. Introduction                                                                                                                  | 117 |
| 4.2. Results                                                                                                                       | 117 |
| 4.2.1. Multiple MAPKs, including p38, are induced by anti-IgM in Ramos                                                             | 117 |
| 4.2.2. Soluble CD40 ligand induces BCL6 through inducing p38                                                                       | 121 |
| 4.2.3. Expression of phosphorylated p38 in tonsil                                                                                  | 125 |
| 4.2.4. Site of action of p38 target genes at the <i>BCL6</i> promoter                                                              | 126 |
| 4.3. Discussion                                                                                                                    | 129 |
| <b>CHAPTER 5. CTCF Involvement in BCL6 regulation</b>                                                                              | 131 |
| 5.1. Introduction                                                                                                                  | 133 |
| 5.2. Results                                                                                                                       | 133 |
| 5.2.1. A putative CTCF binding site on the <i>BCL6</i> exon1                                                                       | 133 |
| 5.2.2. CTCF binds in vitro to the <i>BCL6</i> exon1 regulatory region                                                              | 135 |
| 5.2.3. Methylation of <i>BCL6</i> exon1 does not alter CTCF binding                                                                | 136 |
| 5.2.4. BCL6 and CTCF expression in different lymphoma cell lines                                                                   | 138 |
| 5.2.5. CTCF interacts in vivo with the <i>BCL6</i> exon1 regulatory region                                                         | 140 |
| 5.2.6. CTCF effect on <i>BCL6</i> promoter activity                                                                                | 142 |
| 5.2.7. CTCF effect on BCL6 expression                                                                                              | 146 |
| 5.2.8. CTCF effects on the cell cycle on BCL6 positive and negative cell lines                                                     | 147 |
| 5.3. Discussion                                                                                                                    | 148 |
| <b>CHAPTER 6. Epigenetic modifications and the regulation of <i>BCL6</i> gene</b>                                                  | 153 |
| 6.1. Introduction                                                                                                                  | 155 |

|                                                                   |     |
|-------------------------------------------------------------------|-----|
| <b>6.2.Results</b>                                                | 156 |
| 6.2.1. BCL6 expression is modulated by epigenetic modifications   | 156 |
| 6.2.2. Methylation status of the <i>BCL6</i> regulatory region    | 157 |
| 6.2.3. Histone marks present at <i>BCL6</i> exon1 region          | 159 |
| <b>6.3. Discussion</b>                                            | 161 |
| <b>CHAPTER 7. General discussion</b>                              | 165 |
| 7.1. Biological importance of BCL6                                | 167 |
| 7.2. BCL6 regulation by Imatinib                                  | 168 |
| 7.3. BCL6 and lymphomagenesis                                     | 170 |
| 7.4. Biological Roles of p38 MAPK                                 | 171 |
| 7.5. p38 MAPK and germinal centres                                | 171 |
| 7.6. p38 MAPK , BCL6 and germinal centre derived lymphomas        | 172 |
| 7.7. BCR and CD40 signalling effects on BCL6 expression           | 174 |
| 7.8. Non-lymphoid cell/B-cell interactions may also regulate BCL6 | 176 |
| 7.9. p38 targets and BCL6                                         | 177 |
| 7.10. CTCF regulation of BCL6                                     | 177 |
| 7.11. Epigenetics and <i>BCL6</i>                                 | 179 |
| 7.12. Summary and future work                                     | 182 |
| <b>CHAPTER 8. BIBLIOGRAPHY</b>                                    | 185 |



## FIGURES INDEX

## Page

**GENERAL INTRODUCTION**

|                                                                             |    |
|-----------------------------------------------------------------------------|----|
| Figure 1.1. Stages of B cell differentiation                                | 32 |
| Figure 1.2. Germinal centre reaction                                        | 34 |
| Figure 1.3. Scheme of BCL6 protein domains and protein interacting partners | 36 |
| Figure 1.4. Scheme of several pathways regulated by BCL6                    | 38 |
| Figure 1.5. <i>BCL6</i> gene.                                               | 41 |
| Figure 1.6. BCR-ABL signaling pathways                                      | 45 |
| Figure 1.7. Stress MAPK signaling pathways                                  | 48 |
| Figure 1.8. p38 MAPK signaling pathway                                      | 50 |
| Figure 1.9. CTCF structural domains and interacting partners                | 54 |
| Figure 1.10. CTCF as a “classical” transcriptional factor.                  | 58 |
| Figure 1.11. CTCF insulator activity                                        | 59 |
| Figure 1.12. CTCF regulation of CpG methylation                             | 60 |
| Figure 1.13. CTCF insulation via formation of chromatin loops               | 61 |
| Figure 1.14. Genome wide analysis of CTCF binding sites                     | 64 |

**MATERIAL AND METHODS**

|                                                                                     |    |
|-------------------------------------------------------------------------------------|----|
| Figure 2.1. Scheme depicting the pRS-CTCF vector used for silencing CTCF expression | 81 |
| Figure 2.2. K562 and DG75 are efficiently transfected                               | 83 |
| Figure 2.3. Algorithm used for the bisulfite methylation analysis                   | 96 |

**RESULTS CHAPTER 3**

|                                                                                      |     |
|--------------------------------------------------------------------------------------|-----|
| Figure 3.1. BCL6 is induced in lymphoid blast crisis of CML by Imatinib              | 102 |
| Figure 3.2. Imatinib induces BCL6 expression in CML lymphoid blast crisis cell lines | 103 |
| Figure 3.3. Inhibition of BCR-ABL signalling pathways by Imatinib                    | 105 |
| Figure 3.4. STAT 5 drives BCL6 in CML Lymphoid blast crisis cell lines               | 107 |
| Figure 3.5. m-Tor represses BCL6 expression in Z119 cells                            | 108 |
| Figure 3.6. p38 MAPK induces BCL6 in CML lymphoid blast crisis cell lines            | 109 |
| Figure 3.7. Ectopic expression of BCR-ABL leads to BCL6 down-regulation              | 110 |
| Figure 3.8. STAT5 also regulates BCL6 in germinal centre cells                       | 111 |
| Figure 3.9. p38 upregulates BCL6 in germinal centre cells                            | 112 |
| Figure 3.10. Inhibitors used to study the BCR-ABL signalling pathways                | 114 |

---

## RESULTS CHAPTER 4

|                                                                                      |     |
|--------------------------------------------------------------------------------------|-----|
| Figure 4.1. Multiple MAPKs, including p38, are induced by anti-IgM in Ramos cells    | 118 |
| Figure 4.2. Regulation of BCL6 by different MAPK                                     | 119 |
| Figure 4.3. p38 induces BCL6 expression                                              | 120 |
| Figure 4.4. Soluble CD40 ligand induces BCL6 through inducing p38                    | 122 |
| Figure 4.5. CD40L effects on BCL6 transcription                                      | 124 |
| Figure 4.6. Immunocytochemistry showing phosphorylated p38 and BCL6 expression in GC | 125 |
| Figure 4.7. p38 effect on <i>BCL6</i> promoter activity                              | 128 |

## RESULTS CHAPTER 5

|                                                                                     |     |
|-------------------------------------------------------------------------------------|-----|
| Figure 5.1. Putative CTCF binding site on the <i>BCL6</i> exon 1                    | 134 |
| Figure 5.2. EMSA to analyze in vitro binding of CTCF to <i>BCL6</i> exon 1          | 135 |
| Figure 5.3. CTCF binds <i>BCL6</i> exon1 in a methylation insensitive manner        | 137 |
| Figure 5.4. BCL6 and CTCF expression in lymphoma cell lines                         | 139 |
| Figure 5.5. CTCF interacts in vivo with <i>BCL6</i>                                 | 141 |
| Figure 5.6. Occupancy of the <i>BCL6</i> exon 1 by CTCF and H2A.Z                   | 143 |
| Figure 5.7. CTCF effect on the transcriptional activity of the <i>BCL6</i> promoter | 145 |
| Figure 5.8. CTCF effects on BCL6 expression.                                        | 147 |
| Figure 5.9. CTCF effects on the cell cycle of lymphoma cell lines                   | 149 |

## RESULTS CHAPTER 6

|                                                                                                   |     |
|---------------------------------------------------------------------------------------------------|-----|
| Figure 6.1. Treatment with demethylating agents and HDAC inhibitor agents induces BCL6 expression | 156 |
| Figure 6.2. Methylation status of <i>BCL6</i> exon1 regulatory region                             | 158 |
| Figure 6.3. Binding of modified and variant histones to the <i>BCL6</i> exon1 regulatory Region   | 160 |

## GENERAL DISCUSSION

|                                                                               |     |
|-------------------------------------------------------------------------------|-----|
| Figure 7.1. A proposed model for BCL6 regulation by BCR-ABL in lymphoid cells | 169 |
| Figure 7.2. A proposed model of BCL6 regulation by CTCF                       | 182 |

**TABLES INDEX**

Page

**CHAPTER 2. MATERIAL AND METHODS**

|                                                                                                  |    |
|--------------------------------------------------------------------------------------------------|----|
| Table 2.1. Phenotype, culture conditions, source and references of cell lines used in this study | 75 |
| Table 2.2. Signalling pathways inhibitors and stimulators utilized in this study                 | 77 |
| Table 2.3. Plasmids used in this study                                                           | 82 |
| Table 2.4. Primary antibodies used in this study.                                                | 88 |
| Table 2.5. Table of secondary antibodies used in this study                                      | 89 |
| Table 2.6. Primers used in this study                                                            | 91 |



# **CHAPTER 1**

## ***General introduction***



## CHAPTER 1. General introduction

### 1.1. Outline

Non-Hodgkin's-Lymphomas (NHL) comprise a heterogeneous group of neoplasms that are derived from lymphocytes. Recent surveys and studies performed in Europe have reported that despite the improvements in the diagnosis and in the approaches to treatment, NHL are still an important cause of morbidity and mortality in the European Union with an increase in the mortality rate during the last two decades to 4.4/100000 in men and 2.8/100000 in women (Bosetti et al., 2008).

There is considerable interest in understanding the mechanisms involved in the process of lymphomagenesis. Non-random chromosomal translocations are frequently detected in lymphomas. They commonly place a proto-oncogene under the control of an immunoglobulin gene enhancer to produce high level and constitutive expression. Many important proto-oncogenes have been discovered through the analysis of translocations in lymphomas. For example, c-MYC translocations are characteristic of Burkitt's lymphomas and, cyclin D1 translocations of mantle cell lymphomas. The long arm of chromosome 3 band 27 is one of the most frequent targets of genetic alterations in lymphomas. In 1993, a potent transcriptional repressor named first *BCL5* or *LAZ3* and subsequently *BCL6* was identified in the 3q27 region (Ye et al., 1993). *BCL6* has been shown to play key roles in the development and regulation of the immune system and in lymphomagenesis. However, many aspects of the biology of this oncogene still remain unclear, especially the regulation of the gene in normal B-cells and in lymphomas derived from germinal centre B-cells.

This thesis is concerned with the regulation of the *BCL6* and in this introductory chapter, essential background information on several biological processes relevant for

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the work presented in this thesis will be provided. Firstly, a brief overview of germinal centres will be presented. These are dynamic structures essential for acquired immunity. Next, the characteristics and functions of the BCL6 in normal conditions will be described, followed by its role in lymphomagenesis. Finally, the main biological functions of the p38 MAPK pathway and of the important regulator CTCF will be summarized, since this information will be useful for a better understanding of the latter chapters.

## **1.2. Immune response and germinal centres**

The immune system is responsible for defending the organism from pathogenic agents. The immune system can be separated in three categories: the physical barriers, innate immunity and the adaptive response. The innate system (Delves and Roitt, 2000a) functions to increase the protection imparted by physical barriers, by detecting pathogens and initiating an inflammatory response. Among the principal components of the innate system are soluble proteins such as complement proteins or cytokines, cellular components including leukocytes, and a group of membrane bound receptors and cytoplasmatic proteins, known as Pattern Recognition Receptors, which either recognize specific elements usually conserved and shared by many different microorganisms or, detect cells that have been subject of stresses or damages. Once stimulated, the innate immune system initiates a non-specific inflammatory response against the intruder, and will interact and stimulate the adaptive response (Chaplin, 2010; Turvey and Broide, 2010).

The unique features of the adaptive immune response are specificity and memory. (Bonilla and Oettgen, 2010; Delves and Roitt, 2000b). The adaptive immune system consists of T and B lymphocytes. The former are “cellular immune effectors” whereas the B lymphocytes are “antibody producing cells”. The adaptive response is based on the specific interaction between an antigen and a receptor expressed on T and

B lymphocytes. In contrast with the germline recognition molecules of the innate system, the B and T cell receptors generation requires a complex and sophisticated process that involves the rearrangement of several gene elements that encode for the different components of the T-cell receptor and the B cell receptor (BCR; Immunoglobulin), as well as the introduction of random mutations in the region responsible for the recognition of the antigen (the variable region)(Chaplin, 2010).

The innate and adaptive immune systems work together, the innate system being responsible for the first line of defense providing a rapid but not long lasting, nor specific response, whereas the adaptive immune response will contribute after several days of clonal expansion with a very specific response, and will produce long-lived memory cells which will be able to recognize the pathogen and will generate a very specific, rapid and strong response in case of an eventual reexposition to the same pathogen (Chaplin, 2010; Delves and Roitt, 2000a)

### **1.3. B-cell development**

The characteristic molecular feature of a mature B-cell is the presence of surface immunoglobulin, and the development of B-cells (Figure 1.1) bearing immunoglobulin with a large range of specificities is required for normal immunity. B-cells are produced from bone marrow haematopoietic stem cells, which have undergone several well characterized stages resulting in immunoglobulin gene rearrangement in order to generate a surface B cell receptor (Bonilla and Oettgen, 2010). Early B-cells lack surface or cytoplasmic immunoglobulin (Figure 1.1). Antibody (or immunoglobulin) genes are encoded in DNA by separate gene segments which are joined by a process requiring recombination activating genes (RAG1/2) to produce either a heavy (H) chain or light (L) chain (Figure 1.1).

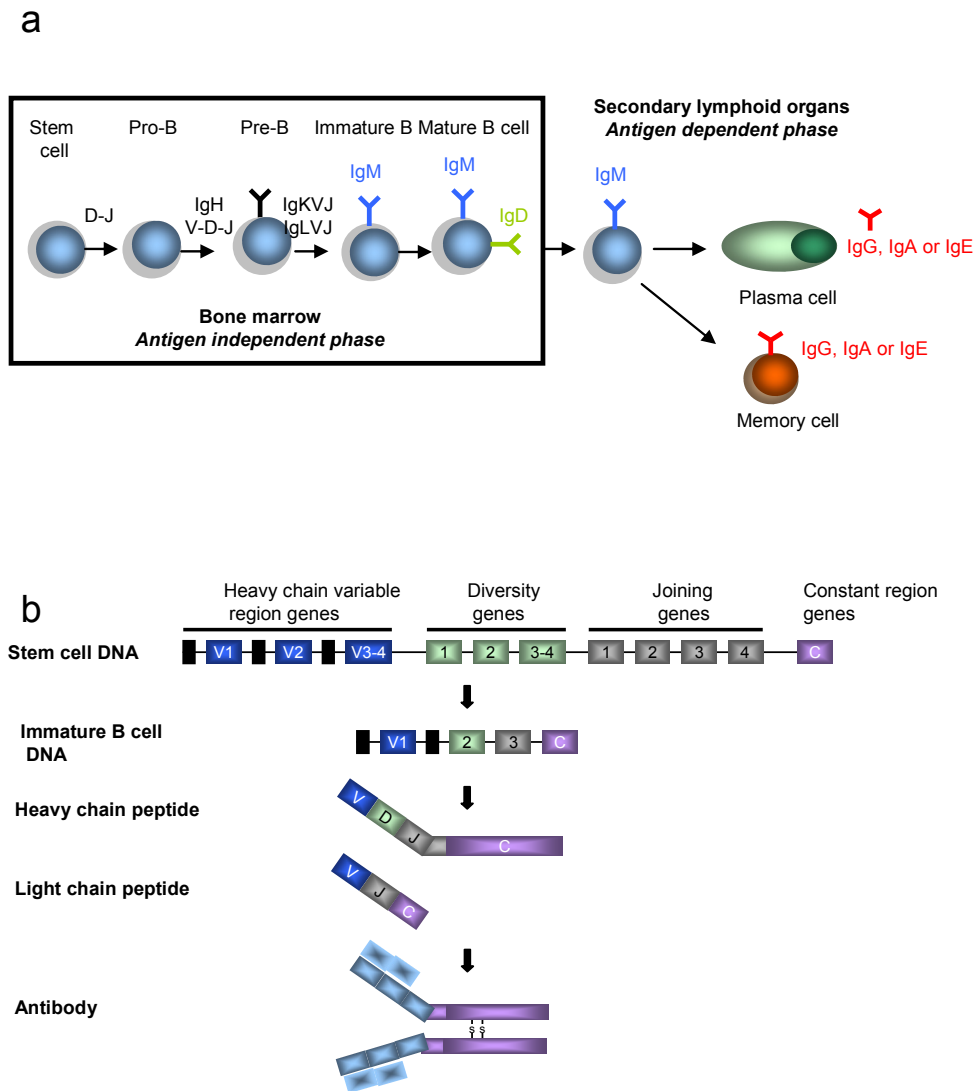


**Figure 1.1. Stages of B cell differentiation.** (a) The development of B-lineage cells can be defined by the differential expression of cell-surface markers and by the rearrangement status of immunoglobulin genes. B cell development can be divided into two phases: the antigen independent and antigen dependent phases. The former takes place in the bone marrow from neonatal through adult life. Mature cells, which express IgM and IgD on its surface move into the periphery where they can be activated by an antigen (*antigen dependent phase*). (b) An antibody is a multi-chain peptide formed with two identical heavy chains and two identical light chains. Each chain consists of a variable region (V), responsible for the antibody specificity for a certain antigen, and a constant region (C). The antibodies are the result of a combination of different V, J and C genes. This combination is essential for the diversification of the antibodies. (This figure is based on Cambier et al, 2007; Delves et al, 2000; Baron, Medical Microbiology 1996)

A complete antibody molecule requires 2 H chains and 2 L chains. B-cells within the bone marrow express a diverse primary repertoire of antibodies but they have low affinity for antigen and only express the  $\mu$  H-chain isotype. B-cells exit the bone marrow and travel to lymph nodes. On encounter with a T-cell dependent antigen the B-cell moves to a follicle where it proliferates intensely to form a germinal centre (McHeyzer-Williams et al., 2001). The germinal centres are therefore dynamic structures within the lymph nodes, and consist of two histologically well differentiated zones with distinct functions (Figure 1.2). The *dark zone* contains centroblasts, and the *light zone* centrocytes, T-cells and follicular dendritic cells. It is believed (Vinuesa et al., 2009) that B-cells proliferate in the dark zone and undergo a process called somatic hypermutation (SHM). SHM requires the enzyme activation induced deaminase, which is responsible for introducing mutations into the immunoglobulin genes in order to produce high affinity antibodies, as well as accomplishing class switch from IgM to IgG or IgA in order to change the effector function. The B-cells then move to the light zone and are selected for the production of high affinity antibodies. Those B-cells that fail selection die by apoptosis whereas those producing high affinity antibodies either return to the dark zone for a further round of SHM, or exit the germinal centre. They leave the germinal centre either as an antibody producing cell (plasma cell) or as a memory B-cell.

Germinal centres, therefore, contain highly proliferating B-cells that are undergoing mutations and for these reasons are predisposed to the formation of malignancies. Indeed, a significant number of B cell lymphomas emerge from these areas (Kuppers et al., 1999) and, the pathogenesis of many autoimmune responses has been lately related to abnormal germinal centre responses (Vinuesa et al., 2009).

Studies using mice bearing homozygous gene disruptions have demonstrated that several B-cell molecules e.g BCL6 (Fukuda et al., 1997) or CD19 (Barrington et al., 2009; Fehr et al., 1998), as well as T-cell e.g. CD40 ligand (Foy et al., 1994) and dendritic cell e.g. lymphotoxin alpha (Matsumoto et al., 1996), are required for germinal centre development.

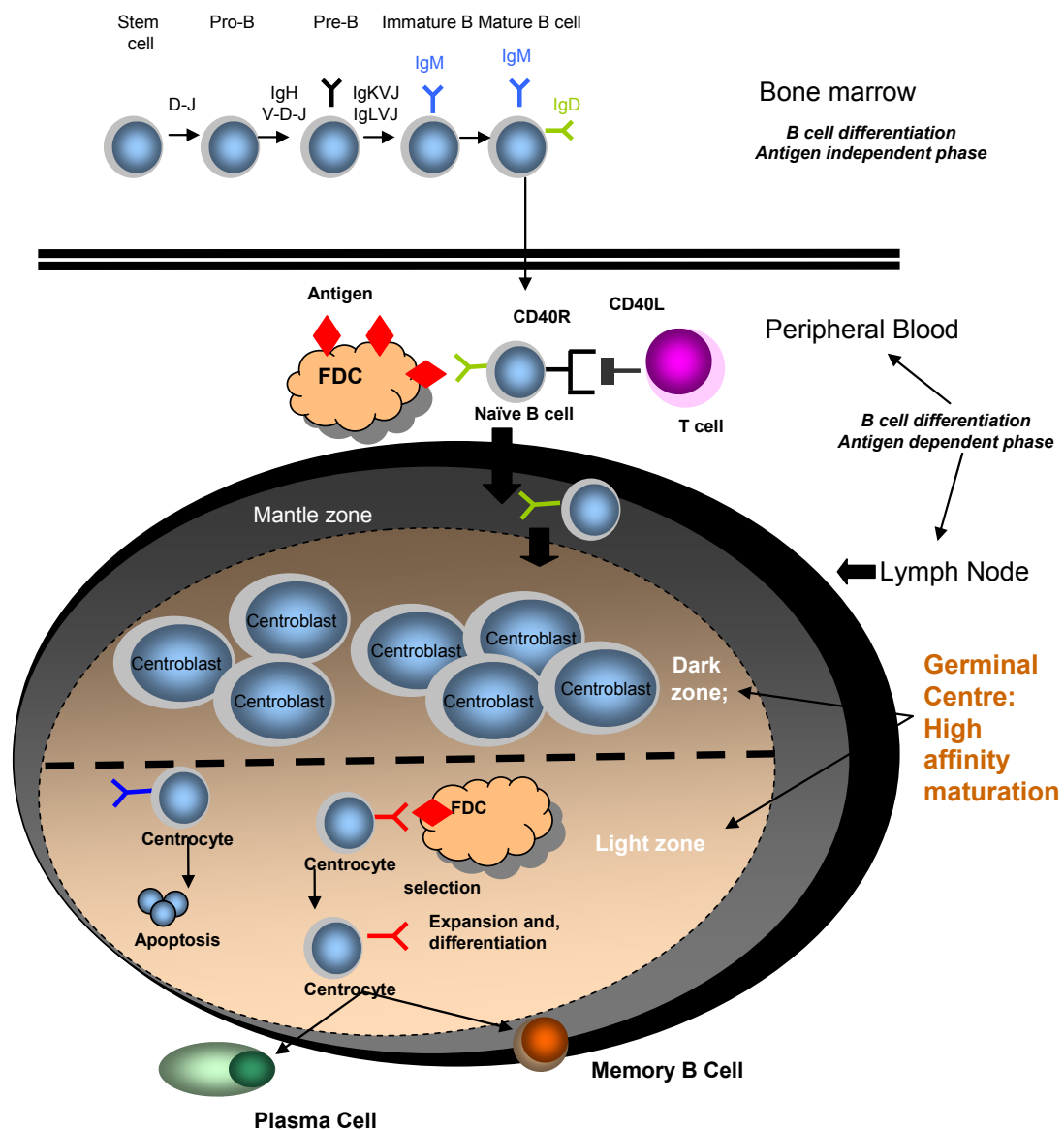


**Figure 1.2. Germinal centre reaction.** This figure illustrates biological processes of the germinal centres (GC). Naïve B cells, when activated by an antigen, are recruited into the follicles within the lymph nodes where they start proliferating to form the germinal centre region. Two areas can be differentiated within the GC: the dark and the light zones. The dark zone is formed with hyperproliferative activated B cells, that are going through the somatic hypermutation process in the immunoglobulin variable genes. These cells are known as centroblasts. The light zone, consists of a number of follicular dendritic cells that have the immunizing antigen on their surface. The B cells within this area, which are known as centrocytes, stop proliferating and are positively selected based on their ability to interact with the antigen presented by the Follicular Dendritic Cells (FDC). Once selected, these cells can differentiate either into plasmatic cells or memory cells. Those cells not capable of interacting or that recognize self antigens undergo apoptosis. (This figure is based on Vinuesa et al, 2009 and Delves et al 2000)

## **1.4. BCL6 is a master transcription factor in germinal centre development and the immune response**

### ***1.4.1. Discovery of BCL6***

The observation that the 3q27 region was frequently disrupted by translocations in a number of patients with non-Hodgkin lymphomas, and that in isolated cases that abnormality was the only cytogenetic finding on the karyotypes, strongly suggested the possibility of a cancer gene being located in that region. Indeed, subsequent subcloning and sequence analysis of that region lead to the identification of the *LAZ3* (now known as *BCL6*) in that region in 1993 (Kerckaert et al., 1993).

### ***1.4.2. BCL6 expression***

BCL6 protein is expressed at high levels in normal lymphoid germinal centre centroblast B cells, in almost all centrocytes cells (Cattoretti et al., 1995), in follicular helper T cells (Tfh) (Bi and Ye, 2010; Mondal et al., 2010) and, although there is less evidence, it seems also be expressed in other T-cells subtypes (Cattoretti et al., 1995) and in macrophages (Mondal et al., 2010). BCL6 protein is also detected in almost all lymphomas with a germinal centre origin (Cattoretti et al., 2005; Onizuka et al., 1995) such as a subgroup of diffuse large cell lymphoma, Burkitt's lymphoma, follicular lymphoma and lymphocyte predominant Hodgkin's lymphoma (Reljic et al., 2000). BCL6 is tightly expressed, and although BCL6 m-RNA is detectable in naïve B cells (Allman et al., 1996), BCL6 protein is not found in naive B-cells or post germinal centre B cells including immunoblasts and normal plasma cells (Cattoretti et al., 1995).

### 1.4.3. Characteristics of BCL6 protein

BCL6 is a 706 amino acid, 95 kDa phospho-protein (Figure 1.3) member of the POZ/POK family of transcription factors, which play important roles in normal development and in cancer (Costoya, 2007; Kawamata et al., 1994).

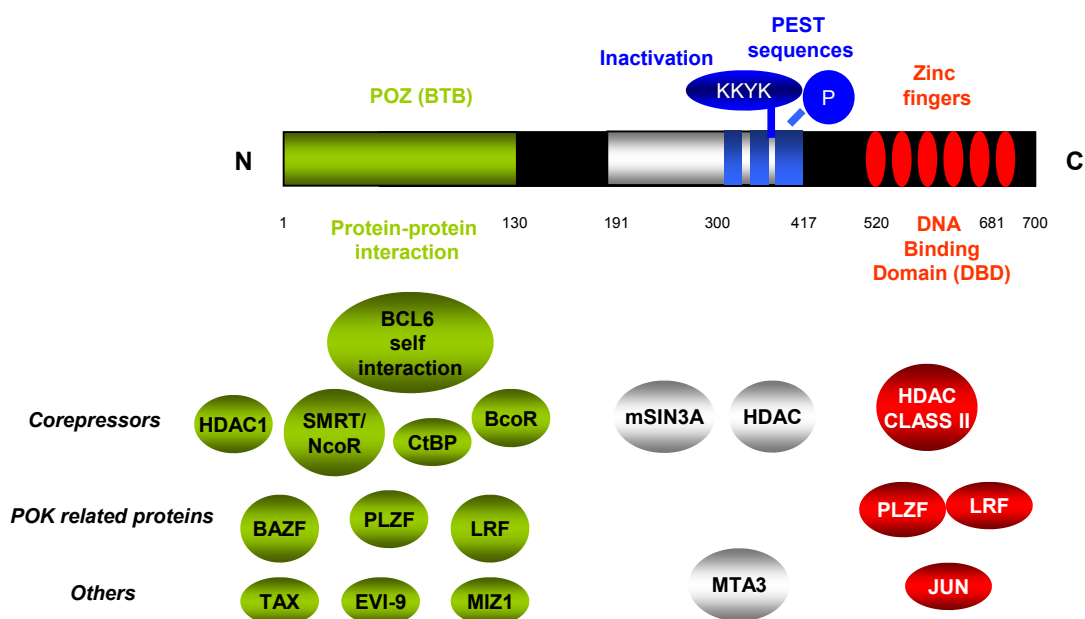


**Figure 1.3. Scheme of BCL6 protein domains and protein interacting partners** The human BCL6 protein, which belongs to the “POK family proteins”, consists of (a) a POZ (BTB) domain (amino acids 1 to 130) at the N-terminal region, which is essential for protein-protein interactions, including several corepressors such as SMRT, NcoR and BcoR (b) a central portion domain with a subdomain spanning from amino acids 370-380 that has been shown to be also important for the repression activity. The central portion has several PEST sequences, that when phosphorylated by the MAPK pathway triggers the BCL6 protein for degradation via the ubiquitin-proteasome pathway. BCL6 KKYK motif (aminoacids 376-379), which is necessary for the acetylation by p300, is located within the PEST sequences region. (c) a C-terminus domain that has six zinc fingers, required for the interaction with the DNA (consensus sequence: TTCCTT(A/C)GAA). The BCL6 partners that interact with known BCL6 domains are shown with the same coloured code (Figure based on Albagli-Curiel, 2003)

These transcription factors characteristically have a number of zinc fingers, six in BCL6, at the C-terminal end that recognize specific regulatory DNA sequences. At the N-terminal end they have a POZ(BTB) domain that is required for protein/protein interaction (Figure 1.3). This later domain interacts with a number of co-repressors such as the nuclear receptor corepressor (NCoR), BCL6 corepressor (BcoR) or the silencing mediator of the retinoid and thyroid hormone receptors (SMRT), to recruit histone deacetylase and accomplish transcriptional repression (Melnick et al., 2002; Mendez et al., 2008; Zhang et al., 2001). BCL6 repression of genes involved in cell proliferation and survival, such as ATR, p53 and cyclin-dependent kinase inhibitor 1A (CDKN1A), depends on the BCL6 interaction with NCoR, SMRT or BCoR (Mendez et al., 2008; Parekh et al., 2008). The POZ(BTB) domain also mediates homo-dimerization, and associates with other proteins such as PLZF or LRF (Ahmad et al., 2003; Albagli-Curiel, 2003).

BCL6 contains a central region that interacts with histone deacetylase (HDAC) and the co-repressor MTA3 in an acetylation-sensitive manner (Mendez et al., 2008; Zhang et al., 2001). In fact, BCL6-MTA3 interaction is required for inhibition of plasma cell differentiation (Mendez et al., 2008). The mid portion of BCL6 also has several PEST sequences that mediate BCL6 degradation (see section 1.4.5) (Niu et al., 1998)

#### **1.4.4. Mechanism of action of BCL6**

BCL6 knockout mice have normal early B-lymphoid development but lack germinal centres and do not have T-cell dependent antibody responses (Cao et al., 1997). Experiments designed to investigate the specific function of BCL6 in B-cell terminal differentiation showed that BCL6 is required to differentiate follicular B cells into germinal centre B cells and to maintain these cells in this state (Baron et al., 2002; Cao et al., 1997; Reljic et al., 2000) .

Previous work (Avantaggiati et al., 1997; Hancock et al., 2005; Harris et al.,

1999; Reljic et al., 2000) suggested that BCL6 binds to STAT (signal transducers and activators of transcription) binding sites and modulates transcription driven by the STAT family of factors, particularly interleukins. An overview of the function of BCL6 is that it modulates the effects of interleukins in order to allow proliferation and prevent differentiation (Avantaggiati et al., 1997; Fearon et al., 2001; Reljic et al., 2000), although the full picture is likely to be more complicated.

Gene expression profiling arrays (Shaffer et al., 2000), genome wide chromatin immunoprecipitation studies together with integrated biochemical and computational approaches (Basso et al., 2010) have led to the identification of a broad spectrum of BCL6 target genes responsible for the control of a number of pathways involved in the normal germinal centre reaction (Figure 1.4).

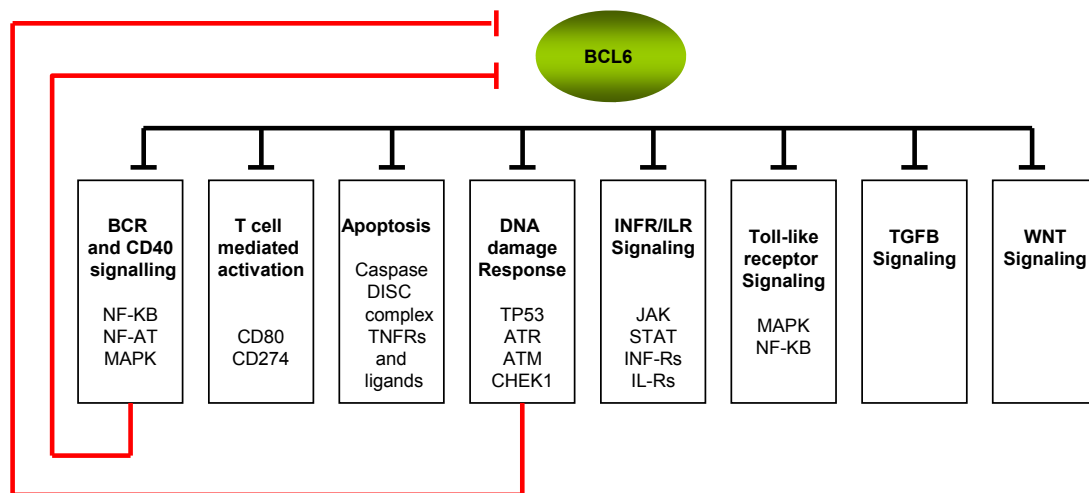


**Figure 1.4. Scheme of several pathways regulated by BCL6.** BCL6 modulates several genes with a variety of functions. In this figure the more representative pathways regulated by BCL6 gene are depicted. Repression of BCL6 by BCR and CD40 signaling pathway and by the DNA damage response is also illustrated. (Figure based on Basso et al, 2010)

These BCL6 target genes were found to be involved in cell cycle arrest, such as the CDKN1A7p21, in apoptosis like BCL2 and also in differentiation such as the TGF  $\beta$  receptor signalling or the WNT signalling. BCL6 also inhibits the Positive Regulatory Domain 1 (PRDM1) gene, also called BLIMP1. This is a zinc finger protein that is essential for the terminal differentiation of B-cells to plasma cells (Crotty et al., 2010; Tunyaplin et al., 2004) (Tunyaplin et al., 2004). Also, BCL6 is capable of suppressing signalling pathways known to downregulate its own expression such as the CD40 or the BCR pathway. Finally, BCL6 directly represses genes required for detecting and responding to DNA damage like p53 (Phan and Dalla-Favera, 2004) or ATR (ataxia-telangiectasia and Rad3 related) (Ranuncolo et al., 2007), allowing therefore the SHM process, required for the high affinity maturation of the antibody. Clearly, BCL6 suppresses important cellular pathways necessary for B cell activation, differentiation and survival in order to protect germinal centre cells from apoptosis and from signals causing a premature exit from the germinal centre before they have completed the antibody high affinity maturation process (Basso et al., 2010).

It has also been suggested that BCL6 might be required to maintain memory B cells in a self-renewing state (Fearon et al., 2001), although the opposite has also been reported (Crotty et al., 2010). Therefore, BCL6 and BLIMP-1 appear to form a system to regulate B-cell terminal differentiation (Crotty et al., 2010). BCL6 is associated with proliferation and BLIMP1 with differentiation.

Mice lacking BCL6 develop a characteristic T systemic inflammatory response suggesting that BCL6 might have a role on T cell regulation (Cao et al., 1997). Indeed, recent studies have shown that BCL6 is both necessary and sufficient to induce CD4<sup>+</sup> cells differentiation towards T<sub>fh</sub> cells, which are essential for the germinal centre reaction (Bi and Ye, 2010). In contrast, BCL6 appears to inhibit the differentiation of T cells into the other CD4<sup>+</sup> T cells subtypes (T<sub>H</sub>2, T<sub>H</sub>1 and T<sub>H</sub>17), which are not directly involved in the germinal centre formation (Crotty et al., 2010). Also, limited evidence suggests that BCL6 favours the formation of both long term memory CD4<sup>+</sup> (Ichii et al.,

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2007) and central CD8<sup>+</sup> memory T cells (D'Cruz et al., 2009; Yoshida et al., 2006).

Preliminary studies indicated that apart from the germinal centre B and T cells, other several tissues, namely skeletal muscle, testicular germ cells and skin epithelium express BCL6 (Bajalica-Lagercrantz et al., 1998). Although the significance of BCL6 expression in cells other than germinal centre B cells is yet not completely clear, it has been proposed that BCL6 might also be important in the terminal differentiation stage in many of these tissues (Bajalica-Lagercrantz et al., 1998). For instance BCL6 was apparently important for the maintenance of a terminally differentiated phenotype in myoblasts, spermatids and keratinocytes. BCL6 seems also to have a protective role from apoptosis due to specific stresses in myoblasts and spermatocytes (Kojima et al., 2001; Kumagai et al., 1999; Yoshida et al., 1996). BCL6 expression was detected in the mammary epithelium, where it has been shown to act as an anti-apoptotic factor and aberrant over-expression was detected in 68% of patients with histologically high-grade ductal breast carcinomas (Logarajah et al., 2003).

Therefore, the role of BCL6 in proliferation and apoptosis remains controversial. BCL6 has been shown to favour proliferation and block apoptosis in some cell types, whereas it seems to induce apoptosis in others (Baron et al., 2002; Hancock et al., 2005; Yamochi et al., 1999). A possible explanation would be that BCL6 can either induce or repress apoptosis depending on the cell type (Albagli-Curiel, 2003; Fukuda et al., 1997; Zhang et al., 2001).

#### **1.4.5. *Transcriptional and post-transcriptional regulation of BCL6***

Despite the importance of BCL6 expression for the germinal centre formation, little is known about BCL6 transcriptional regulation. The *BCL6* gene is composed of 10 exons spread over 26 kb of genomic DNA (Figure 1.5). Two different mRNA splicing variants have been reported depending on content or not of the exon 2. The translation start site has been identified in exon 3 (Kawamata et al., 1994).

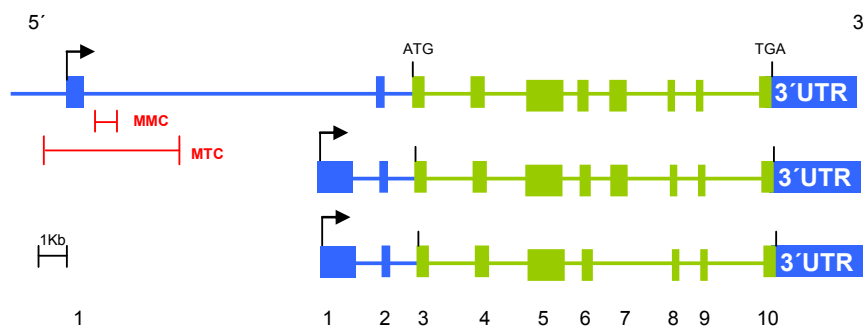


**Figure 1.5. *BCL6* gene.** This figure shows the genomic organization of the *BCL6* gene. Protein coding exons are represented in green, whilst the non coding ones appear in blue. Two different mRNA isoforms, with different transcription start site, which encode for identical *BCL6* protein, and a third variant with absence of exon 7 have been reported. The functional meaning of this third variant is still unknown. The major Translocation Cluster (MTC) is indicated. The Major Mutations Cluster, according to Migliazza et al 1995 is also illustrated. (*This figure was based on NCBI Entrez Gene; Shen et al, 2008; Bernardin et al; 1997*)

Previous studies have reported that the *BCL6* promoter spans a 1.5 kb region (Fukuda et al., 1995) and contains different putative response elements, a TATA binding protein site and also *BCL6* binding sites (Fukuda et al., 1995). However, the *BCL6* regulatory region has been incompletely characterized. The first intron-exon boundary contains *BCL6* regulatory elements which have been demonstrated to play important roles in the transcriptional regulation of *BCL6*. One of these regions, named the ES region, located within exon 1, contains two consensus nucleotides sequence (BSA1A and BSA1B) for *BCL6* and it has been confirmed that *BCL6* binds to these regions repressing its own transcription (Kikuchi et al., 2000). The effect of occupancy of *BCL6* binding sites within exon 1 by *BCL6*, therefore, defines a negative autoregulatory circuit. These *BCL6* binding sites are mutated in some cases of diffuse large cell lymphoma and the mutations disrupt the negative autoregulatory circuit thereby allowing prolonged *BCL6* expression, which is likely to promote lymphomagenesis (Gaidano et al., 2003). Clearly there must be additional control mechanisms that allow sustained *BCL6* expression.

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BLIMP-1, an essential regulator of plasma cell differentiation, directly represses BCL6 transcription, allowing differentiation into plasmatic cells (Cimmino et al., 2008). BCL6 transcription is also repressed by the CD40/NF- $\kappa$ B/IRF4 pathway. This regulatory mechanism is disrupted due to chromosomal translocations and point mutations in a subset of Diffuse Large B cell Lymphomas (Saito et al., 2007). Additionally, our laboratory has recently identified a DNase I hypersensitive site located 4.4Kb upstream the transcriptional initiation site of the *BCL6* gene, that binds a repressive complex formed with the ZEB1 (zinc finger E-box-binding homeobox 1) and the corepressor CtBP (C-terminal binding protein). As ZEB1 protein expression is relatively low in germinal centres cells, it is likely that ZEB1 expression is necessary for repressing BCL6 expression in naive B-cells (Papadopoulou et al., 2010).

Growth factors activate the STAT family of latent transcription factors. BCL6 transcription is driven by STAT5 in tonsillar B-cells (Scheeren et al., 2005). STAT3 has also been shown to drive BCL6 in germinal centre cells (Arguni et al., 2006).

A variety of post-translational modifications of BCL6 have been described. In the B-cell line, Ramos, activation of the B-cell receptor induces mitogen-activated protein (MAP) kinase mediated phosphorylation of the BCL6 PEST sequences, which in turn, targets BCL6 for rapid degradation by the ubiquitin-proteasome pathway (Niu et al., 1998). Additionally, BCL6 is inhibited through p300-mediated acetylation (Bereshchenko et al., 2002). Finally, it has been recently reported that BCL6 can be post-transcriptionally inhibited by the microRNA miR-127 (Saito et al., 2006).

#### **1.4.6. *BCL6* deregulation and lymphomas**

*BCL6* is a frequent target of chromosomal translocations with many different partners, the immunoglobulin (Ig) heavy chain locus being the most common. These translocations often cluster in a 4 kb region on the *BCL6* gene known as the major breakpoint region (MBR) or major translocation cluster (MTC) (Figure 1.5), spanning the

promoter, the first non-coding exon and the 5' part of the first intron (Albagli-Curiel, 2003). As a result of these rearrangements transcription of the unaltered *BCL6* coding region is controlled by the regulatory sequences of the partner gene (promoter substitution), leading to deregulated *BCL6* expression (Akasaka et al., 2000). Despite repeated efforts it has not been possible to determine whether the partner involved in *BCL6* translocations determine the clinical outcome (Ohno, 2006). However, it has been suggested that those lymphomas with non-IgH/*BCL6* translocations have a worse outcome than those with IgH/*BCL6* translocations (Ohno, 2006).

The *BCL6* locus, in common with the immunoglobulin and with some other non-immunoglobulin loci, is targeted by activation induced deaminase (AID), which is required for the somatic hypermutation (SHM) process. It has been proposed that the SHM machinery is involved in the generation of both non-IgG and IgG/*BCL6* translocations (Ohno, 2006). Also, as a result of the AID action, the *BCL6* locus acquires point mutations (Capello et al., 2000; Chen et al., 1998; Jardin and Sahota, 2005; Mateo et al., 2001; Migliazza et al., 1995; Pasqualucci et al., 1998; Szereday et al., 2000; Vitolo et al., 2002). Mutations spanning the first non-coding exon and clustering in the 5' region of the first intron have been found in about 10% of diffuse large B-cell lymphomas. Analysis of the region surrounding exon 1 showed a negative autoregulatory circuit: *BCL6* bound to a high affinity site in exon 1 that caused its own transcription to be switched off (Pasqualucci et al., 2003; Wang et al., 2002). *BCL6* gene mutations affecting the *BCL6* binding site in exon1 have been found frequently among diffuse large cell lymphoma (DLBCL)-derived alleles but not in normal germinal centre B-cells (Pasqualucci et al., 2003). Thus, the finding of mutations within the *BCL6* binding sites within exon 1 (BSE 1) mutations (see above) specifically restricted to DLBCL suggests selection of these mutations in lymphomas probably due to disruption of the negative autoregulatory circuit (Capello et al., 2000; Gaidano et al., 2003; Szereday et al., 2000; Wagner and Kaeda, 2005).

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BCL6 expression is a prognostic marker in DLBCL. DLBCL can be classified attending to the cell origin in two subtypes: the Germinal Centre B (GCB) like type, and the Activated B Cell like (ABC) type. The former one shows a germinal centre gene expression pattern showing therefore high levels of BCL6 expression, and has been shown to have long survival compared to the ABC ones. Moreover, patients with DLBCL expressing high levels of BCL6 mRNA and protein, have a favourable outcome when compared with those that do not express it (Lossos et al., 2004; Lossos et al., 2001; Ohno, 2006; Rosenwald et al., 2002), and this difference is maintained with modern treatments that include the anti-CD20 monoclonal antibody, Rituximab (Lenz et al., 2008). Although the molecular mechanisms underlying the causes of the different behaviour of these two subtypes of lymphomas are not completely understood yet, it has been recently proposed that different modulation of signalling pathways and their target genes might explain these differences (Sarosiek et al., 2009).

This thesis analyses the regulation of BCL6. Our work led me to focus on two mechanisms: p38 mitogen activated protein kinase (MAPK) signalling and the DNA binding factor CTCF. These are very different but both centre their effects on exon 1 of *BCL6*. In the following sections scientific background of BCR-ABL, p38 MAPK and CTCF will be outlined.

### 1.5. BCR-ABL involvement in BCL6 regulation

The *BCR-ABL* oncogene is the result of a balanced translocation between chromosomes 9 and 22 t(9;22), that gives rise to the Philadelphia chromosome (Ph). Attending to the breakpoint on the *BCR* gene on chromosome 22, three different isoforms of the BCR-ABL have been identified, which encode a p190, p210 or p230 fusion proteins. Almost all patients with chronic myeloid leukemia and around 20% of

patients with Ph<sup>+</sup> acute lymphoblastic leukemia have the p210 isoform, whereas the p190 is detected in the rest of Ph<sup>+</sup> ALL and may also be detected, at a low levels, in patients with CML (Hazlehurst et al; 2009).

The BCR-ABL oncoprotein plays key roles in the pathogenesis of the CML, and most of its effects are performed through the stimulation of pre-existing signalling pathways such as the RAS/RAF/MEK, JAK/STAT and PI3K/AKT signaling pathways (Steelman et al., 2004) (Figure 1.6). Hyperactivation of these pathways by BCR-ABL, leads to an hyperproliferative state.

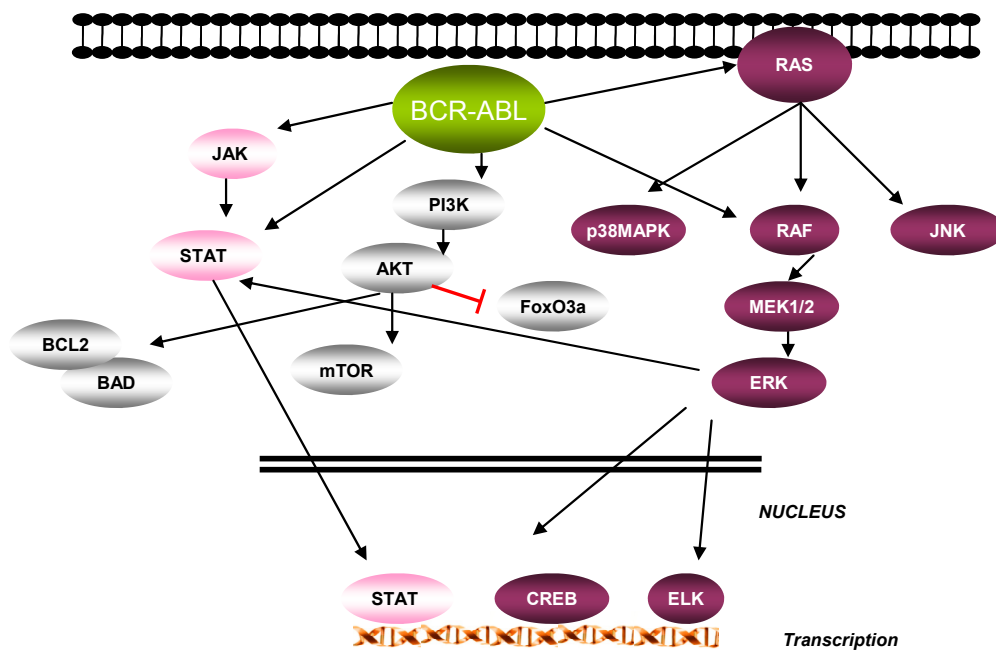


**Figure 1.6. BCR-ABL signaling pathways.** This figure shows a simplified diagram of the principle signaling pathways downstream BCR-ABL, including the PI3K/AKT(grey), JAK/STAT(pink) or the Ras/MEK/MAPK (deep red) pathways. (This figure was based on Steelman et al, 2004 and Deininger et al, 2000)

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The BCR-ABL oncoprotein activity can be successfully inhibited by the signal transduction inhibitor Imatinib mesylate (STI571), by directly binding to the site of tyrosine kinase activity, and preventing its activity (O'Brien and Deininger, 2003).

Expression profiling studies of genes transcriptionally regulated by BCR-ABL using differential display to compare the mRNA profile of the lymphoblastic CML cell line BV173 treated with Imatinib with that of untreated control cells (Deininger et al., 2000b), showed for the first time that BCL6 mRNA and protein were upregulated upon inhibition of BCR-ABL tyrosine kinase in these cells. In agreement with these results, Mattos et al (Fernandez de Mattos et al., 2004) have also demonstrated that BCL6 is upregulated in BV173 cell line on treatment with imatinib.

There is one implication of these works for the biology of BCL6: the BV173 cell line cultured in the presence and absence of imatinib might be a good model system for analysing the regulation of BCL6.

#### **1.6. MAPK involvement in BCL6 regulation**

Signalling pathways are required for the co-ordination of a vast number of biological processes, including cellular proliferation, differentiation and death. Mitogen activated protein kinases (MAPK) constitute a family of complex signal transduction pathways that plays key roles in a number of biological systems, including the haematopoietic and the immune system (Geest and Coffey, 2009; Huang et al., 2009; Wagner and Nebreda, 2009). Deregulation of these pathways have been observed in a number of diseases comprising several autoimmune diseases, like rheumatoid arthritis (Lin et al., 2009; Mbalaviele et al., 2006), systemic lupus erythematosus (Wong et al., 2009) and in different kinds of cancers such as breast cancer (Chen et al., 2009), pancreatic cancer (Adachi et al., 2010), hepatocellular carcinoma (Hsieh et al., 2007) or hematologic malignancies (Feng et al., 2009; Plataniias, 2003).

### 1.6.1. MAPK family

Six different subfamilies of MAPK have been identified: the extracellular signal-regulated kinases (ERK 1 and 2), c-Jun NH<sub>2</sub>-terminal kinases (JNK 1, 2 and 3), p38 (p38  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ), ERK3, ERK5 and ERK7 (Geest and Coffey, 2009). The JNK and the p38 pathway constitute the stress-activated pathways, since both are frequently activated by cellular stresses such as, ultraviolet irradiation, cytokines, heat shock proteins, or osmotic shock protein (Nebreda and Porras, 2000) (Figure 1.7). Parallel co-activation of the JNK and p38 pathway induced by the same stimuli has been reported in many cases.

Four different isoforms of p38 have been identified; the p38 $\alpha$ , p38 $\beta$ , p38 $\gamma$  and p38 $\delta$  each encoded by different genes; MAPK14, MAPK 11, MAPK 12 and MAPK 13, respectively. These p38 isoforms share only 60% identity and are differently expressed in the body. p38 $\alpha$  is highly and ubiquitously expressed, and most of the p38 functions described in the literature refer to this isoform. p38 $\beta$  can also be detected in a number of cell types, but usually at low levels, and its specific role is not well understood. In contrast, the  $\gamma$  and the  $\delta$  isoforms are only expressed in certain cell types where they perform very specific functions (Ono and Han, 2000; Wagner and Nebreda, 2009).

### 1.6.2. p38 functions in normal and cancer cells

Once activated, each MAPK phosphorylates different target proteins, usually in a cell type and context dependent manner, including transcription factors, tyrosine kinases and even components of the translational machinery, leading to a complex and sophisticated signalling network with important functions in different biological processes (Figure 1.7).

Some of the important transcription factor targets of p38 are p53, ETS like gene 1 (ELK1), the myocyte-specific enhancer factor 2 (MEF2), STAT 1 and 3, and C/EBP $\beta$ . (Cuenda and Rousseau, 2007; Nebreda and Porras, 2000; Ono and Han, 2000; Wagner

and Nebreda, 2009; Xu et al., 2003) Many protein kinases such as the p38-Regulated/Activated Kinase (PRAK) or certain MAPK-activated protein kinases or MAP kinase-interacting serine/threonine kinase 1 and 2 (MNK1 and MNK2) are also activated by p38 phosphorylation. In addition p38 has other targets substrates like the heat shock proteins (Cuenda and Rousseau, 2007; Nebreda and Porras, 2000; Ono and Han, 2000; Wagner and Nebreda, 2009; Xu et al., 2003) (Figures 1.7 and 1.8).

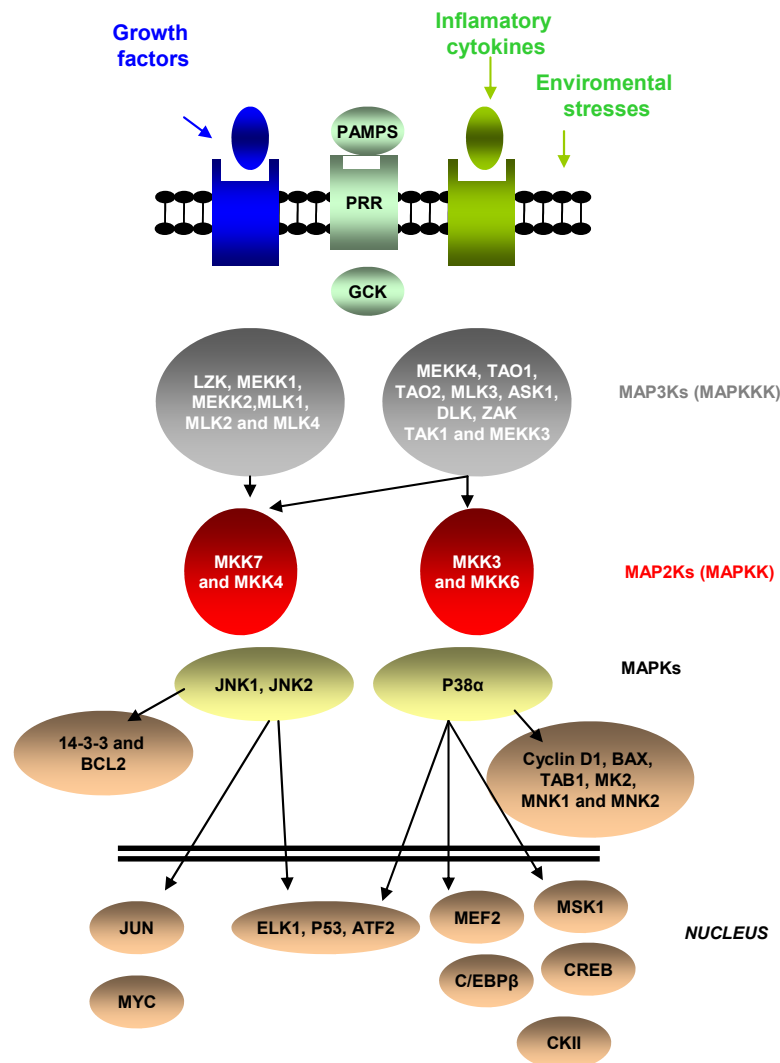


**Figure 1.7. Stress MAPK signalling pathways.** A number of different inflammatory cytokines, growth factors and environmental stresses, including genotoxic agents, toxins, ultraviolet irradiation among others, are responsible for the activation of the *stress*-MAPK signalling pathway. This figure illustrates the main MAP2K and MAP3K upstream activators of the p38 and JNK pathways. Also, some of the principal p38 and JNK targets are depicted. (This figure is based on Wagner et al, 2009 and Zhong et al, 2007)

p38 can either induce or inhibit cell proliferation and these opposing effects are likely to be tissue and context specific, and may also depend on both the level of kinase activity and interaction with co-stimulatory pathways (Wagner and Nebreda, 2009). Thus, p38 antiproliferative effects were demonstrated in a number of different cell types such as cardiomyocytes, hepatocytes, lung cells, fibroblasts but, on the other hand a p38 mediated proliferative effect has been shown in certain haematopoietic and cancer cells (Platanias, 2003; Wagner and Nebreda, 2009).

Interestingly, p38MAPK was shown to modulate cell cycle checkpoint controls, and it is therefore possible that deficient p38 function might lead to a hyperproliferative status (Bulavin et al., 2004). In fact, p38 was shown to inhibit cyclin D in different cell types (Lavoie et al., 1996). p38 is also capable of inducing apoptosis in a variety of models, but it can also be anti-apoptotic. The various p38 effects might, at least in some cases, be mediated through different p38 isoforms, since the p38 $\beta$  form seems to preferentially inhibit apoptosis whereas the p38 $\alpha$  form has been shown to have strong pro-apoptotic effects in different model systems (Kaiser et al., 2004; Nemoto et al., 1998; Silva et al., 2006).

### 1.6.3. *p38 and the immune system*

p38 plays key roles in the immune system (Huang et al., 2009). On the one hand, the activation of the germinal centre kinase GCK/p38-JNK pathways seems to be crucial for an adequate systemic inflammatory response (SIRS) (Zhong et al., 2009). The SIRS is a body response, frequently triggered by infections, characterized by organ failure and systemic inflammation, which is due to a massive release of proinflammatory cytokines. Pattern recognition receptors (PRR), which play a pivotal role in the innate immune response, are responsible for identifying invading pathogens, through their expression of molecular motifs called pathogen-associated molecular patterns (PAMPs). Stimulation of the PRR receptors initiates the SIRS response, through the activation of several signalling cascades.

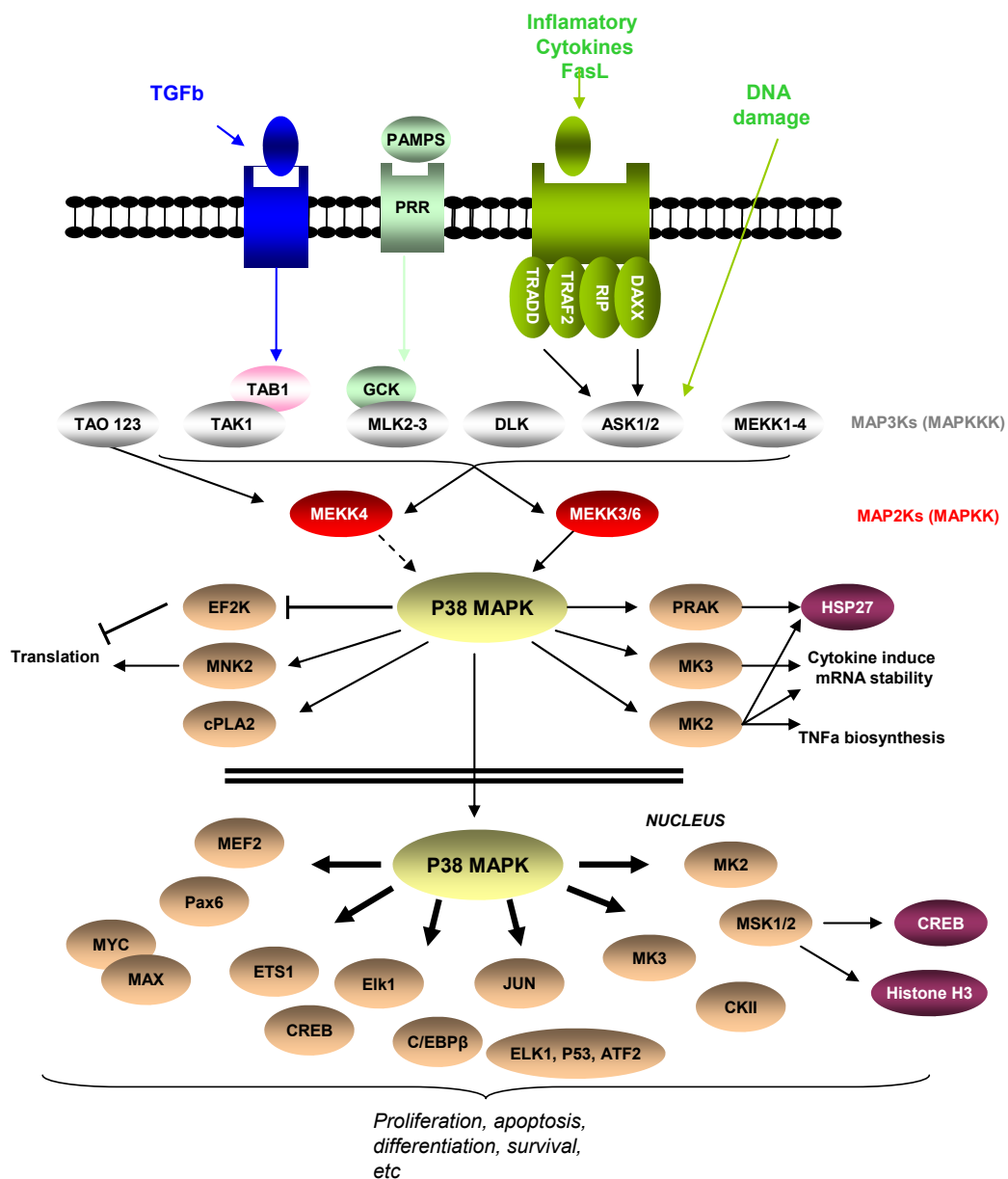


**Figure 1.8. p38 MAPK signaling pathway.** p38 MAPKs are implicated in a wide variety of biological processes including physiological responses to stress and inflammation, regulation of proliferation and differentiation. Studies performed in animal models have revealed that those functions are performed in a cell type and cell context dependent manner. A number of different inflammatory cytokines, growth factors and environmental stresses, including genotoxic agents, toxins, ultraviolet irradiation among others, are responsible for the activation of this pathway. This figure illustrates the principal p38 targets. (*This figure is based on Wagner et al, 2009 ; Zhong et al, 2007; Cuadrado and Nebreda; 2010; Cell signaling*)

Germinal Centre Kinase (GCK) is the best characterized member of a group of protein kinases, the GCKs, that has been implicated in a wide range of cellular functions such as proliferation, apoptosis and inflammation. GCK can be activated by certain PAMPs like the LPS, PGN or the bacteria flagellin. Once stimulated, the GCK recruits the MLK2 and MLK3, which in turn activates the JNK and p38 signaling pathway (Zhong and Kyriakis, 2007) (Figure 1.8), that contribute to the induction of cytokines, which play key roles in the systemic inflammatory response (Zhong et al., 2009)

The JNK and the p38 pathway were implicated in the production of several cytokines, such as INF- $\gamma$ , which is important for Th1 differentiation (Chi et al., 2004) or TNF $\alpha$ , IL-1 and IL-6, which are important mediators of the angiogenic and inflammatory response (Kumar et al., 2003). p38 regulation of the inflammatory response also involves other mechanisms, such as modulation of the NF- $\kappa$ B pathway (Wagner and Nebreda, 2009). Recently, p38 was also shown to be required for germinal centre formation, as mice bearing homozygous disruptions of the p38 activator MEKK1 have defective germinal centre formation and, as a consequence, deficient production of antibodies (Gallagher et al., 2007).

#### 1.6.4. p38 and cancer

p38 has an uncertain role in cancer pathogenesis; on the one hand it has been reported to act as a tumour suppressor, since it is able to induce apoptosis and stop proliferation in some models, but it is also involved in important oncogenic processes such as angiogenesis. It is becoming clear that the p38 role in cancer is completely depends on the tumour type (Wagner and Nebreda, 2009).

p38 role in angiogenesis and metastasis has been widely studied. p38 seems to be necessary for the invasiveness of different cell cancer types, such as the human hepatocellular carcinoma (Hsieh et al., 2007), head and neck squamous cell carcinoma (Junttila et al., 2007), glioma (Demuth et al., 2007) and lung (Matsuo et al., 2006). Matrix

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restructuration by cancer cells can be induced by p38 through induction of different metalloproteinases (Kumar et al., 2010; Zhou et al., 2007).

In contrast, p38 tumour suppressor activity has been demonstrated in both mice models and human cell experiments. Overexpression of the p38 inhibitor protein phosphatase 1D (PPM1D) led to an increased sensitivity to tumourigenesis, and this effect was reversed by enforced expression of a constitutively active form of p38 MAPK. In agreement, inhibition of mammary gland proliferation is observed upon PPM1D genetic silencing (Bulavin et al., 2004). Another line of evidence that supports a tumour suppressor function for p38, is based on a decrease in apoptosis and senescence processes, after inhibition of Gadd45 $\alpha$ /MEKK4, which is an upstream activator pathway of JNK and p38 MAPK (Tront et al., 2006). Also, loss of function studies performed in mice, have shown that the p38 $\alpha$  has a protective effect against Kras-associated lung carcinogenesis (Ventura et al., 2007). Finally, p38 can inhibit cell growth by controlling the expression of cyclins (Lavoie et al., 1996), or can induce a G2/M checkpoint by phosphorylating p53 (Bulavin et al., 1999; Huang et al., 1999; She et al., 2000), or by phosphorylating and inhibiting the phosphatase Cdc25B (Bulavin et al., 2001).

#### **1.6.5. p38 and haematological malignancies**

p38MAPK has been involved in the pathogenesis of different haematological malignancies including leukemias myelodysplastic syndromes and several lymphoproliferative diseases (Feng et al., 2009). Constitutive activation of the p38 targets, c-Jun and JunB has been reported in Hodgkin lymphoma cells, and might be responsible for the proliferation of those cells (Mathas et al., 2002). P38 appears to be required for the TNF $\alpha$  mediated proliferation of non-Hodgkin lymphoma cells (Kotlyarov et al., 1999). Also, p38MAPK pathway has been shown to play key roles in the transformation of follicular lymphomas to the more aggressive diffuse large B cell lymphoma, and indeed, inhibition of this pathway led to apoptosis and inhibition of cell

growth in a subset of t(14;18) cell lines. Moreover, lymphoma proliferation was also arrested in NOD-SCID mice upon treatment with p38 inhibitors (Elenitoba-Johnson et al., 2003; Lin et al., 2004).

p38 MAPK seems also to be involved in the proliferation of multiple myeloma cells induced by growth factors secreted by the marrow microenvironment, such as IL-6, IL-10, insulin-like growth factor 1 and granulocytic colony-stimulating factor (Nguyen et al., 2006). In fact, inhibition of p38 MAPK synergizes with the anti-myeloma effect of PS-341 (the proteasome inhibitor also called Bortezomib) (Hideshima et al., 2004).

p38MAPK has been shown to be inactivated in a certain lymphoma cell lines upon treatment with the monoclonal antibody anti-CD20 Rituximab, and in fact, inhibition of the p38 MAPK in those cells mimicked the Rituximab effect inducing apoptosis (Bonavida, 2007). On the contrary, the apoptosis induced by Rituximab in chronic lymphocytic leukemic cells requires activation of the p38MAPK pathway (Pedersen et al., 2002).

Many p38 MAPK inhibitors have been developed and even tested in phase I clinical trials. Unfortunately these drugs have severe side effects (Yong et al., 2009). Also, as p38 has been shown to have ambivalent roles in carcinogenesis, it is still a matter of concern whether the use of these drugs might be beneficial for some cancer types, but not for others (Wagner and Nebreda, 2009).

### **1.7. CTCF as a potential regulator of BCL6**

The CCCTC-binding factor (CTCF) has been implicated in various regulatory functions. It is a well established chromatin regulator that plays key roles in inducing and supporting chromatin interactions. Over the last decade a substantial amount of information related to the CTCF functions have been gathered together (Ohlsson et al.,

2010b; Phillips and Corces, 2009). It is involved in epigenetic regulation, which is not only crucial for normal cell development but is also implicated in many cancer processes. The features and functions of CTCF will be summarized in the following sections, paying special attention to its involvement in lymphoid cell regulation, since we have found that CTCF is a potential regulator of the *BCL6* gene.

### 1.7.1. CTCF structural and functional domains

The CTCF protein consists of several domains: the N-terminal domain, a central DNA binding domain composed of eleven zinc fingers, and a C-terminal domain (Figure 1.9.).

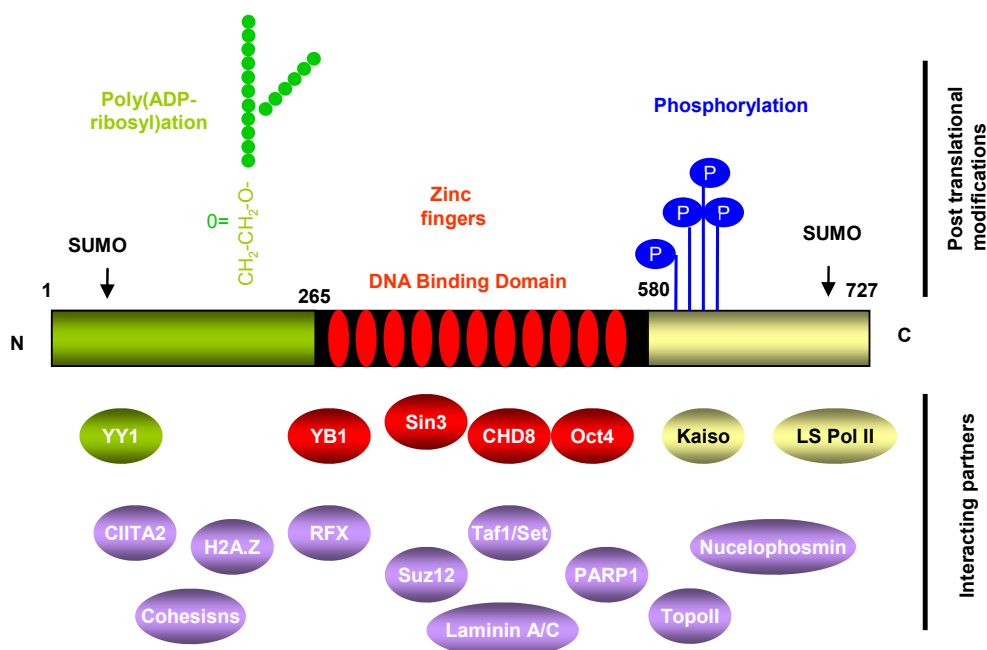


**Figure 1.9. CTCF structural domains and interacting partners.** The figure shows the N-terminal domain (spanning from amino acid 1 to 265), the central domain, which consists of 11 zinc fingers (amino acids 268 to 580) and the C-terminal domain (580-727). The main post-translational modifications are also depicted including, phosphorylation, SUMOylation and poly(ADP-ribosylation). The CTCF partners that interact with known CTCF domains are shown with the same coloured code. Other interacting proteins are shown in mauve, as the interacting domains are not mapped on the CTCF protein (*This figure is based on Ohlsson et al, 2010b*)

The central zinc finger domain is responsible for the recognition and interaction with DNA. In fact, different combinations of individual zinc fingers enable CTCF to bind to many different DNA sequences (Filippova et al., 1996; Jothi et al., 2008). This particular characteristic may explain why CTCF has been implicated in a number of different functions such as cell proliferation, differentiation, apoptosis, transcriptional repression/activation, chromatin insulation, imprinting and chromosome X inactivation (Ohlsson et al., 2010b). The CTCF zinc finger domain interacts with DNA, but it also interacts with other nuclear proteins (Ohlsson et al., 2001). The zinc finger domain together with sequences located on both the N and C terminal domains are considered to be essential for CTCF transcriptional repression (Lutz et al., 2000). The N- and C-terminal regions do not share significant similarity to any other protein and do not form classical domains but unstructured regions (Martinez and Miranda, 2010).

#### ***1.7.2. CTCF postranslational modifications and interacting partners***

CTCF functions are known to be modulated through several post-translational modifications and by the interaction with different proteins (Figure 1.9).

The (S(604)KKEDS(609)S(610)DS(612)E (SKKEDSSDSE) motif located at the CTCF C-terminal domain is known to be phosphorylated at serine-residues, and this modification has been related to reduced transcriptional repression by CTCF (Klenova et al., 2001). Furthermore, mutations of these serines overcome CTCF mediated growth arrest. Phosphorylation of these residues has been attributed to the protein kinase CK2 (formerly casein kinase II) (Klenova et al., 2001), and this kinase has been reported to be regulated by the p38 MAPK (Sayed et al., 2000).

Another relevant CTCF modification is SUMOylation, which occurs at both the N and C-terminus of CTCF. This modification does not affect CTCF DNA binding ability but has been shown to be important for CTCF repressive function at the c-MYC promoter (MacPherson et al., 2009).

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One of the most interesting modifications that the CTCF protein undergoes is poly(ADP-ribosyl)ation by poly(ADP-ribose) polymerase 1 (PARP1) (Farrar et al., 2010; Klenova and Ohlsson, 2005; Yu et al., 2004). This enzyme plays a key role in different cell processes such as DNA repair, cell proliferation, and genomic instability. CTCF has been shown to directly interact with, and possibly activate, PARP-1 (Farrar et al., 2010; Guastafierro et al., 2008). Poly(ADP-ribosyl)ation regulates CTCF insulator and chromatin barrier functions (Yu et al 2004; Witcher and Emerson 2009) (see below for further details).

CTCF is known to interact with a great number of proteins involved in many different functions, such as transcription enzymes (RNA polymerase II large subunit), transcriptional regulatory factors (Oct4, YY1, etc), genome integrity mediators (PARP-1), nuclear architectural proteins (cohesins, nucleophosmin/B23, etc.) and chromatin constituents, like the variant histone H2A.Z (for review see (Ohlsson et al., 2010b; Zlatanova and Caiafa, 2009b). CTCF interaction partners are depicted in [Figure 1.9](#).

### ***1.7.3. CTCF intracellular localization and expression***

CTCF is a transcriptional regulator that is highly conserved from *Drosophila* to human and ubiquitously expressed (Filippova et al., 1998; Klenova et al., 1993).

CTCF is located in the nucleus (Klenova et al., 1993) and its distribution during the cell cycle is dynamic (Burke et al., 2005; Klenova et al., 1998). The different CTCF functions seem to depend on specific localization in different nuclear compartments, such as the nuclear matrix (Dunn et al., 2003), nucleolar periphery (Yusufzai and Felsenfeld, 2004; Yusufzai et al., 2004) , nucleolus (Torrano et al., 2006) and nuclear membrane (Guelen et al., 2008).

Studies performed on hematopoietic models have shown that in the myeloid lineage CTCF expression progressively decreases during the differentiation process (Delgado et al., 1999). Work in our laboratory demonstrated the CTCF nucleolar

localization in several experimental model systems. In myeloid cells, localization of CTCF to the nucleolus was associated with cell growth inhibition and differentiation (Torrano et al., 2006). Expression of CTCF has also been shown to vary during normal murine T- cell differentiation, with the highest levels being found in immature single positive T cells (Heath et al., 2008).

#### **1.7.4. CTCF molecular functions**

CTCF is an important regulator that plays key roles in global chromatin structure and, therefore, in the organization of the entire genome (Ohlsson et al., 2010b). Over the past two decades progress has been made in our knowledge of precise mechanisms through which CTCF regulates chromatin structure and molecular interactions. CTCF can either function as a transcriptional repressor or activator and is capable of acting as an enhancer blocking protein or as a barrier element. These molecular functions are summarized below.

##### ***a. Transcriptional repression or activation***

The first function described for CTCF was the transcriptional repression of the oncogene *c-MYC* both in humans and in chicken (Filippova et al., 1996; Klenova et al., 1993; Lobanenko et al., 1990) (Figure 1.10). Since then, a number of other genes with different functions have been also shown to be repressed by CTCF (Filippova, 2008), such as the chicken lysozyme gene (Burcin et al., 1997), the proximal exonic region of the hTERT gene (Renaud et al., 2005) or a silencer region in the HLA-DRB1 gene (Arnold et al., 2000). However, CTCF has also been involved in the transcriptional upregulation of different genes like the amyloid precursor protein (*APP*) gene (Vostrov and Quitschke, 1997; Yang et al., 1999) (Figure 1.10).

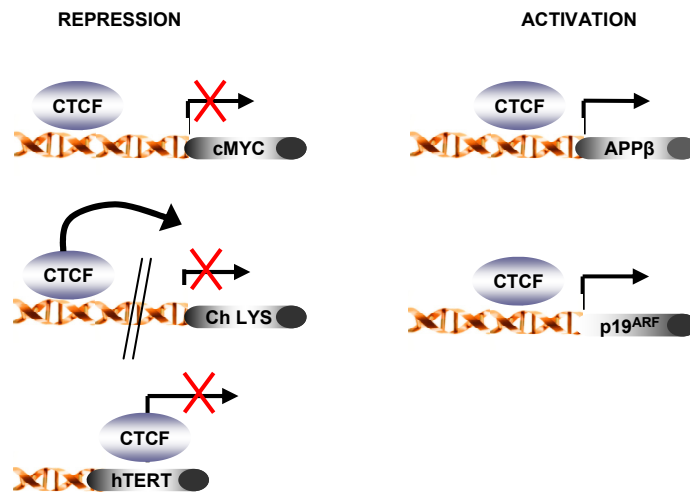


**Figure 1.10. CTCF as a “classical” transcriptional factor.** CTCF has been shown to mediate both repression and activation of transcription of a number of genes. Left panel shows some genes negatively regulated by CTCF including the *cMYC*, the *Ch-lysozyme* and the *hTERT*. The right panel, depicts some genes activated by CTCF, such as *APPβ* and *p19<sup>ARF</sup>*

CTCF has been found to regulate transcription of several genes implicated in the cell cycle control including *p19<sup>ARF</sup>*, the human retinoblastoma (RB) gene, PIM1, the Polo-Kinase, *p16<sup>INK4a</sup>*, *p27<sup>KIP1</sup>* among others (De La Rosa-Velazquez et al., 2007; Filippova, 2008; Klenova et al., 2002; Recillas-Targa et al., 2006) .

#### **b. Chromatin insulation**

Chromatin insulators can be defined as DNA regulatory sequences that establish boundaries between genes. They control gene interactions and prevent inappropriate regulatory influences between neighboring genes. Insulators may act either as enhancer blockers or as barrier elements (Gaszner and Felsenfeld, 2006; Wallace and Felsenfeld, 2007). The enhancer blocking elements are usually located between promoters and enhancers, limiting the interactions between them and, therefore restricting the enhancer’s activity. The barrier elements act mainly as

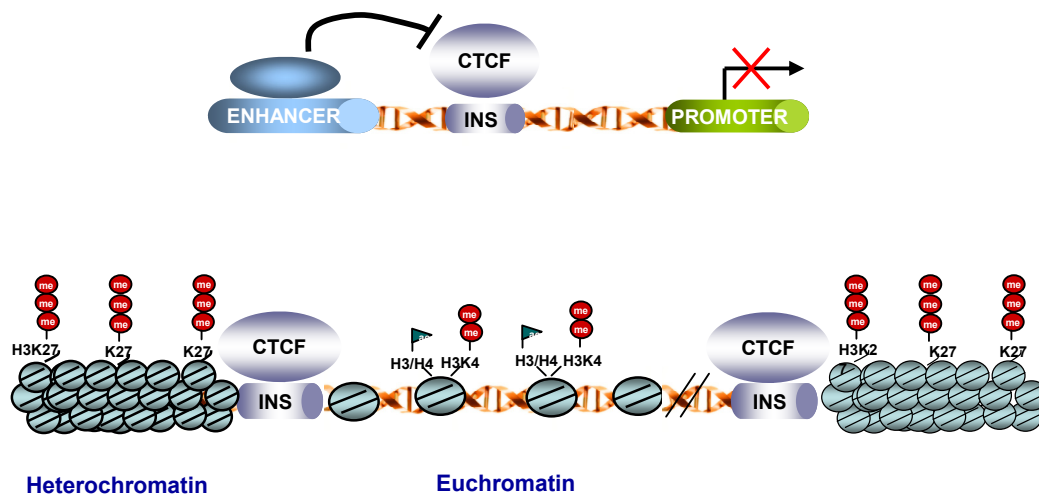


**Figure 1.11. CTCF insulator activity.** DNA insulators are represented by grey cylinders,. Nucleosomes are shown as blue circles. The enhancer blocking function is illustrated in the top panel. Enhancer blockers are usually located between promoters and enhancers. The bottom panel depicts the CTCF barrier function. The barrier elements inhibit the spreading of inactive chromatin (heterochromatin). CTCF is the only insulator binding protein described so far in vertebrates.

boundaries impairing the spreading of heterochromatin into euchromatin, or sometimes restraining the advance of the methylation front. Insulators may harbor both properties (Gaszner and Felsenfeld, 2006; Recillas-Targa et al., 2006).

Several lines of evidence have shown that CTCF is required for both enhancer blocking and barrier function (Figure 1.11). Studies performed on the chicken beta-globin cluster demonstrated the enhancer blocking activity of CTCF. Other insulator regions at the mouse and human beta globin locus were later on identified (Bell et al., 1999; Farrell et al., 2002; Saitoh et al., 2000). Subsequently, CTCF was found to mediate enhancer blocking activity at the Igf2/H19 locus. Moreover, CTCF plays key roles in the epigenetic modifications that are required for the genomic imprinting of that locus (Bell and Felsenfeld, 2000; Hark et al., 2000; Kanduri et al., 2000) reviewed in (Filippova, 2008). This function is regulated through DNA methylation which interferes with CTCF binding, eliminating CTCF-mediated enhancer blocking activity. More

recently, genome wide studies showed a global insulation role for CTCF (reviewed in (Ohlsson et al., 2010a; Phillips and Corces, 2009)).

CTCF has been shown to interact with different chromatin barrier regions, acting as a physical boundary and leading to a variety of effects which are mainly context-dependent. CTCF delimits chromatin domains with opposite functions. In support for this notion, a comparison between the CTCF binding sites (CTS) of CD4<sup>+</sup>, HeLa and Jurkatt T cells showed that CTCF was preferentially bound to boundary regions between active and repressive chromatin domains, marked by histone H2A lysine acetylation and histone H3 lysine 27 trimethylation (Cuddapah et al., 2009). Also, the variant histone H2A.Z was shown to co-localize with CTCF genome wide (Barski et al., 2007). Thus, CTCF seems to be acting as a physical barrier controlling the histone modifications and therefore the chromatin status, and this is made in a cell-dependent manner (Barski et al., 2007; Bartkuhn and Renkawitz, 2008; Cuddapah et al., 2009). Finally, CTCF was also shown to prevent spreading of DNA methylation (Figure 1.12) and maintain DNA methylation free zones. By this mechanism, CTCF protects promoters from epigenetic silencing (Filippova, 2008; Recillas-Targa et al., 2006).

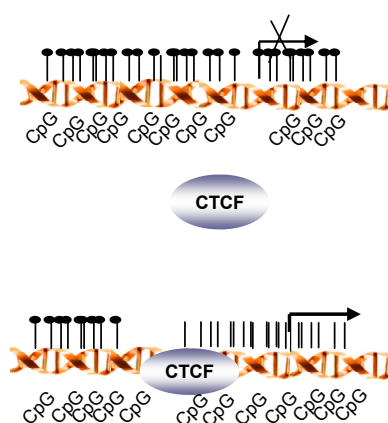


**Figure 1.12. CTCF regulation of CpG methylation.** In some genes, CpG methylation prevents CTCF binding (top panel). CTCF has been demonstrated to block the progression of the CpG methylation front, sustaining methylation free zones (bottom panel). Black circles indicate methylated CpGs, whereas black bars show unmethylated CpGs (Modified from Recillas-Targa et al, 2006 and Filippova et al, 2008)

The study of the CTCF functions has provided new insights on the mechanisms that control the genomic organization. CTCF seems to be essential in the long distance chromosomal interactions some times even between different chromosomes, through the formation and maybe stabilization of loops and hubs. These structures will then control the access of the transcription machinery (Figure 1.13) (Kurukuti et al., 2006; Phillips and Corces, 2009; Splinter et al., 2006; Yoon et al., 2007; Zlatanova and Caiafa, 2009a)

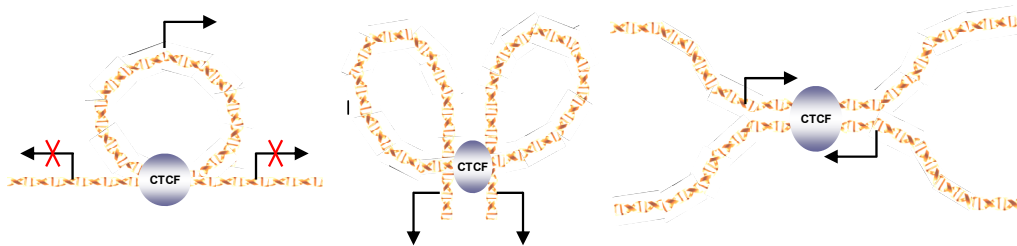


**Figure 1.13. CTCF insulation via formation of chromatin loops.** This figure shows three examples of how CTCF intervenes in the genomic organization by the formation of loops/hubs. CTCF is capable of inducing loop domains that isolate genes from the effects of other close genes (left figure). CTCF is also able to induce the formation of a chromatin hub, where regulatory elements or coregulated genes are brought close together (middle figure). CTCF appears to mediate interchromosomal interactions by bringing nearer genes or/and regulatory regions located on different chromosomes (right panel) (*Modified from Williams and Flavell; 2008*)

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#### ***1.7.5. CTCF role in cell proliferation, differentiation and apoptosis***

Several lines of evidence indicate that CTCF blocks cell proliferation, probably through the regulation of genes involved in cell cycle control (Rasko et al., 2001). Indeed, in a variety of cell model systems including the Burkitt lymphoma cell line Raji, enforced CTCF expression lead on a substantial inhibition of the cell proliferation rate (Rasko et al., 2001; Torrano et al., 2005). It is interesting that CTCF effect on proliferation does not depend on the stage of the cell cycle, being able to arrest cells at any stage of cell cycle (Rasko et al., 2001). This anti-proliferative function is known to be dependent on the phosphorylation of CTCF at the C-terminal domains (El-Kady and Klenova, 2005; Klenova et al., 2001). In lymphoid cells CTCF is also involved in the regulation of cell cycle progression. Cell growth inhibition induced by BCR stimulation in the WEHI-231 mouse B-cell line was mediated through CTCF (Qi et al., 2003). In fact, enhanced expression on CTCF in these cells reproduced the effects of the BCR stimulation (Qi et al., 2003). There may also be a T-cell role for CTCF. Studies performed in mice have shown that CTCF is essential for the proliferation of alpha-beta T cells (Heath et al., 2008). Thymocytes from CTCF knockout mice were smaller than those of wild-type animals and blocked in the cell cycle. Thus CTCF is a crucial positive regulator of cell growth and proliferation in alpha-beta T cells (Heath et al., 2008). These data on T cells are opposite to those described in B cells which reinforce the idea that CTCF function is context dependent.

Several studies have underlined the importance of CTCF on the differentiation processes of different cell types. Our group has demonstrated that CTCF specifically induce the erythroid differentiation in a pluripotent cell line (Torrano et al., 2005). CTCF is also essential for the T cells differentiation in the thymus (Heath et al., 2008; Ribeiro de Almeida et al., 2009), and in contrast enforced expression of CTCF has been associated to an impaired differentiation process of dendritic cells (Koesters et al., 2007). CTCF therefore, seems able to both enhance and inhibit proliferation depending on the cellular context.

CTCF also has cell context dependent effects on apoptosis. CTCF was shown to cause apoptosis in the WEHI-231 B cell line (Qi et al., 2003) and also in a subset of dendritic cells (Koesters et al., 2007). However, decreased expression of CTCF was followed by apoptosis, and high levels of CTCF in breast cancer cells and tumours were found associated with resistance to apoptosis (Docquier et al., 2005)

#### **1.7.6. Genome wide analysis of CTCF binding sites.**

Different approaches that included chromatin immunoprecipitation, combined with microarray hybridization (ChIP-chip), high throughput sequencing (ChIP-seq) and computational analysis have made it possible to identify between ~14,000 to ~ 40,000 CTCF binding sites (CTS), depending on both the cell type and the organism analyzed (Barski et al., 2007; Boyle et al., 2008; Cuddapah et al., 2009; Chen et al., 2008; Jothi et al., 2008; Kim et al., 2007; Xi et al., 2007; Xie et al., 2007). Remarkably, CTCF binding sites are not only located promoter regions (20%) but also in introns (22%), exons (12%) and in intergenic regions (46%) (Kim et al., 2007) (Figure 1.14.). As a result of these studies, consensus motifs for the CTS have been identified in a variety of organisms (Figure 1.14.) (recently reviewed in (Ohlsson et al., 2010b; Phillips and Corces, 2009)

Over the last few decades, it has become clear that CTCF plays key roles in the distribution, organization and regulation of the entire genome. In agreement with this idea, CTS have been found across the human genome linker regions between nucleosomes (Fu et al., 2008). Linker regions are known to be involved in the positioning of the nucleosomes, suggesting a role for CTCF in nucleosome repositioning (reviewed in (Ohlsson et al., 2010b). The exact role of CTCF in this regard is not completely understood yet, but one possibility is that CTCF modulates nucleosome movement through its interaction with chromatin remodelers such as CHD8 (Ishihara et al., 2006).

a



b

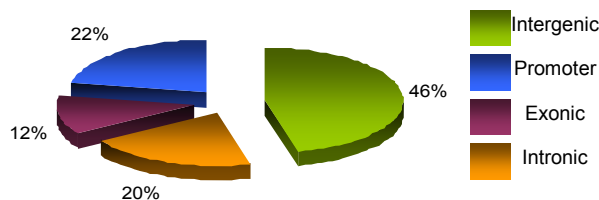


**Figure 1.14. Genome wide analysis of CTCF binding sites.** (a) Illustration of a CTCF consensus sequence binding motif generated from ChIP on chip analysis (Kim et al, 2007). The size of each letter illustrates the relative frequency of a specific nucleotide at each position. Other CTCF consensus sequences from ChIP seq data are reviewed in Ohlsson et al, 2010b. (b) The pie chart shows the frequency of location of the CTCF binding sites within exons, introns, promoters (within 2.5 Kb of transcriptional start sites) as well as in intergenic regions. (This figure is based on Kim et al, 2007)

Complexes with other proteins are required for the three dimensional organization of the genome mediated by CTCF (Ohlsson et al., 2010b). It is known that the location of each gene within chromosome territories in the nucleus is not random, and this is important for gene regulation and function (Takizawa et al., 2008). Various studies (Kurukuti et al., 2006; Splinter et al., 2006) have suggested that these interactions might be stabilized through CTCF-cohesin complexes. Cohesin is a multisubunit protein complex that mediates sister chromatid cohesion during mitosis, facilitates the repair of damaged DNA and has important, but not well established functions in the control of gene expression (Peters et al., 2008). In several independent studies cohesin proteins were found to co-localize extensively with CTCF (Parelho et al., 2008; Rubio et al., 2008; Stedman et al., 2008; Wendt et al., 2008) (reviewed in (Wendt and Peters, 2009). About 1,800 cohesin/CTCF sites were identified in immature lymphocytes (B3 pre-B cells) and thymocytes (Parelho et al., 2008).

CTCF-cohesin complexes have been proposed to play a regulatory role in CTCF-mediated loop formation in the immunoglobulin heavy chain (Igh) locus, and this process is believed to be necessary for V(D)J gene recombination to produce immunoglobulin H- and L-chains. CTCF *in vivo* binding to the 3' regulatory region of the IgH locus was demonstrated in B cells at different stages of differentiation (Garrett et al., 2005). By ChIP-chip analysis it was found more than 50 CTCF-binding sites throughout Igh locus in pro-B and pre-B cells (Degner et al., 2009). In contrast, certain cohesin subunits colocalized with CTCF through the IgH locus preferentially in pro-B cells. Lineage and stage-specific cohesin subunits recruitment to CTCF sites during B lymphocyte development was found, suggesting that the differential binding of cohesins to CTCF sites may promote multiple loop formation and thus effective V(D)J recombination within the locus (Degner et al., 2009).

#### 1.7.7. CTCF in human cancer

Over the last decade a number of mechanisms explaining the CTCF participation in malignant cell transformation have been considered.

Firstly, the human *CTCF* gene is located on the long arm of chromosome 16 (16q22.1), a chromosomal region frequently associated with loss of heterozygosity in breast and prostate cancer and Wilm's tumor (Klenova et al., 2002). Interestingly, CTCF has been considered essential for the control of numerous cancer associated genes such as *c-myc*, *p53*, *p16*, *RB*, *p19 ARF*, *BRCA-1*, *TERT*, and *IGF2* (De La Rosa-Velazquez et al., 2007; Filippova, 2008; Klenova et al., 2002; Renaud et al., 2005; Soto-Reyes and Recillas-Targa, 2010; Witcher and Emerson, 2009). Loss of CTCF binding to the regulatory regions of certain tumour suppressor genes such as *RB* or the *p16* genes has been shown to induce an aberrant methylation and cause reduced expression of those genes essential for the control of the cell cycle and proliferation (De La Rosa-

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Velazquez et al., 2007; Witcher and Emerson, 2009). CTCF involvement in the epigenetic regulation of the human p53 gene has been recently reported. In this case CTCF has the ability to maintain the p53 promoter in a local chromatin conformation by protecting this region against suppressor histone marks (Soto-Reyes and Recillas-Targa, 2010). There are other mechanisms altering CTCF function. Several point mutations at the zinc finger domain of CTCF have been identified in breast, prostate and Wilm's tumours, which interfere with the recognition of specific DNA targets. For example, the H345R substitution was predominately found in prostate cancer whereas the R448Q mutation was detected in Wilm's tumor (Filippova et al., 2002; Klenova et al., 2002). Also, loss of imprinting due to aberrant methylation of CTS in the IGF2/H19 locus has been implicated in Wilm's tumour and colorectal cancer (Filippova, 2008). Mutations of the target sequence can impede CTCF binding. A single mutation at one of the CTCF binding sites at the H19 ICR locus was sufficient for interfering with CTCF function, despite the presence of other CTS at the same locus (Pant et al., 2004). This mutation might explain why the H19ICR locus has an aberrant methylation pattern in a variety of cancers (Tycko, 1999).

Abnormal CTCF localization in the cytoplasm might be another mechanism of tumorigenesis. Cytoplasmic CTCF was detected on an elevated percentage of patients with breast cancer (Rakha et al., 2004). It is not yet known how the abnormal localization of CTCF might be implicated in the tumor progression or development, but it may be associated with abnormal regulation of tumor suppressor genes (Recillas-Targa et al., 2006).

Two different CTCF forms can be identified depending on the poly(ADP-ribosyl)ation status; CTCF-180 kDa is poly(ADP-ribosyl)ated and is the predominant form detected in normal breast tissues (Docquier et al., 2009). In the contrary the CTCF-130 kDa was the only form identified in breast tumor tissues. The CTCF-130 kDa form was predominantly detected in proliferating cells (S-Phase) and CTCF-180 kDa in non-

proliferating cells. In fact the poly(ADP-ribosyl)ated form of CTCF was directly demonstrated to be impeding cell division (Docquier et al., 2009; Farrar et al., 2010).

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## 1.8. Aims

The signalling pathways regulating BCL6 are not known in detail, but are very important because tight control of BCL6 expression is required for normal immunity. Deregulated expression occurs in a subgroup of lymphomas despite the presence of an unarranged *BCL6* locus and one cause of this is disruption of cis-acting control elements in exon 1. The main goal of this project was to find out how signalling pathways controlling BCL6 expression are integrated. For this purpose we established the following aims:

1. To investigate BCL6 regulation in lymphoid Ph+ cell lines. Professor Melo's group at the Hammersmith Hospital whilst investigating the effects of the tyrosine kinase inhibitor, Imatinib, in chronic myeloid leukaemia found that BCL-6 mRNA and protein were upregulated upon inhibition of Bcr-Abl tyrosine kinase in different lymphoid blast crisis cell lines. Therefore, we will use these lymphoid blast crisis cell lines cultured in the presence and absence of imatinib to investigate the regulation of BCL6.

2. To extend our results on BCL6 regulation to Burkitt lymphoma cell. In order to demonstrate relevance to the germinal centre biology, we will use human Burkitt lymphoma cell lines (Ramos and DG75), which are representative of the germinal centre stage of development, to confirm and extend our findings with the BCR-ABL expressing lymphoid cell lines.

3. To explore the BCL6 regulation by MAPK and BCR signalling pathways. We will also investigate how the MAPK signalling pathways which have been shown to be important for the regulation of BCL6 are modulated by the CD40 and the BCR signalling pathways, which are known to play essential roles in the germinal centre reaction.

4. To determine the involvement of CTCF in BCL6 regulation. Using bioinformatics we discovered a CTCF binding site in *BCL6* exon 1. In order to determine the biological relevance of CTCF in the BCL6 regulation we will investigate CTCF in vitro and in vivo binding to BCL6 in several BCL6 positive and negative cell lines. We will also characterize the pattern of expression of CTCF in human lymphoma cell lines. As CTCF has been implicated in the epigenetic regulation of several genes we will finally study the epigenetic regulation of BCL6 by CTCF.



## **CHAPTER 2**

### ***Material and Methods***



## CHAPTER 2. Material and methods

### 2.1. Cell culture methods

#### 2.1.1 *Cell lines culture*

Cell lines were grown in either RPMI-1640 or DMEM (Lonza) supplemented with 10 to 20% fetal calf serum (Lonza), 2% L-glutamine and 5% penicillin/streptomycin and maintained at 37° C in a humidified 5% CO<sub>2</sub> atmosphere. Cells were either purchased or donated by groups within the Department of Haematology. [Table 2.1](#) provides a list of cell lines used in this study. Cells were maintained at the appropriate density (see table 2.1)

#### 2.1.2 *Primary cells*

Frozen mononuclear cells from a patient with lymphoid blast crisis of chronic myeloid leukaemia (CML) were kindly donated by Hospital Marques de Valdecilla (Haematology Department; Santander; Spain). Cells were thawed out using a thawing solution (DMEM medium with DNase I (Calbiochem) and preservative free heparin (Monoparin, CP Pharmaceutical Ltd)). 10<sup>7</sup> cells were cultured in 1 ml of FCS-free-medium (Iscove's Modified Dulbecco's medium (Sigma, St Louis, MO), 1% penicillin-streptomycin-l-glutamine and serum substitute (BIT:Stem Cell technologies)) and kept in culture overnight before performing the experiment (Hamilton et al., 2006).

#### 2.1.3. *Cell counting, cell cycle and viability.*

**Cell counting** was performed using a haemocytometer. For ChiP assays, where a very accurate measure was required in order to obtain reproducible results, we used

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the Countess® Automated Cell Counter (Invitrogen) following the manufacturer's instructions.

**Cell viability** was assessed by the dye exclusion test with Trypan Blue staining (Sigma).

**Cell cycle:** Cell cycle measurement was carried out using propidium iodide staining.  $1 \times 10^6$  cells were harvested, and resuspended in buffer (0,1%NP-40; 0,5mg/ml RNase A; PBS) and incubated at room temperature for 30 minutes. Propidium iodide (0,05 x mg/ml) was added and cells were immediately analyzed by flow cytometry using the FACs Canto II. The analysis was performed in linear scale for cell cycle experiments.

## 2.2. Purification of primary lymphoma cells using a FAC-Sorter

Lymph nodes biopsies of patients with lymphomas were provided by the Haematology Department of the Hospital Universitario Marqués de Valdecilla after diagnosis was made. Informed consent was obtained from every patient and approval to perform this study was obtained from the local research ethics committee. Single cell suspensions were obtained from the lymph node by physical disruption using a 20 gauge (0.9mm) needle attached to a sterile plastic syringe. These cells were then separated in the FACS Aria (BD) using the FCS/SSC parameters and two to four specific monoclonal antibodies against surface markers specific of each type of lymphoma. The separated cells were then used to obtain genomic DNA.

**Table 2.1.** Phenotype, culture conditions, source and references of cell lines used in this study.

| CELL LINE NAME | PHENOTYPE                                           | CULTURE MEDIUM | SOURCE                                     | REFERENCES                                    |
|----------------|-----------------------------------------------------|----------------|--------------------------------------------|-----------------------------------------------|
| RAMOS          | Human Burkitt's Lymphoma VEB negative               | RPMI-10F       |                                            | Ben Bassat et al, 1997                        |
| DG75           | Human Burkitt's Lymphoma VEB negative               | RPMI-10F       |                                            | Gasparini et al, 2005<br>Martinez et al, 1993 |
| BJAB           | Human Burkitt's Lymphoma VEB negative               | RPMI-10F       |                                            | Klein et al, 1975                             |
| RAJI           | Human Burkitt's Lymphoma VEB positive               | RPMI-10F       | Pr. Leon U. Cantabria                      | Epstein et al, 1966;<br>Epstein et al, 1965   |
| MUTU III       | Human Burkitt's Lymphoma VEB negative               | RPMI-10F       | Dr. Noémi Nagy (MTC) Karolinska Institute. | Nagy et al, 2000                              |
| TOLEDO         | DLBCL                                               | RPMI-20F       | Dr. A Piris CNIO (Madrid)                  | Martinez et al, 1993                          |
| LYO3           | DLBCL                                               | RPMI-20F       | Dr. A Piris CNIO (Madrid)                  | Martinez et al, 1993                          |
| K562           | CML Erythroleukaemia BC (p210 BCR-ABL)              | RPMI-10F       | Pr. JV Melo University of Adelaide         | Koeffler et al, 1980;<br>Klein et al, 1976    |
| BV173          | CML Lymphoid BC (p210 BCR-ABL)                      | RPMI-10F       | Pr. JV Melo U of Adelaide                  | Pegoraro et al, 1983                          |
| Nalm-1         | CML Lymphoid BC (p210 BCR-ABL)                      | RPMI-20F       |                                            |                                               |
| Z119           | PRE-B-ALL Ph+ (p190 BCR-ABL)                        | RPMI-20F       | Pr. JV Melo U of Adelaide                  | Estrov et al, 1996                            |
| Z-33           | PRE-B-ALL Ph+ (p190 BCR-ABL)                        | RPMI-20F       | Pr. JV Melo U of Adelaide                  |                                               |
| TOM-1          | PRE-B-ALL Ph+(p190 BCR-ABL)                         | RPMI-20F       | Pr. JV Melo U of Adelaide                  | Blood et al, 1987                             |
| MV(4;11)       | Biphenotypic B-myelomonocytic leukemia with t(4;11) | RPMI-10F       | Pr. JV Melo U of Adelaide                  | Lange et al, 1987                             |
| HEK293T        | Human EmbrYonic Kidney                              | DMEM-10F       | *                                          | Pear et al; 1993                              |

\*These cell lines were already in the laboratory.

Abbreviations:

F: Fetal Calf serum

EVB: Epstein Barr Virus

Burkitt's: Lymphoma: B cell Lymphoma

DLBCL: Diffuse Large B Cell Lymphoma

BC CML: Blast Crisis of Chronic Myeloid Leukaemia

ALL Ph+: Acute Lymphoblastic Leukaemia with Philadelphia chromosome (which is the result of a balanced translocation between chromosomes 9 and 22)

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### **2.3. Fluorescence In Situ Hybridization (FISH)**

Cultured cell lines or primary cell lines were firstly washed with PBS. The pellet is then carefully resuspended by vortexing adding drop by drop up to 8 ml of pre-warmed 0,075 M KCl. Samples were incubated for 30 minutes at 37°C, centrifuged and carefully resuspended by vortexing and adding drop by drop up to 8 ml of cold Carnoy's solution (75% methanol and 25% of glacial acetic acid(45%)). Between two and five additional washes with Carnoy's were performed in order to clear all debris. Samples were then incubated on ice for 10 minutes and a drop of the sample was placed on a slide and allowed to air dry. Five µl of a prediluted- probe (LSI *BCL6* (3q27) 32-191016; VYSIS) was put directly on the slide and covered with a cover-slide. The slide was placed in a block at 85°C for 3 minutes, covered with parafilm and inserted on a humid box that was placed on an incubator at 37°C for 18 hours. The day after, the parafilm was removed, the cover slip was carefully removed by incubating it for 4 minutes in a Coplin Jar containing prewarmed (72°C) hybridization solution (2XSSC (NaCl 3M y NaHCO<sub>3</sub> 0.3M)/0.3% NP-40). The slide was then placed in another Coplin Jar containing hybridization solution and washed for 4 minutes. Slides were then allowed to air dry completely. 10 µl of Vectashield (Vector Laboratories) were added and a cover slip was placed on top. Fluorescence signal was analyzed using a Fluorescent Microscope (Nikon Eclipse 50i).

### **2.4. Drug treatments**

#### ***2.4.1. Signaling pathways inhibitors***

The BCR-ABL cell lines, Z119 and BV173, and the germinal centre cell lines, Ramos and DG75, and primary cells were incubated with inhibitors of either the

MEK/MAPK or of the PI3K/AKT pathways at the concentrations and for the lengths of time indicated (Table 2.2). The ERK 1/2 pathway was inhibited using the MEK1/2 inhibitors U0126 or/and PD98059 (Calbiochem). SB 202190 (Calbiochem) was used to inhibit the p38 MAPK pathway., LY294002 (Calbiochem) and Rapamycin (Cell Signalling Technology) were used to respectively inhibit the PI3K and the m-TOR pathways. Where indicated, Imatinib (0.5-5 $\mu$ M; kindly provided by Professor Junia Melo) was included in the cell suspension. Stock solutions of the inhibitors were prepared with either DMSO or PBS. The final concentration of DMSO in all the experiments was below 0.8%.

**Table 2.2.** Signalling pathways inhibitors and stimulators utilized in this study

| SIGNALING PATHWAY | INHIBITOR        | COMPANY          | DOSE           | REFERENCE                                   |
|-------------------|------------------|------------------|----------------|---------------------------------------------|
| MEK 1/2           | UO126            | Calbiochem       | 10-100 $\mu$ M | Favata et al, 1998<br>Promega Notes         |
| MEK 1/2           | PD98059          | Calbiochem       | 10-100 $\mu$ M | Han et al,2001<br>Promega Notes             |
| P38MAPK           | SB202190         | Calbiochem       | 10-50 $\mu$ M  | Nemoto et al ,1998<br>Gauduchon et al; 2005 |
| JNK               | JNK inhibitor II | Calbiochem       | 5-20 $\mu$ M   | Han et al, 2001<br>Bennet et al, 2001       |
| PI3K              | LY294002         | Calbiochem       | 10-50 $\mu$ M  | Paunescu et al, 2000<br>Ireton et al, 1996  |
| mTOR              | Rapamycin        | Cell Signaling   | 5-50 nM        | Sin et al, 2006                             |
| TARGET MOLECULE   | STIMULATORS      | COMPANY          | DOSE           | REFERENCE                                   |
| BCR               | Anti-IgM         | Southern Biotech | 10 $\mu$ g/ml  | Niu et al, 1998                             |
| CD40 Receptor     | Soluble CD40     | Biovision        | 0.1-20 ng/ml   | Biovision                                   |
| P38MAPK           | Anysomicin       | Sigma            | 50-100 ng/ml   | Hong et al, 2007                            |

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#### 2.4.2. CD40 and BCR stimulation

Ramos cells were preincubated with different inhibitors of the MEK/MAPK pathway and/or treated afterwards with anti-IgM (Southern Biotech; 10 µg/ml) as indicated.

For the CD40 experiments, Ramos cells were treated with CD40 ligand (Biovision; 0.1-20ng/ml.) or cultured on a mono-layer (80-90% confluent and 30Gy irradiated) of a mouse fibroblast cell line either stable transfected with human CD40 ligand (mCD 40L), or non transfected for up to 24 hours.

#### 2.4.3. Anisomycin and Cycloheximide

To assess the phospho-p38 effect on the *BCL6* promoter activity, 1 µg/ml of anisomycin (Sigma) was added to the medium 2 hours before obtaining the lysates. To determine whether the p38 effect required *de novo* protein synthesis or was mediated by phosphorylation of an existing pathway, the protein synthesis inhibitor cycloheximide (20µg/ml) was added 2 hours before the anisomycin.

#### 2.4.4. Demethylation treatment

In order to study the effects of different epigenetic modifications, cells were treated with 5-aza-2'-deoxycytidine (5-azadC) and, in some cases with Trichostatin A (TSA). Two and a half million cells were seeded on 10 ml R10F and incubated with or without 1µM freshly made 5-azadC (stock diluted in DMSO and stored at -80°C; Sigma) for 72 hours. For 5-azadC and TSA co-treatments, cells were treated with 1µM 5-azadC for 72 hours after which TSA (300 nM) was added for another 72 hours. Cell pellets were used for RNA analysis.

## 2.5. Construction of plasmid vectors

All constructs used in this study were verified by DNA sequencing and/or restriction enzyme digestion

**2.5.1. Dominant negative STAT5.** The pORSVI plasmid containing dominant negative Stat 5 (DN STAT5)(694F) was a kind gift from Professor Junia Melo

### 2.5.2. Luciferase Reporter Vectors

The pGL3 basic and the pRL null vectors were purchased from Promega. The (-1296)*BCL6*pGL3 reporter vector containing a section of the *BCL6* proximal promoter region were donated by Professor Eric Lam (Hammersmith Hospital) (Fernandez de Mattos et al., 2004). The *BCL6*(-4.9/+0.5)pGL3, *BCL6*(-4.9/+0.2)pGL3, *BCL6*(-4.3/+0.5) and the *BCL6*(-4.3/+0.2)pGL3 reporter vectors were generated by Dr. Vasiliki Papadopoulou (Department of Haematology, Hammersmith Hospital) (Papadopoulou et al., 2010).

The *BCL6* Exon-1pGL3 and *BCL6*ΔExon 1pGL3, -4.9/Δ+0.2pGL3 were generated as follows. The *BCL6* gene regions spanning from -4904 to the +239 (in relation to the *BCL6* transcription start site) were amplified using a Expand Long Template PCR System (Roche) in a final reaction volume of 50 µl (30 ng of genomic DNA, 300 nM of each primer, 200 nM of each dNTP and 0.4µl of the DNA Polymerase), in a thermocycler using a “Long template program” (95°C 2min; [94° 20 s; 62°C 30 s; 68°C 10min] 10 cycles; [94° 20 sec; 62°C 40 s; 68°C 10 min-increasing 20 sec per cycle] 25 cycles; 68°C 9 min).

The *BCL6* gene regions spanning from +239 to +533 were amplified using Phusion<sup>R</sup> High Fidelity Taq on a final volume of 50 µl containing 200 ng of genomic DNA, 300 nM of each primer, 200 nM of each dNTP and 1X Phusion Buffer and 0.06 units of Phusion Taq. PCR conditions were 98°C 45s followed of 40 cycles (98°C 5s; 2-

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step 72°C 45 s) and 72°C 10 min. Restriction enzyme recognition sites were added to the primers for subsequent sub-cloning.

The PCR products were mixed with loading buffer [0.005% (w:v) bromophenol blue 2% and 30% (v:v) glycerol 100%) and separated on a 1 to 1.5% agarose gels with 0.5 µg/ml of ethidium bromide in TBE buffer for 1 hour at 100V. 1 Kb DNA ladder and 100 bp ladder (Invitrogen) were used as weight markers. The insert fragments were then purified using the QIAquick Gel Extraction Kit (Qiagen) following the producer's protocol, and digested overnight with an excess of restriction enzymes. Hind III and Xho I (New England Biolabs) were used to digest the 270 bp Exon 1 WT and mutant *BCL6* fragments and NheI and Bgl II (New England Biolabs) for the digestion of the (-4904/+533) mutant *BCL6* fragment.

The pGL3 Basic vector was digested overnight with the same restriction enzymes and purified, using the QIAquick Gel Extraction Kit. In order to avoid self-ligation, 5'phosphates of the linearized vector were removed using 10 Units of Shrimp Alkaline Phosphatase per µg of vector, incubating at 37°C for 15 minutes. Then heat inactivation was performed for 15 additional minutes at 37°C. In order to calculate the concentrations of both the insert and the vector, a small amount of both components were run on a 1% agarose gel. Then, vector and insert were ligated using T4 DNA ligase at 16°C O/N. Three different molar ratios were used (1:1; 1:3 and 1:5). The ligated material was transformed into competent *E. Coli*. Briefly, 1 vial of competent *E. Coli* cells (One Shot TOP10 Chemical competent *E. Coli* cells; Invitrogen) was thawed on ice. 2 µl of the ligated material was added into one vial of competent bacterial cells and mixed gently. The vial was incubated on ice for 30 minutes, and then incubated at 42°C for 30 sec. After incubating the vial for two minutes on ice, 250 µl of SOC medium was added to the vial, and then the vial was placed on a 37°C incubator with horizontal shaking for an hour. 100 µl of each transformation was then plated on an a pre-warmed ampicillin-agar plate and left on an 37°C incubator O/N. Plasmids were then isolated from grown

colonies using Wizard Plus SV minipreps DNA purification system and screened by restriction digestion.

Plasmids, were then purified using the PureLink™ HiPure Plasmid DNA Purification Ki (Invitrogen), following manufacturer's instructions recommendations.

### 2.5.3. CTCF vectors

CTCF expression vectors used were: pCDNA-CTCF (Torrano et al., 2005), kindly provided by Dr Klenova (Essex University, UK) and three different pEGFP-CTCF constructs (pEGFP-CTCF-Full Length, pEGFP-CTCF-Zinc Finger, pEGFP-CTCF-C Terminal) (Burke et al., 2005; Torrano et al., 2006), kindly provided by Dr R. Renkawitz (Justus-Lieby University, Germany). For silencing CTCF expression the pRS-CTCF containing a shRNA CTCF on the pRETRO Super vector (Brummelkamp et al., 2002) (Figure 2.1), generated by Veronica Torrano, was used.

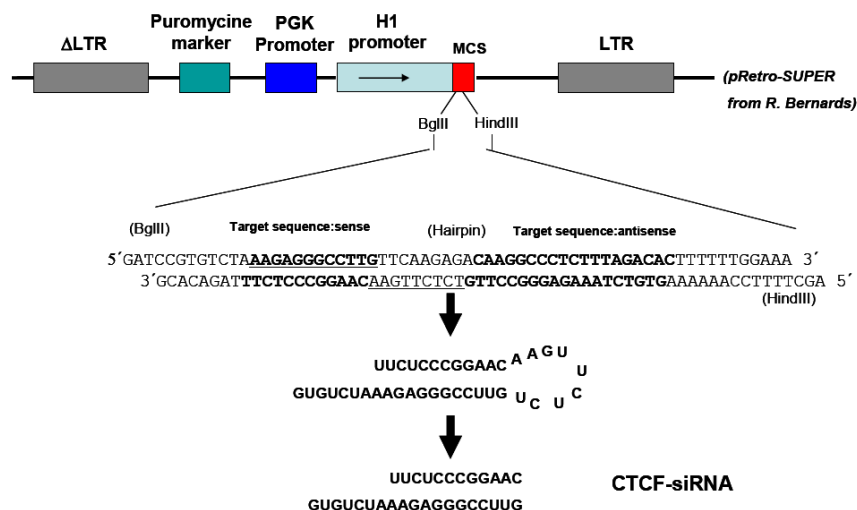


Figure 2.1. Scheme depicting the pRS-CTCF vector used for silencing CTCF expression.

CTCF target sequencing was described in Ishihara et al, 2006.

#### 2.5.4. pGMT- vector

For subcloning the bisulfite treated DNA sequences the pGEM-T easy vector (Promega) was used. For ligation 2 µl of the PCR product, 0.5 µl (25 ng) of vector, 1 µl of T4 DNA Ligase (3 Weiss Units/µl) and 5 µl of 2X Rapid ligation buffer, and 2.5 µl of distilled autoclaved water were used. Ligation reaction was performed at room temperature for 1:30 h. Transformation was carried out using DH5α Competent cells (Invitrogen). Minipreps of 10-30 colonies of each condition were carried out. The restriction enzyme Pvu I (Fermentas) was used to verify the insertion of the PCR fragment on the vector. Five to ten minipreps were then submitted for sequencing using the SP6 primer.

**Table 2.3.** Plasmids used in this study

| PLASMID NAME             | PLASMID TYPE | REFERENCE                | ORIGIN           |
|--------------------------|--------------|--------------------------|------------------|
| pORSVI DN STAT5(694F)    | Expression   | Sonoyama et al, 2002     | Pr. JV Melo      |
| Mig p210 BCR-ABL plasmid | Expression   | Quackenbush et al, 2000  | Pr. JV Melo      |
| Mig R1                   | Expression   | Quackenbush et al, 2000  | Pr. JV Melo      |
| pGL3 Basic               | Reporter     | Promega                  | Commercial       |
| pRL Null                 | Reporter     | Promega                  | Commercial       |
| -1296BCL6pGL3            | Reporter     | Mattos et al             | Pr. E Lam        |
| BCL6(-4.9/+0.5)pGL3      | Reporter     | Papadopoulou et al, 2010 | Dr. SD Wagner    |
| BCL6(-4.9/+0.2)pGL3      | Reporter     | Papadopoulou et al, 2010 | Dr. SD Wagner    |
| BCL6(-4.3/+0.5)pGL3      | Reporter     | Papadopoulou et al, 2010 | Dr. SD Wagner    |
| BCL6(-4.3/+0.2)pGL3      | Reporter     | Papadopoulou et al, 2010 | Dr. SD Wagner    |
| BCL6(-4.3/Δ+0.2)pGL3     | Reporter     | Battle et al , 2009      |                  |
| BCL6(+5/+0.2)pGL3        | Reporter     | Battle et al , 2009      |                  |
| BCL6(+5/Δ+0.2)pGL3       | Reporter     | Battle et al , 2009      |                  |
| pcDNA3                   | Expression   | Invitrogen               | Commercial       |
| pcDNA-hCTCF              | Expression   | Torrano et al , 2005     | Dr. E Klenova    |
| pEGFP-C2                 | Expression   | Clontech                 | Commercial       |
| pEGFP-CTCF-FL            | Expression   | Torrano et al, 2006      | Dr. R. Renkawitz |
| pEGFP-CTCF-ZF            | Expression   | Torrano et al, 2006      | Dr. R. Renkawitz |
| pEGFP-CTCF-CT            | Expression   | Torrano et al, 2006      | Dr. R. Renkawitz |
| pGEM T-Easy vector       |              | Promega                  | Commercial       |

## 2.6. Transfections

**2.6.1. DG75, MUTU III, BV173 and Z119 cells transfections:** 2 to  $10 \times 10^6$  cells were transfected by Nucleofection (Amaxa GmbH, Cologne, Germany) following the company's recommendations. Briefly, after centrifugation, cells were resuspended in 100  $\mu$ l of specific transfection buffer (solution T for BV173; solution L for Z119; solution V for MUTU III and DG75). Two to ten  $\mu$ g of plasmid are added and then nucleofected with specific electrical setting (T-16 program for BV173 and Z119 cells; 0-006 program for DG75 and MUTU III cells). 0.5  $\mu$ g of the GFP (Green fluorescent protein)-Max vector was cotransfected to monitor transfection efficiency. After Nucleofection, cells were immediately transferred into pre-warmed complete RPMI 1640 medium, left overnight and analyzed the day after for transfection efficiency by flow cytometry. Cells were left in culture up to 72 hours.

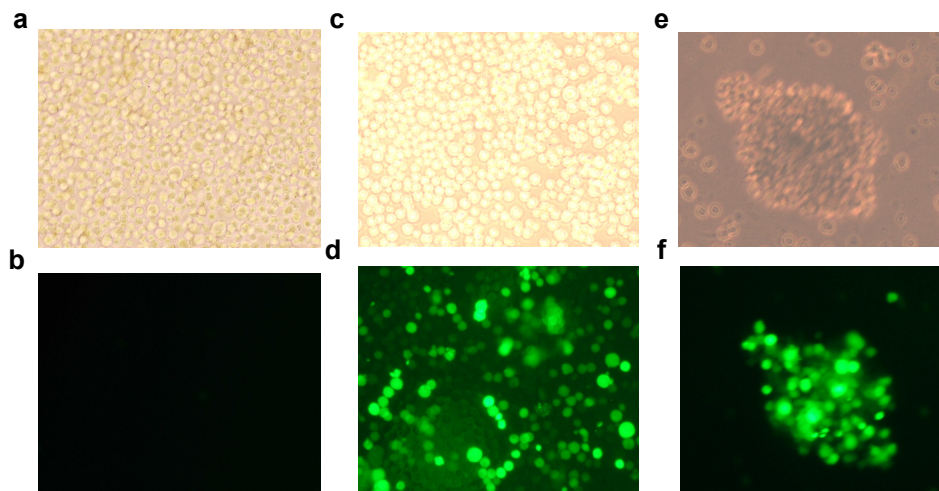


**Figure 2.2. K562 and DG75 are efficiently transfected (a,b,c and d) K562 cells and (e,f) Dg75** 48 hours after transfection with an empty vector (a,b) or a GFP-max vector (c,d,e and f). GFP expression was analyzed by an inverted immunofluorescence microscope . GFP expression was used to assess transfection efficiency

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**2.6.2. K562 cells transfections:** Ten million cells were also transfected by nucleofection (Amaxa GmbH, Cologne, Germany) as describe above but instead of using the Amaxa Nucleofector solution buffer, we used 100 µl of MIRUS Reagent (Ingenio Electroporation solution). Six µg of plasmids were used per nine million cells. The T-16 program was used for nucleofection. Transfection efficiency was examined using an immunofluorescence microscope.

**2.6.3. 293T cells transfections:**

For **luciferase assays**, 293T cells were transfected using Lipofectamine 2000 (Invitrogen), following producer's instructions. One day before transfection 800000 cells were plated on 6 well plates. Before transfection normal medium was replaced with medium without antibiotics. Five µl of Lipofectamine 2000 were resuspended in 250 µl of RPMI without FCS or antibiotics on an eppendorf tube and on a different eppendorf 4 µg plasmids were resuspended in 250 µl RPMI. After 5 minutes at room temperature, diluted DNA and diluted lipofectamine were combined and incubated for 20 minutes at room temperature. The resultant mixture was added to the cells. Four hours later normal medium was added, and cells were incubated for 20 additional hours.

For **Electrophoretic Mobility assays**, 293T cells were transfected using jetPEI (Genycell Biotech). One day before transfection 800000 cells were plated on 6 well plates. Ten µl of jetPEI were resuspended in 250 µl of NaCl 150mM on an eppendorf tube and 5 µg of the pEGFP vector or the pEGFP-CTCF plasmid were resuspended in 250 µl of NaCl 150 mM on a different tube. Both tubes were vortexed and incubated for 5 minutes at room temperature. Then diluted DNA and diluted lipofectamine were combined and incubated for 20 additional minutes at room temperature. The resultant mixture was added to the cells. Two hours later normal medium was added, and cells were incubated for 36 additional hours. Cells were then harvested and washed with cold PBS. Transfection efficiency was analyzed by immunofluorescence microscopy, based

on the GFP expression.

## **2.7. Luciferase reporter assays**

**2.7.1. In DG75, Z119 and BV173 cells:** 4 µg of the pGL3 basic vector (Promega) or equimolar concentrations of any of the different BCL6pGL3 constructs, were cotransfected together with 7 µg of either the dnSTAT5 plasmid, or the CTCF vectors and 0.6µg of the pRL reporter vector (Promega) into ten million cells using the Amaxa Nucleofector system as described above. pRL was used as a transfection control. Cells were then washed with phosphate-buffered saline (PBS), resuspended in 50-100µl of 1X passive lysis buffer (promega) and further lysis was performed by several freeze-thaw cycles. Then samples were centrifuged (12000 rpm, 15 min at 4°C) and 20 µl of the supernatants were assayed for luciferase activity using the Dual-Glo Luciferase Reporter System following the manufacturer instructions (Promega).

**2.7.2. K562 cells:** K562 cells were co-transfected with 0.5 µg of the pRL-Null transfection control vector (Promega) and with either 4 µg of the pGL3 basic vector (Promega) or with equimolar concentrations of different BCL6pGL3 constructs, and with 7 µg of different CTCF constructs using the nucleofector Amaxa Nucleofector system as described earlier. After 52 hours of transfection cells were harvested, washed using PBS and resuspended in 120 µl of 1X passive lysis buffer and further lysis was performed by several freeze-thaw cycles. Five µl of the lysates were used to assess luciferase activity using the Dual-Glo Luciferase Reporter System in a Luminometer TD 20/20 Turner Designs.

**2.7.3. 293T cells:** 293T cells were transfected with 0.4 µg of PRL vector and, with either 3.5 µg of the PGL3 basic vector or equimolar concentrations of any of the different BCL6pGL3 constructs. Cells were harvested 18-24 hours later and lysed on 120 µl of 1X passive lysis buffer and by three freeze-thaw cycles. Twenty to 50 µl of the

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lysates were used to measure the luciferase activity as described above.

## 2.8. Protein analysis

### 2.8.1. *Western blot analysis*

Cells (2 to  $10 \times 10^6$ ) were centrifuged at 1500 rpm for 5 minutes, washed once in cold PBS and resuspended in RIPA lysis buffer (1% nonidet NP-40, 100 mM NaCl, 20 mM Tris-HCl (pH 7.4), 10mM NaF, 1mM orthovanadate and complete protease and phosphatase inhibitors cocktail (Sigma)). Lysates were then incubated on ice for 30 minutes and centrifuged (12000rpm, 20 minutes at 4°C). The supernatant was placed on a new tube and frozen at -70°C. One to 10 µl of the lysates were used for protein concentration by either Bradford method or Bicinchoninic acid (BCA) kit (Sigma). 3X WB Loading buffer (100 mM Tris pH 6.8; 4% SDS; 30% glycerol; 200mM DTT, 0.03% bromo phenol blue (BPB) were mixed with 25 to 100 µg of protein in a final volume of 30µl, boiled for 4 minutes and separated by vertical electrophoresis (MiniProtean cubette, BioRad) on a 1XTGS buffer (25mM Trizma; 192 mM glycine and 1% (w:v) SDS. The percentages of the SDS- Polyacrylamide gels varied from 7.5 to 10%, attending to the weight of the protein to be analyzed. Proteins were transferred onto either a PVDF membrane (Immobilon-P, Millpore, Bedford, MA) or a nitrocellulose membrane (Schleicher and Schuell) for 1 hour at 175V using the humid Mini-Protean IITM system (Biorad) on transfer buffer (25mM Trizma base; 192mM glycine and 20% methanol).

The membrane was blocked, either in 2-5% skimmed milk or 2-5% BSA, in 1XTBS (Tris-Borate acid-SDS) buffer pH 7.4 for an hour at RT on a shaker, and then incubated O/N with the primary antibody (table 2.4) diluted on either 2-5% skimmed milk or 2-5% BSA 1XTBS buffer. The day after, the membranes were sequentially washed three times for 10 minutes at RT using wash Buffer (1XTBS-T), followed by incubation for an additional hour at RT with the secondary antibodies (table 2.5), conjugated either

with HRP or with infrared dyes, on a 1XTBS-1%SDS solution. Specific bands were visualized either using the enhanced chemiluminescence system (ECL, GE Healthcare)/developer or the Odyssey imaging system (LI-COR) which detects infrared fluorescence.

In order to re-incubate with additional antibodies, membranes were washed with wash buffer for 5 minutes, incubated with Restore Plus Stripping buffer (Pierce) for 10-20 minutes at 37°C and for 5 extra minutes at RT and washed three times for 5 minutes with wash buffer.

For protein loading control, the blots were restained with either anti-GAPDH, anti-ABL or anti-Actin antibody. Specifications and dilutions used of the different antibodies included in this study are shown in [table 2.5](#).

Comercial positive controls for JNK and phospho-p38 were purchased from Santa Cruz Biotechnology. Anti-IgM treated Ramos was used as a positive control for Erk1/2.

### **2.8.2. Immunofluorescence assays.**

A suspension of  $5 \times 10^5$  cells was centrifuged at 1.500 rpm, resuspended in 10  $\mu$ l of PBS, placed on a micro-slide (Thermo-Scientific) and allowed to dry. Cells were then fixed with either cold methanol for 10 minutes at -20°C or 3.7% paraformaldehyde in PBS for 10 minutes at RT and, in some cases, permeabilized with 0.5% triton X-100. Slides were washed 3 times for 5 minutes in cold 1%FCS-PBS, incubated with the primary antibody for 1 hour at RT, washed 3 times for 5 minutes in cold 1%FCS-PBS and incubated with the second antibody for 1h at room temperature. Following further washing slides were mounted with the anti-fading Vectashield medium (Vector Laboratories) with DAPI to visualize the nucleus. Cells were analyzed on immunofluorescence microscope (Zeiss).

**Table 2.4.** Primary antibodies used in this study

| PRIMARY ANTIBODY                                  | TYPE                                         | COMPANY                    | Aplication | CATALOG NUMBER | DILUTION |
|---------------------------------------------------|----------------------------------------------|----------------------------|------------|----------------|----------|
| Anti-BCL6 N-3                                     | Rabbit polyclonal                            | Santa Cruz Biotechnology   | WB         | sc-858         | 1:100    |
| Anti-STAT5 (c-17)                                 | Rabbit polyclonal                            | Santa Cruz Biotechnology   | WB         | sc-835         | 1:250    |
| Anti-Phospho-STAT5(Tyr694)                        | Rabbit polyclonal                            | Cell Signalling Technology | WB         | #9351          | 1:500    |
| Anti-phospho p38 MAPK (Tyr180/Tyr182)             | Rabbit Monoclonal                            | Cell Signalling Technology | WB         | #9215          | 1:1000   |
| Anti-p38 MAPK                                     | Rabbit Polyclonal                            | Cell Signalling Technology | WB         | #9212          | 1:1000   |
| Anti-Phospho-SAPK/JNK (Thr183/Tyr185) (81E11)     | Rabbit Monoclonal                            | Cell Signalling Technology | WB         | #4668          | 1:1000   |
| Anti-SAPK/JNK                                     | Rabbit Polyclonal                            | Cell Signalling Technology | WB         | #9252          | 1:1000   |
| Anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) | Rabbit Polyclonal                            | Cell Signalling Technology | WB         | #9101          | 1:1000   |
| anti-p42/44 MAPK (ERK1/2)                         | Rabbit Polyclonal                            | Cell Signalling Technology | WB         | #9102          | 1:1000   |
| Anti-phospho-c-Abl (Tyr412) (247C7)               | Rabbit Monoclonal                            | Cell Signalling Technology | WB         | #2865          | 1:1000   |
| Anti-c-Abl (24-11)                                | Mouse Monoclonal                             | Santa Cruz Biotechnology   | WB         | Sc-23          | 1:100    |
| Anti-IRF4                                         | Rabbit Monoclonal                            | Cell Signalling Technology | WB         | #4964          | 1:1000   |
| Anti-BCL-xL (54H6)                                | Rabbit Monoclonal                            | Cell Signalling Technology | WB         | #2764          | 1:1000   |
| Anti-CTCF                                         | Mouse Monoclonal                             | BD Biosciences             | WB<br>EMSA | 612148         | 1:250    |
| Anti-CTCF                                         | Mixture of 9 different monoclonal antibodies | Pugacheva et al, 2005      | CHIP       |                | 1:20     |
| anti-Actin (I-19)                                 | Mouse Polyclonal                             | Santa Cruz Biotechnology   | WB<br>EMSA | sc-1616        | 1:1000   |
| Anti-GAPDH                                        | Rabbit Monoclonal                            | Cell Signalling Technology | WB         | #2118          | 1:2000   |
| Anti-H3 AC                                        | Rabbit Polyclonal                            | Millpore                   | CHIP       | 06-599         | 1:20     |
| Anti H3K4me2                                      | Rabitt Polyclonal                            | Abcam                      | CHIP       | 32356          | 1:20     |
| Anti H2A.Z                                        | Rabbit Polyclonal                            | Abcam                      | CHIP       | 4147           | 1:20     |

**Table 2.5.** Table of secondary antibodies used in this study

| SECONDARY ANTIBODY                                 | COMPANY                    | CATALOG NUMBER | DILUTION |
|----------------------------------------------------|----------------------------|----------------|----------|
| Anti mouse IgG HRP-linked secondary antibody       | Cell Signalling Technology | #7076          | 1:2000   |
| Anti-Rabbit HRP-linked secondary antibody          | Cell Signalling Technology | #7074          | 1:2000   |
| IRDye 800CW Goat Anti-Mouse Secondary Antibody     | LI-COR Biosciences         | 926-32210      | 1:1000   |
| IRDye 680CW Donkey Anti-Mouse Secondary Antibody   | LI-COR Biosciences         | 926-32222      | 1:10000  |
| IRDye 680 Donkey Anti-Rabbit Secondary Antibody    | LI-COR Biosciences         | 926-32223      | 1:10000  |
| IRDye 800 Goat Anti-Rabbit Secondary Antibody      | LI-COR Biosciences         | 926-32213      | 1:10000  |
| Goat Anti-Mouse IgG (H+L), DyLight 680 Conjugated  | Thermo Scientific          | 35518          | 1:10000  |
| Goat Anti-Mouse IgG (H+L), DyLight 800 Conjugated  | Thermo Scientific          | 35521          | 1:10000  |
| Goat Anti-Rabbit IgG (H+L), DyLight 680 Conjugated | Thermo Scientific          | 35568          | 1:10000  |
| Goat Anti-Rabbit IgG (H+L), DyLight 800 Conjugated | Thermo Scientific          | 35571          | 1:10000  |

## 2.9. RNA analysis by reverse transcription (RT) and real-time PCR

Total RNA was isolated by using the RNeasy kit (Qiagen) and DNase I treated (Invitrogen). 2 µg RNA were mixed with a master-mix solution containing 1XBuffer RT, 0.5mM of each dNTPs, Oligo dT-Primer 1 µM, RNase inhibitor 10 Units/20 µl reaction, RNase free water and reverse transcribed by 4U/20 µl reaction of the omniscript reverse transcriptase (Qiagen) at 37°C for 1h. cDNA samples were stored at -20°C. The cDNA obtained was then used in real time quantitative PCR analysis using either Taqman or SYBR-Green approaches.

**Taqman:** Primers (Table 2.6) and probes mixtures were purchased from Applied Biosystems. Amplifications were performed on an ABI Prism 7700 Sequence

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Detector (Applied Biosystems) for 10 minutes at 95°C, 2 minutes at 50°C, followed by 40 cycles of 15 seconds at 95°C and 1 min at 60°C. Single reactions contained 12.5 µl of Taqman buffer 2X; 1.25 µl of Primer-Probe mix; 4µl of cDNA and 7.25 µl of water. Triplicates of all conditions were performed.

**SYBR green:** Primers were designed using the primer 3 program according to PCR standard guidelines (length 18 to 25 bp; GC content 40 to 65%; no G at a 5' end; avoiding secondary structures; T<sub>m</sub>: 50 to 65°C) and properly tested using a pool control cDNA sample. A PCR mix was prepared (44 µl final volume, used for two duplicate reactions) containing 22 µl of 2X SYBR® Green RT-PCR Reaction Mix, 0.3 µM of specific primers, 2 µl of cDNA and 17.4 µl of distilled water. Twenty µl per well were loaded on a 96 well plate. PCR was performed on a Bio-Rad iCycler (Bio-Rad) thermocycler as follows: 95°C 10 min; [95°C 5 min; 55-65°C 30 s] 40 cycles; 95°C 1 min; 55°C 1 min; for melting curve [55°C 10 s decreasing by half a degree each cycle] 80 cycles.

Results were analyzed and expressed by comparative DeltaDeltaCt ( $\Delta\Delta C_t$ ) method:

$$*\text{delta}CT_1 = CT \text{ (target condition 1)} - CT \text{ (normalizer condition 1; S14 or HRTP1)}.$$

$$\text{delta}CT_2 = CT \text{ (target condition 2)} - CT \text{ (normalizer condition 2; S14 or HRTP1)}.$$

$$** \text{ delta delta } CT: \text{delta } Ct_1 - \text{delta } Ct_2$$

$$*** \text{ comparative expression level} = 2^{-\Delta\Delta C_t}$$

$$**** \text{ Standard deviation} = \sqrt{(SD_1^2 + SD_2^2)}$$

**Table 2.6.** Primers used in this study.

| Name                     | Primers                                                                                 | RS              | Application                  |
|--------------------------|-----------------------------------------------------------------------------------------|-----------------|------------------------------|
| <b>Myc N</b>             | 5'-ACAAGGAGGTGGCTGGAAC-3'<br>3'-GCGTTCAGGTTTGCAGAAAG-5'                                 |                 | ChIP                         |
| <b>H.1 Gombert</b>       | 5'-CAACGCAACACAGGATATGG-3'<br>3'-TTCCCCTCCTGGCTTTTAGT-5'                                |                 | CHIP                         |
| <b>CTCF<sub>1</sub></b>  | 5'-GGACCTGAAGCCAAAGAACA-3'<br>3'-AAGTTTCGGACTCCTCCACA-5'                                |                 | ChIP<br>EMSA                 |
| <b>CTCF<sub>2</sub></b>  | 5'-GGACCTGAAGCCAAAGAACA-3'<br>3'-AAGTTTCGGACTCCTCCACA-5'                                |                 | RT-PCR                       |
| <b>BCL6 4.9 to +Δ0.5</b> | 5'-GCTAGCCAGCTCCTTTCCACTCCTCTCG-3<br>5'-TCAGATCTAGTGTCCGGCCTTTCTAGAACTTTGCGTATAGCCAC-3' | Nhe1<br>BglII   | Cloning<br>luciferase vector |
| <b>BCL6 +2/+0.5</b>      | 5'-GCCCCTCGAGCCTCGAACC GG-3'<br>5'-GATCAAGCTTAGTGTCCGGCCTTTCTAGAACTTTCTTGATCACC-3'      | XhoI<br>HindIII | Cloning<br>luciferase vector |
| <b>BCL6 +2/Δ+0.5</b>     | 5'-GCCCCTCGAGCCTCGAACC GG-3'<br>5'-TCAAGCTTAGTGTCCGGCCTTTCTAGAACTTTGCGTATAGCCAC-3       | XhoI<br>HindIII | Cloning<br>luciferase vector |
| <b>BCL6 (Taqman)</b>     | <i>(Hs00153368_m1) Applied Biosystems</i>                                               |                 | RT-PCR                       |
| <b>BCL6 (E3-E4)</b>      | 5'-AGAGCCCATAAACGGTCCT-3'<br>3'-AGTGTCCACAACATGCTCCA-5'                                 |                 | RT-PCR                       |
| <b>HRTF-1 (Taqman)</b>   | <i>(hs99999909_m1) Applied Biosystems</i>                                               |                 | RT-PCR                       |
| <b>RPS14</b>             | 5'-TCACCGCCCTACACATCAAAC-3<br>3'-CTGCGAGTGCTGTCAGAGG-5                                  |                 | RT-PCR                       |
| <b>NFκB</b>              | 5'-AGTTGAGGGGACTTTCCAGGC-3'<br>5'-GCCTGGGAAAGTCCCCTCAACT-3'                             |                 | EMSA                         |
| <b>PU.1</b>              | 5'-GCTGCTTCTGGTCCAAACAT-3'<br>3'-CCCTCCTACTCATCCCTGAA-5'                                |                 | ChIP                         |
| <b>PU.2</b>              | 5'-CTGCACTGCAATTGCCTTTA-3'<br>3'-GCACTACACGCAGCACAGTC-5                                 |                 | ChIP                         |
| <b>PU.3</b>              | 5'-CACTTCAGCAAAAGTCCAAGG-3'<br>3'-CGTGCCTTTTCTTTCCCTATC-5'                              |                 | ChIP                         |
| <b>PU.4</b>              | 5'-AACCTCTCGCTCCCTTTTGT-3'<br>3'-TGACATAAGAGGGGGAGTGC-5                                 |                 | ChIP                         |
| <b>BSP8</b>              | 5'-GGAGGGAGAGTTATTTTAGGT-3'<br>5'-ACAACAACAACAATAATCACCT-3'                             |                 | Bisulfite assay              |

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## 2.10. Chromatin Immunoprecipitation (ChIP) assays

ChIP assays were performed using a modified version of the Upstate Biotechnology protocol. Briefly, between  $5 \times 10^7$  to  $10^8$  cells (Niu et al., 2003) were harvested, resuspended in 1% formaldehyde/ PBS and incubated for 10 minutes in agitation. Glycine was added to a final concentration of 0.125 mM and incubated for 10 additional minutes. Cells were centrifuged, washed twice with cold PBS and resuspended in 200  $\mu$ l of lysis buffer (10mM EDTA, 50mM Tris-Cl pH 8 and 1% SDS) containing protease inhibitors cocktail (1:100; Roche), homogenized by passing the lysate 5 times through a blunt 20 gauge needle and incubated 10 minutes on ice. Lysates were then sonicated using a Bioruptor (Diagenode) at the maximum setting for 10 minutes (30 seconds on-30 seconds off) for three times on ice cold water, leading to fragments between 250-1000 bp. Lysates were then centrifuged for 10 minutes at 10000 rpm and supernatant was afterwards diluted 10 times with the dilution buffer (2mM EDTA, 150mM NaCl, 20mM Tris-HCl pH 8, 1% Triton X 100, 0.01% SDS and protease inhibitors cocktail). Ten percent of this solution was used as INPUT control. Antibodies were added to the diluted lysates and incubated for 18 hours with rotation at 4°C. A mixture of 9 different monoclonal antibodies (Pugacheva et al., 2005) was used to immunoprecipitate CTCF. Characteristics of all antibodies employed in this study are detailed in [Table 2.4](#). In every experiment one sample without antibody was incorporated as a negative control. Seventy five  $\mu$ l of “Dynabeads® Protein G” were mixed with 35  $\mu$ l of Sperm DNA salmon (Upstate), incubated 1 h at 4°C, added to the antibody-chromatin mix solution and incubated for 4 additional hours at 4°C with rotation. Afterwards beads were sequentially washed for 5 minutes with rotation using 1 ml of Low salt wash Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 1% Triton X-100, 20 mM Tris-HCl pH 8 and 150 mM NaCl), High salt wash Buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 1% Triton X-100, 20 mM Tris-HCl pH 8 and 500 mM NaCl), LiCl wash Buffer (0.25M LiCl, 1% NP-40, 1% NaDOC, 1mM EDTA, 10mM Tris-HCl pH 8) and

two washes with 1 X TE buffer. Protein-DNA complexes were then eluted using freshly prepared elution buffer (1% SDS and 0.1 M NaHCO<sub>3</sub>) for 30 minutes at 65° and then cross link was reverted by addition of 5M NaCl to the elutes and incubation at 65°C overnight. Beads were removed using a magnet and lysates were treated with proteinase K at 45°C for 3 hours. DNA was purified using “Wizard® SV PCR Clean-Up system” (Promega), using 120 µl of H<sub>2</sub>O for elution.

Eluted DNA was then analyzed by real time-PCR. PCR was performed in duplicates with equal amounts of specific antibody immunoprecipitated sample, control (sample with no antibody) and Input. The CTCF target site oligonucleotides used were firstly optimized using genomic DNA as template. The primer sequences utilized are detailed in [Table 2.6](#). The -5.9 Kb upstream region (H1site) on the c-myc gene (Gombert et al., 2003) was used as a negative control. The Myc N CTCF Binding site was used as a positive control (Ohlsson et al., 2001). The comparative cycle threshold approach was used for the data analysis as described above (Fitzpatrick et al., 2007). The relative enrichment of a particular sequence was obtained using the following formula:

$$2^{(CT_{\text{Input}} - CT_{\text{immunoprecipitated sample}})}$$

where CT is the number of cycles needed to rise the threshold. Fluorescent levels obtained by amplification of the “non-antibody sample” were used to normalize the fluorescent levels of the rest of the samples.

## 2.11. [Electrophoretic mobility shift assay \(EMSA\)](#)

### 2.11.1. [Preparation of radiolabelled probes](#)

**T4 method:** Oligonucleotides were stored at a 100 µM stock solution. 1 µl of each oligonucleotide stock solution was mixed with 98 µl of annealing buffer (10mM Tris; 1mMEDTA; 50mM NaCl pH 8), and boiled for 5 minutes letting cold down at room

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temperature for 8 hours. In order to radiolabel the probe 10 µl of the template DNA was mixed with 1 µl of T<sub>4</sub> polynucleotide kinase (PNK), 2 µl of 10X T4 PNK buffer and 2 µl of the radio-isotope <sup>32</sup>P, and incubated for 30 minutes at 37°C. To eliminate labelled nucleotides not incorporated to the DNA probe the reaction was passed through a Illustra Probe Quant column (GE Healthcare) following manufacturer's instructions. 1 µl of the elution was diluted with 19 µl of TE buffer, and of this 1 µl was used for a 20 µl final EMSA volume reaction, so the final amount used per reaction was 0.05 pmole of the <sup>32</sup>P-labelled probe.

**PCR method:** A 198 bp radioactive labelled probe was obtained by PCR from genomic DNA. Primers sequences are detailed on [table 2.6](#). PCR was performed in a final volume of 50 µl containing 3 µl of the [α-<sup>32</sup>P]dCTP (Hartman Analytic), 200 ng of genomic DNA, 0.2 mM each dNTP, 0.5 µM of each primer and 1 U of DFS-Taq DNA Polymerase (Bioron) with the following PCR conditions: 95°C for 2 min, 40 cycles (95°C 30s, 65°C 30s and 72°C 30s) and 72°C 1min. The efficiency and quality of the PCR was assessed by running on a gel the product of a PCR which didn't contain radioisotope that was performed in parallel with the radioactive one. The PCR fragments were purified using "Wizard® SV PCR Clean-Up system" and eluted with 50 µl of distilled water.

**2.11.2. Nuclear extracts:** Nuclear extracts were obtained from transfected HEK293T cells as described earlier. Cells were resuspended with Buffer 1 (HEPES 10mM; KCl 10mM; EDTA pH8 0.25mM, EGTA-K pH 8 0.125M, Spermidine 0.5 mM, NP40 0.1%, DTT 1mM and protease inhibitors 1:100) vortex and incubated 10 minutes on ice. Cells were then centrifuged 5 min at 1500 rpm. Supernatant was removed and pellet was resuspended in 50 µl of buffer 2 (HEPES 20mM; NaCl 0.4M; EDTA 0.25 mM, MgCl<sub>2</sub> 1.5mM, DTT 0.5 mM and proteinase inhibitors 1: 100) and incubated 1 hour with

rotation at 4°C. Samples were afterwards centrifuged for 20 minutes at 15000 rpm. Supernatant was then placed on a clean tube and frozen at -70°C until used.

**2.11.3. EMSA reaction:** Binding reactions were performed by mixing 1 µl of the nuclear extract with EMSA buffer (1.5 µg of poli-dIdC, 20 mM HEPES pH 7.5, 50 mM KCL, 5% Glycerol, 0.175mM EDTA) and with either water or cold competitor or 1 µl of anti-CTCF antibody (Table 2.4), or 1 µl of the anti-Actin antibody (Table 2.5) and incubated on ice for 10 minutes. Later 1 µl of the probe was added to each condition and the resulting mixture was incubated for 30 additional minutes. Samples were electrophoresed on a vertical non-denaturing 4% poly-acrylamide gel (160V for 2 hours) in 0.5X Tris-borate-EDTA (TBE) buffer. Gels were fixed using 10% acetic buffer for 10 minutes and then dried for 30 minutes (BioRad). Bands were obtained by exposing an X-ray film for 18-24 hours.

#### 2.11.4. Preparation of methylated probes

Probes were methylated using the Sss I methyltransferase (New England Biolabs) as follows: Five µl of the purified probe were incubated with 3 µl of NEB buffer 2, 3 µl (12U) of Sssl (3 µl of H<sub>2</sub>O in the case of the control probe), 1 µl of SAM (S-adenosylmethionine; 1 µl of H<sub>2</sub>O in the case of the control probe) 32mM and 18 µl of distilled water for 4 hours at 37°. After this process 3 µl of NEB buffer 2, 3 µl (12U) of the Sssl (3 µl of H<sub>2</sub>O in the case of the control probe), 1 µl of SAM 32mM (S-adenosylmethionine; 1 µl of H<sub>2</sub>O in the case of the control probe) and 23 µl of distilled water were additionally added and incubated for 4 hours at 37°C. This process was repeated again after 4 hours. The reaction was ended by heating at 65°C for 20 minutes. Probes were afterwards purified using the Wizard® SV PCR Clean-Up system", eluting with 50 µl of H<sub>2</sub>O. The New England Biolab Program (<http://tools.neb.com/NEBcutter2>) was used to find which CpG methylation sensitive enzymes were adequate to verify the

methylation status of both the control and Sss-I treated probe. Digestions with 1,5 U of SmaI or HinP1I or (Hin6I) and PmlI (Eco71I) and 5 µl of the purified probe were left overnight and run on a vertical non-denaturing 8% poly-acrylamide gel for 2:30h in 0.5X TBE buffer.

## 2.12. Bisulfite Genomic Sequencing

Genomic DNA was obtained from lymphoma cell lines and samples from lymphoma patients (Figure 2.3) using the DNeasy Blood & Tissue Kit (Qiagen; ref: 69506). Bisulfite conversion was performed using the commercial kit EZ DNA Methylation-Gold Kit Zymo Research Cat No D5005 following manufacturers instructions (Figure 2.3).

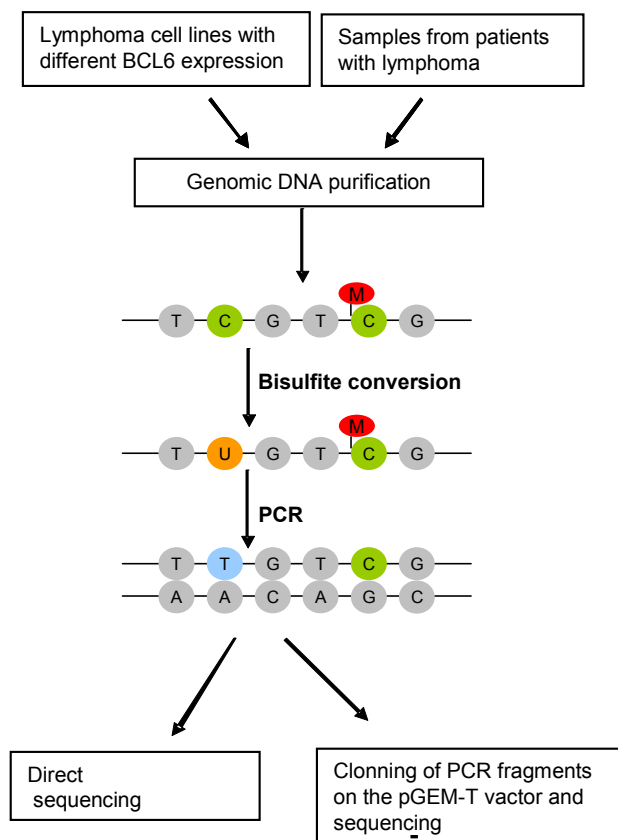


Figure 2.3. Algorithm used for the bisulfite methylation analysis.

Briefly 500 ng of genomic DNA were mixed on a PCR tube with 130 µl of CT conversion reagent. Tubes were placed on a thermal cycler and the following sequential steps were followed: 98°C 10 minutes, 64°C 2.5h. The resulting product was loaded on a Zymo-Spin™ IC Column containing 600 µl of M-Binding Buffer. After inversion of the tube samples were centrifuged for 10 minutes and then 100 µl of M-Wash Buffer were added to the column. After centrifuging, samples were incubated for 20 minutes at room temperature with 200 µl of M-Desulphonation Buffer. Columns were washed twice using the M-wash buffer. Ten µl of M-Elution Buffer were used to elute the bisulfite treated DNA. These samples were kept at -70° until used.

BSP primers were designed using the methyl primer express software (Applied Biosystems). The specific oligonucleotides were optimized using bisulfite treated genomic DNA from K562 cell line. The sequences of the oligonucleotides are specified on [Table 2.6](#). Five µl of the bisulfite modified DNA was amplified using the Expand High Fidelity PCR System (Roche; ref: 11 732 650 001). PCR conditions were as follows: 95°C for 2 min, 40 cycles (95°C 30s, 59°C 30s and 72°C 30s) and 72°C 1 min. PCR products were in some cases sent for direct sequence. In other cases the PCR products were cloned into the pGEM-T easy vector as described earlier.



## **CHAPTER 3**

***CML lymphoid blast crisis cell lines  
as model systems to define the effects  
of BCR-ABL mediated signaling on  
BCL6 expression***



## CHAPTER 3. CML lymphoid blast crisis cell lines as model systems to define the effects of BCR-ABL mediated signalling on BCL6 expression.

### 3.1. INTRODUCTION

The *BCR-ABL* oncogene is the result of a balanced translocation between chromosomes 9 and 22 t(9;22), that gives rise to a BCR-ABL fusion oncoprotein with constitutive tyrosin kinase activity. Professor Melo's group at the Hammersmith Hospital, whilst investigating the effects of the BCR-ABL tyrosine kinase inhibitor, Imatinib in BCR-ABL positive cells have unexpectedly found a relationship between BCL6 and BCR-ABL. This was derived from expression profiling of genes transcriptionally regulated by BCR-ABL using differential display to compare the mRNA profile of the lymphoblastic CML cell line BV173 treated with Imatinib, with that of untreated control cells (Deininger et al., 2000a). As a result, BCL6 mRNA and protein were found to be up-regulated upon inhibition of BCR-ABL tyrosine kinase in these cells. On the basis of this result, they investigated whether the pattern of BCL6 mRNA up-regulation was restricted to BV173 cells. They tested 14 CML or Ph-positive ALL cell lines exposed or not to 1µM imatinib for 24 hours by northern blotting. BCL6 was found to be consistently upregulated in 7 out of 8 Ph-positive *lymphoid lines* tested upon BCR-ABL inhibition (Figure 3.1.). The only exception was SD1, a cell line previously shown to be resistant to Imatinib. In contrast, BCL6 repression by BCR-ABL was confirmed when Imatinib was withdrawn. Furthermore, when BCL6 expression was tested in the imatinib-resistant subline BV173-r8 its expression was found not to be up-regulated.

In confirmation of these preliminary results, Walker et al, (Walker et al., 2007) and Mattos et al (Fernandez de Mattos et al., 2004) have also demonstrated that BCL6

is upregulated in BV173 cell line on treatment with Imatinib appears to be, therefore a suitable model system for analysing the regulation of BCL6 expression.

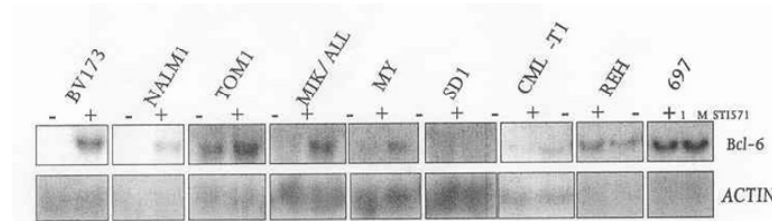


**Figure 3.1. BCL6 is induced in lymphoid blast crisis of CML by Imatinib.** Northern showing BCL6 mRNA expression (upper panel) in contrast to a control gene (lower panel) in seven BCR-ABL positive cell lines (BV173, Nalm1, Tom1, MIK/ALL, My,SD1 and CML-T1) and two BCR-ABL negative (REH and 697) when treated (+) or non treated (-) with 1 $\mu$ M Imatinib1 for 24h. *This is a result from the thesis of Sara de Almeida Deus Vieira.*

For this reason, we used the BV173 cell line and other lymphoid BCR-ABL expressing cell lines, in the presence and absence of Imatinib, to investigate the signalling pathways involved in the regulation of BCL6 and to determine how the pathways controlling BCL6 expression are integrated.

### 3.2. RESULTS

#### 3.2.1. Imatinib induces BCL6 expression in CML lymphoid blast crisis cell lines and primary cells

In order to find out if the results of (Deininger et al., 2000b) and (Fernandez de Mattos et al., 2004) could be extended various cell lines were treated with different concentrations of Imatinib for different lengths of time. All BCR-ABL lymphoid blast crisis cell lines tested showed induction of BCL6 protein expression on treatment with 0.5  $\mu$ M of Imatinib (Figure 3.2.a), and this was largely due to increases in BCL6 transcription (Figure 3.2.b). The BCR-ABL myeloid cell lines tested showed no induction of BCL6.

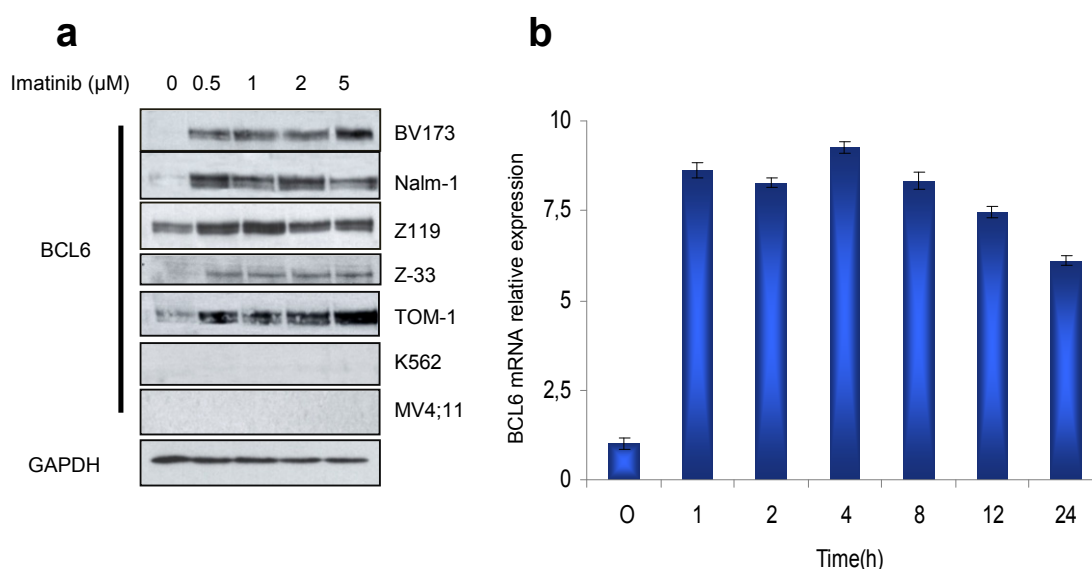


**Figure 3.2. Imatinib induces BCL6 expression in CML lymphoid blast crisis cell lines** (a) BCL6 expression in two lymphoid blast crisis of CML Ph+ cell lines with a p210 BCR-ABL transcript (BV173, Nalm-1), three acute lymphoblastic leukemia Ph+ cell lines with a p190 BCR-ABL transcript (Z-33, Z119, Tom-1), 1 erythroid blast crisis of CML cell line (K562) and 1 acute myelogenous leukemia Ph negative (MV4;11) after treatment with the indicated amounts of Imatinib for 24 hours. BCL6 expression was analyzed by Western blot. GAPDH was used as loading control (b) RT-qPCR analysis for the BCL6 was performed on BV173 cells after treatment with 1  $\mu$ M of Imatinib for different lengths of time as indicated. Values are expressed as BCL6/HPRT ratios. Triplicates of all conditions were performed.

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To examine whether this phenomenon was restricted, or not, to cell lines, primary cells from a patient with lymphoid blast crisis of CML were investigated. These cells were predicted to have normal sensitivity to Imatinib because sequencing showed that there were no mutations on the BCR-ABL kinase domain. Sequencing was carried out by Natalie Rossi (Department of Haematology; Hammersmith Hospital). These cells also demonstrated increased BCL6 expression on treatment with Imatinib, demonstrating that the effects I observed were not simply a feature of transformed cell lines (See below Figure 3.6.c).

BV173 and Z119 cell lines were selected to carry out further work. Both cell lines represent different diseases and express different BCR-ABL transcripts: BV173 derives from a lymphoid blast crisis of CML and expresses a p210 BCR-ABL transcript (Pegoraro et al., 1983) whilst Z119 is an acute lymphoblastic leukaemia cell line that displays a p190 transcript (Estrov et al., 1996). BV173 has low basal BCL6 expression but Z119 has intermediate BCL6 basal expression (Figure 3.2.a).

### ***3.2.2. BCR-ABL downstream pathways as potential regulators of BCL6***

The PI3K/Akt/FoxO3a pathway has already been implicated in BCL6 regulation in the BV173 cell line (Fernandez de Mattos et al., 2004). Other important targets of BCR-ABL such as ERK1/2 or STAT5 have been previously associated with the regulation of BCL6 in other cell lines (Niu et al., 1998; Scheeren et al., 2005; Walker et al., 2007) and, therefore, other major BCR-ABL signaling pathways might also contribute to the regulation of BCL6 in lymphoid blast crisis cells.

Several downstream BCR-ABL pathways, including the Ras/Raf/MEK, the PI3K/AKT/mTOR and the JAK/STAT5, have been widely investigated (Steelman et al., 2004). In order to investigate candidate molecules regulating BCL6, these major BCR-ABL pathways were analyzed (Figure 3.3). In agreement with previous results (Parmar et al., 2004; Steelman et al., 2004), we found that Imatinib reduced phosphorylation of

STAT5 and AKT, but that p38 MAPK was relatively resistant to the effects of this drug (Figure 3.3). In BV173 and Z119, despite BCR-ABL, there was no detectable phospho-ERK1/2 or phospho-JNK. This is surprising because the ERK1/2 pathway has been implicated in BCR-ABL mediated transformation (Cortez et al., 1997). However, there is also evidence that ERK1/2 is not active in primary cells from CML patients (Platanias, 2003; Towatari et al., 1997). Therefore, the effects of STAT5, PI3K/AKT/mTOR and p38 MAPK on BCL6 expression were investigated in more detail.

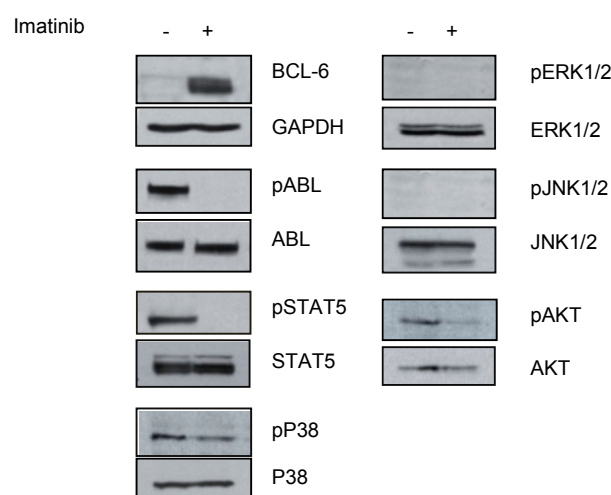


**Figure 3.3. Inhibition of BCR-ABL signalling pathways by Imatinib.** Activation of BCR-ABL, STAT5, P38, ERK1/2, JNK1/JNK2 and AKT on BV173 cells in basal conditions and after 6h of treatment with 1  $\mu$ M Imatinib were analyzed by western blot using specific phospho-antibodies.

### 3.2.3. STAT 5 upregulates BCL6 in CML Lymphoid blast crisis cell lines.

STAT5 upregulates BCL6 in primary tonsillar B-cells (Scheeren et al., 2005) but it appears that it downregulates BCL6 in cell lines (Walker et al., 2007). It is known that BCR-ABL phosphorylates STAT5 (Steelman et al., 2004) and consistent with this finding western analysis showed that Imatinib led to dephosphorylation of STAT5 (Figure 3.4.a).

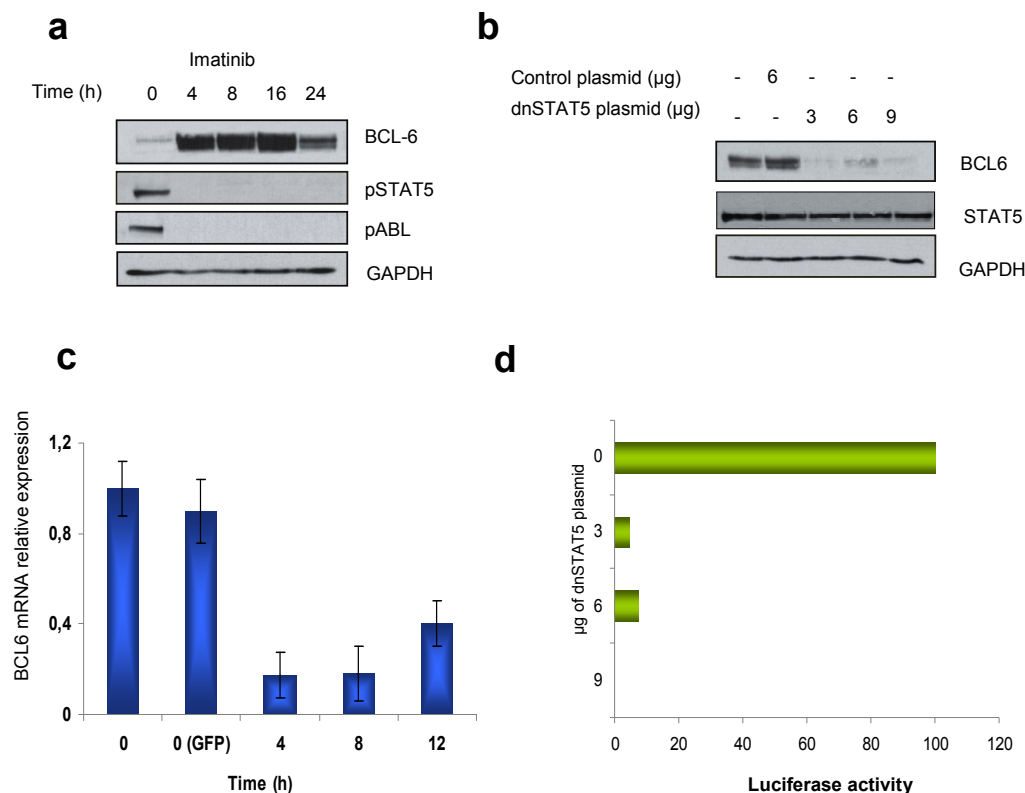


**Figure 3.4. STAT 5 induces BCL6 in CML lymphoid blast crisis cell lines** (a) Effect of Imatinib on BCL6 and pSTAT5 expression in BV173 cells when exposed to 5 µM Imatinib for the indicated lengths of time (b) BCL6 protein expression in Z119 cells after transfection of the indicated amounts of a truncated form of STAT5 analyzed by western blot. (c) RT-qPCR showing BCL6 mRNA expression normalised to HPRT after transfection of 6 µg of a dominant negative STAT5 at different time-points (Column 1: untransfected; Column 2: transfected with GFP-max control plasmid; Columns 3,4 and 5: harvested at 4, 8 and 12 hours respectively) (d) Reporter activity in Z119 cells after co-transfection with -1296Bcl6PgI3 (*BCL6* promoter reporter construct) and different amounts of a dominant negative form of STAT5 (Upper Bar :-1296Bcl6PgI3 vector alone; The rest of the bars show -1296Bcl6PgI3 vector together with 3, 6 and 9 µg of the dominant STAT5 plasmid respectively).

These results raise the possibility that induction of BCL6 expression by Imatinib occurs through dephosphorylation of STAT5 in these cells. In order to pursue this idea, Z119 cells, which have detectable basal expression of BCL6, were transiently transfected with a mammalian expression vector encoding a dominant negative mutant of STAT5. Interestingly, both mRNA and protein levels of BCL6 were constantly downregulated by the dominant negative STAT5 (Figures 3.4.c and 3.4.b). Moreover reporter assays using a construct containing the *BCL6* promoter region (-

1296BCL6pGL3), which includes several STAT5 binding sites (Scheeren et al., 2005), showed a remarkable decrease in the promoter activity when Z119 cells transfected with the dominant negative STAT5 (Figure 3.4.d).

In conclusion, these results suggest that the BCL6 increased expression observed in Lymphoid Ph<sup>+</sup> cell lines after treatment with Imatinib is not due to the STAT5 pathway, since STAT5 becomes dephosphorylated and therefore inactive upon treatment with imatinib. Also, the fact that BCL6 is not expressed in BCR-ABL cells in basal conditions despite the constitutive activation of STAT5, which induces BCL6 in basal conditions, shows that additional strong repressive pathways must be inhibiting BCL6 expression in these cells.

#### **3.2.4. *m-Tor represses BCL6 expression in Z119 cells.***

The Phosphatidylinositol 3-kinase (PI3K) pathway is another major pathway downstream BCR-ABL. Aberrant activation of this pathway has been detected in several cancers, and has been demonstrated to be involved in cell growth and survival of cancer cells (Engelman, 2009). The LY294002 is a highly specific PI3K inhibitor. In order to investigate whether the PI3K pathway was mediating the Imatinib effect on BCL6 expression, the LY294002 inhibitor was employed in the Z119 cell line. Western analysis showed a transient increase of BCL6 protein expression after 30 minutes of treatment in Z119 cells (Figure 3.5.a).

The mammalian target of rapamycin (mTOR) is one of the principal targets downstream the PI3K/AKT pathway (Engelman, Nat reviews; 2009). For this reason we aimed to examine whether the Imatinib effect on BCL6 expression was performed through the PI3K/AKT/mTOR pathway. Z119 cells were treated with rapamycin, and once again these cells showed a transient upregulation of BCL6 expression after 4 hours of treatment (Figure 3.5.b). Therefore, inhibition of mTOR can in part mimic the

effects of Imatinib, implying that phosphorylated mTOR contributes to inhibit BCL6 expression.

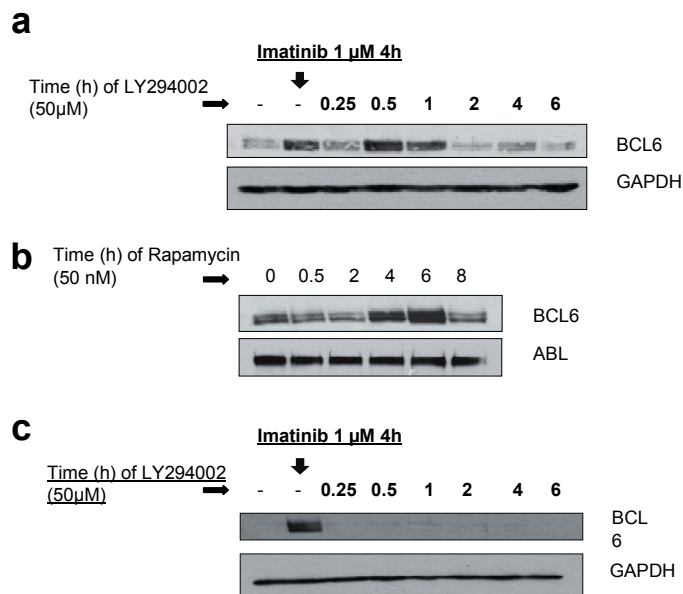


**Figure 3.5. m-Tor represses BCL6 expression in Z119 cells** (a) Western displaying BCL6 expression in Z119 cells basal conditions (Lane 1), after treatment with 1  $\mu$ M Imatinib for 4 hours (Lane 2) or with 50  $\mu$ M LY294002 for different lengths of time (Lanes 3 to 8). GAPDH was used as loading control (b) BCL6 expression in Z119 cells in basal conditions and after treatment with 50nM Rapamycin for the indicated lengths of time, were analyzed by western blot. ABL was used as a loading control (c) BCL6 expression in BV173 cells in basal conditions (lane 1), after treatment with 1 $\mu$ M Imatinib for 4 hours (lane 2) and with 50 $\mu$ M of LY294002 at different time points. GAPDH or ABL were used as loading control

Unfortunately, such effect on BCL6 expression was not observed when the BV173 cell line was treated with any of the PI3K Inhibitor (Figure 3.5.c). Since the Z119 and BV173 cell lines represent two different diseases (Ph+ Acute Lymphoblastic Leukemia and lymphoid blast crisis of CML respectively), further studies will be required to determine whether the PI3K/mTOR outcome is specific of a the Z119 cell line, or is an effect observed in other Ph+ ALL cell lines.

### 3.2.5. p38 MAPK induces BCL6 in CML lymphoid blast crisis cell lines

We reasoned that, because p38 MAPK failed to be dephosphorylated by Imatinib, it was a candidate protein responsible for the Imatinib effect on BCL6 expression. Utilising Z119, a cell line showing basal expression of BCL6, we found that a specific inhibitor of p38 MAPK, SB202190, reduced BCL6 expression (Figure 3.6.a) under basal conditions, supporting this idea. A prediction derived from this finding is that Imatinib and SB202190 will have opposing effects on BCL6 expression.

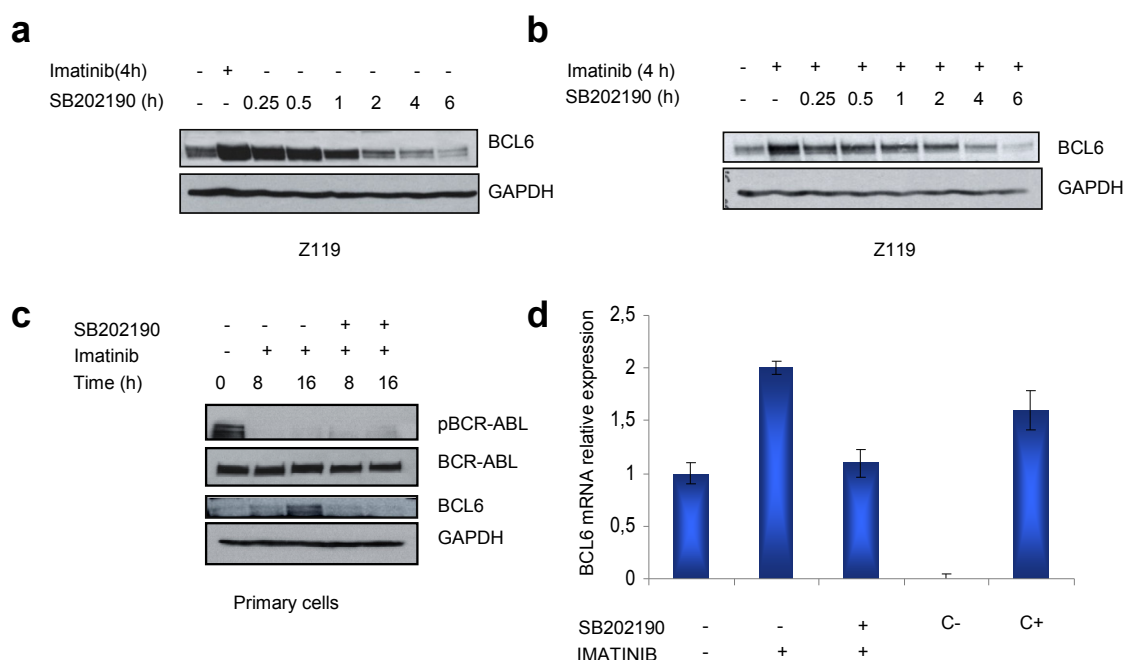


**Figure 3.6. p38 MAPK induces BCL6 in CML lymphoid blast crisis cell lines**(a and b) BCL6 expression analyzed by western blot in Z119 cells after treatment with 10  $\mu$ M SB202190 for different lengths of time (a) and after treatment with SB202190 for the same lengths of time in combination with 1  $\mu$ M of Imatinib for 4 hours (b). GAPDH was used as a loading control (c) Cells from a patient with lymphoid blast crisis of CML were analyzed for BCL6 expression and phospho BCR-ABL activity by western blotting in basal conditions after treatment with Imatinib for 8 and 16 hours, and after co-treatment with SB202190 and Imatinib for 8 hours and 16 hours (d) RT-qPCR showing BCL6 mRNA expression normalised to HPRT in primary CML cells in basal conditions (Column 1), after 4 hours of Imatinib (Column 2), and after treatment with SB202190 for 6 hours and 1 $\mu$ M Imatinib for 4 hours (Column 3). Column 4 and 5 show BCL6 relative expression in the non BCL6 expressing cell line 293T (C-) and in the BCL6 expressing cell line Ramos (C+), respectively.



repression in Ph<sup>+</sup> cells is a direct effect of BCR-ABL but also that the same signalling pathways that operate in the BCR-ABL cell lines might also be active in the germinal centre cells. Next the effects of manipulating STAT5 and p38 in Ramos or DG75 lymphoma cells were investigated.

### 3.2.7. STAT5 also regulates BCL6 in germinal centre cells.

To examine the STAT5 contribution to BCL6 expression in germinal centre cell lines we decided to inhibit STAT5 function using a dominant negative form of STAT5 in Ramos and DG75 cells. Transfection efficiency assessed by FACS was between 20-30% in Ramos cells and 70-90% in DG75 cells after 8 hours of transfection (Figure 3.8.a).

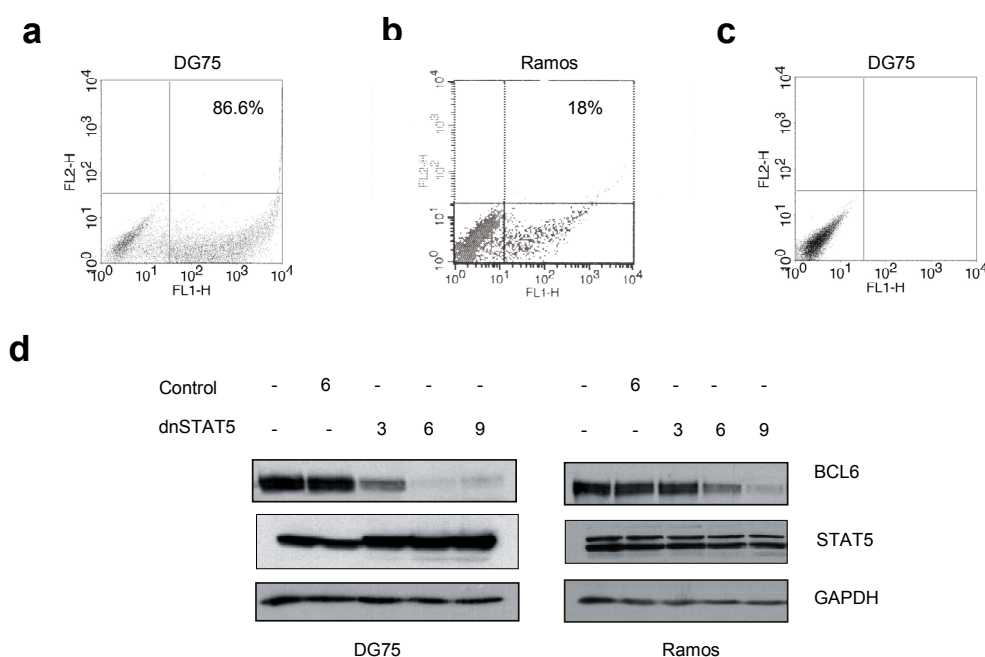


**Figure 3.8. STAT5 also regulates BCL6 in germinal centre cells** (a, b) Flow cytometry analysis of GFP expression after 18 hours of transfection with a GFP-Max vector in DG75 and Ramos cells respectively. The number in the top left quadrant indicate the percentage of cells expressing GFP (c) Plot showing GFP expression in untransfected DG75 cells. (d) BCL6 expression in two different Burkitt cell lines, DG75 (Left panel) and Ramos (Right panel) was analyzed by western after transfection co-transfection with GFP-max and with various doses of a truncated form of STAT5 as indicated.

Western blots showed that BCL6 downregulation could be induced by dnSTAT5 in both DG75 and Ramos cell lines (Figure 3.8.b). All results - in CML and B-cell lines - are consistent with previous reports (Scheeren et al., 2005) that STAT5 contributes to inducing BCL6 in germinal centre cells.

### 3.2.8. p38 is important for BCL6 regulation in germinal centre cells.

To find out the effects of manipulating p38 on basal BCL6 expression in the germinal centre cells the p38 inhibitor SB202190 was employed. BCL6 protein downregulation was observed in both DG75 and Ramos cells after treatment with this inhibitor (Figure 3.9), indicating that the p38MAPK pathway is also implicated in the BCL6 regulation in germinal center cells.

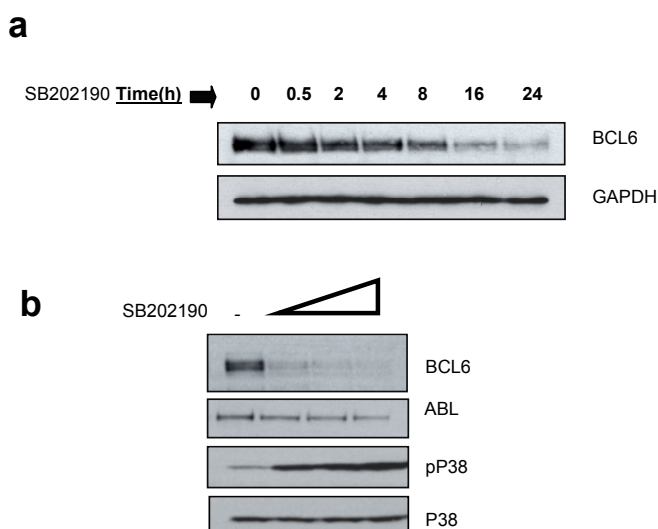


**Figure 3.9. p38 upregulates BCL6 in germinal centre cells (a)** BCL6 expression in DG75 cells after treatment with 20  $\mu$ M SB202190 for different lengths of time. **(b)** Western analysis for BCL6, phospho p38 and p38 were carried out in Ramos cells after treatment for 6 hours with increasing concentrations of the the p38 inhibitor SB202190 (Lane 1: untreated: Lane 2: 10  $\mu$ M; Lane 3: 20  $\mu$ M and Lane 4: 50  $\mu$ M).

As previously reported SB202190 increased p38 phosphorylation (Oster et al., 2008; Wang et al., 2000), maybe because this inhibitor inhibits p38 inhibition but phosphorylation by proteins in the MAPK signalling cascade continues.

### 3.3. DISCUSSION

Much remains to be discovered about the signalling pathways regulating BCL6, and they are likely to be very important in determining entry and exit from germinal centres and also in the causation of germinal centre derived lymphomas.

BCL6 has been shown to be upregulated in the CML lymphoid blast crisis cell line BV173 when treated with the BCR-ABL tyrosine kinase inhibitor Imatinib mesylate (STI571). We and others have shown that BCL6 upregulation is not only observed in lymphoid blast crisis of CML but it is also induced in Ph<sup>+</sup> acute leukaemia cell lines (Figure 3.2.a), indicating that BCL6 expression induced by Imatinib is a general phenomenon of BCR-ABL cells bearing a lymphoid phenotype.

Collectively the data suggests that these different BCR-ABL lymphoid cell lines cultured in the presence and absence of Imatinib could be used as inducible systems for analysing the regulation of BCL6 transcription.

Among the signalling pathways downstream of BCR-ABL are MAPK, JNK/STAT and the PI3K pathways. The individual effects of each of these pathways can be analyzed by using commercial available specific inhibitors (Figure 3 10). Dissection of these pathways demonstrated that in CML lymphoid blast crisis and ALL Ph<sup>+</sup> cell lines, BCL6 regulation is the result of the concerted action of a number of different signalling pathways. STAT5 was shown to be a strong BCL6 up-regulator. However, given that STAT5 is inhibited by Imatinib, STAT5 can not be responsible for the increase in expression observed as a consequence of Imatinib treatment. Here the function of the

p38 MAPK pathway in driving BCL6 expression in ALL and CML BCR-ABL positive cells upon treatment with Imatinib was shown for the first time.

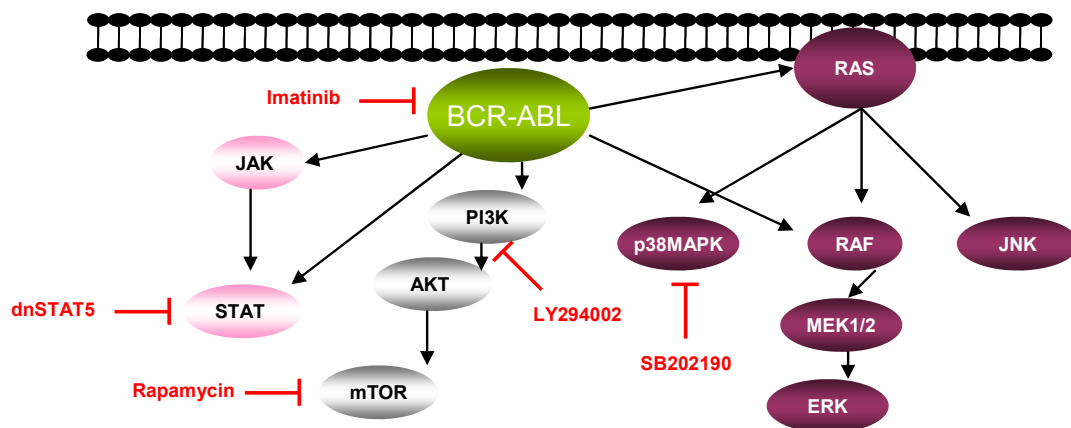


Figure 3.10. Illustration to show the inhibitors used to study the *BCR-ABL* signaling pathways

Transfection of a BCR-ABL p210 vector produced a significantly decrease in the BCL6 protein expression in germinal centre cells. Consequently, one prediction would be that the same pathways running in the CML cells might be also mediating the BCL6 regulation in the germinal centre cells. Once again, both STAT5 and p38 were shown to be inducers of BCL6 in the germinal representative cell line, Ramos. However, they act under different circumstances. The effect of STAT5 appears to be overwhelmed by negative pathways in CML cells, and in addition it is effectively dephosphorylated by Imatinib demonstrating that it cannot mediate the Imatinib effect that prompted this line of research. Phosphorylated p38, on the other hand, also causes induction of BCL6, but in distinction to other signalling molecules downstream of BCR-ABL, it is not dephosphorylated by Imatinib, and is thus available to induce BCL6. Our major conclusion is that p38 is an important and previously unrecognised inducer of BCL6. We will investigate mechanisms of regulation of p38 in germinal centre B-cell lines in the next chapter.

## **CHAPTER 4**

***CD40 and B-cell receptor signalling  
induce MAPK family members that  
can either induce or repress BCL6  
expression***



## CHAPTER 4. CD40 and B-Cell receptor signalling induce MAPK family members that can either induce or repress BCL6 expression

### 4.1. INTRODUCTION

The regulation of BCL6 is still not completely understood but is of great interest, because disruption of normal BCL6 regulation is an important mechanism in lymphomagenesis. Using BCR-ABL positive lymphoid cells as model system, it was demonstrated in Chapter 3 that different pathways seem to be working in a co-ordinated way to regulate BCL6. Among these pathways, p38 MAPK was an important enhancer of BCL6 expression.

B-cell receptor (BCR) stimulation in germinal centre cells is known to induce BCL6 protein degradation by a mechanism involving the ERK1/2 MAPK signalling pathway (Niu et al., 1998). On the other hand, activation of the CD40 signalling leads to a decrease in BCL6 expression by activation of the NF- $\kappa$ B/IRF4 pathway. Therefore, different pathways are involved in BCL6 regulation, and in this chapter the effects of these stimuli on MAPK signalling and BCL6 expression was characterised.

### 4.2. RESULTS

#### *4.2.1. Multiple MAPKs, including p38, are induced by anti-IgM in Ramos*

BCL6 regulation in germinal centre cells was analyzed in the Ramos cell line which is derived from a human Burkitt's lymphoma, a lymphoma originated from the germinal centre. Ramos cell line is EBV negative, does not have rearranged the BCL6 locus and express the IgM on its surface (Benjamin et al., 1982). Altogether, these

characteristics indicate that Ramos cells are representative of the GC and appropriate for studying the BCL6 regulation and function.

Westerns to find out how BCR signalling alters phosphorylation of MAPK proteins showed increased p38 phosphorylation as well as ERK1/2 phosphorylation (Figure 4.1.a). It, therefore, appeared that BCR signalling activated pathways that both induce and repress BCL6. In order to test this in more detail I used the specific p38 inhibitor, SB202190, together with anti-IgM antibody and found that this combination reduced BCL6 expression further (Figure 4.1.b).

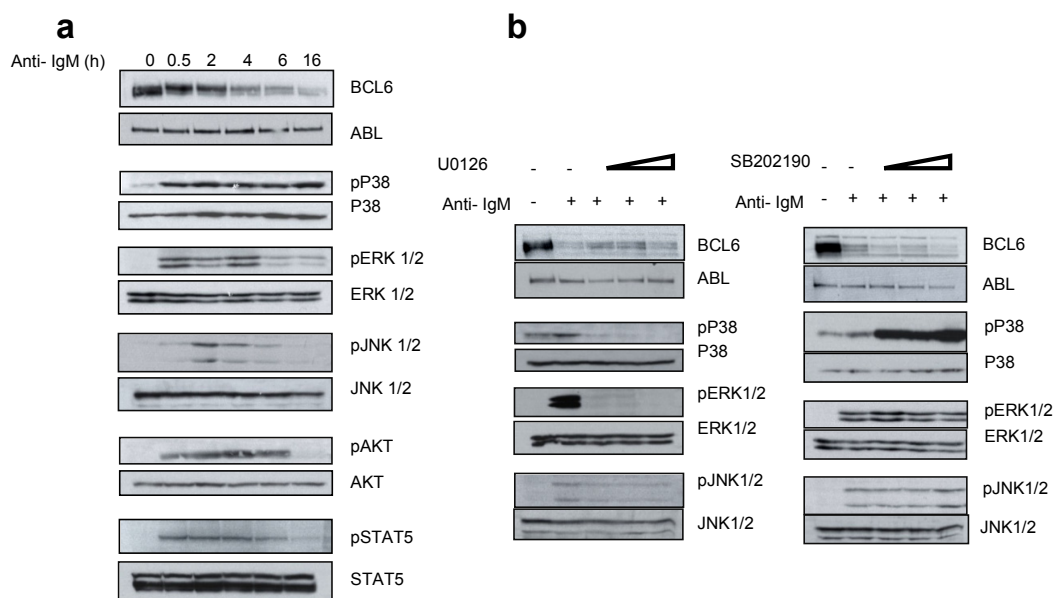


**Figure 4.1. Multiple MAPKs, including p38, are induced by anti-IgM in Ramos (a)** Ramos cells were exposed to 10 µg/ml of anti-IgM for different lengths of time.. **(b)** BCL6 expression on Ramos cells was analyzed by western blott in basal conditions (Lane 1), after treatment with 10 µg/ml of anti –IgM for 6 hours (Lane 2) or after treatment with 10 µg/ml of anti –IgM for 6 hours after 30 minutes of pre-incubation with increasing concentrations of either the MEK1/2 inhibitor U0126 (right panel) or the p38 inhibitor, SB202190 (Lane 3:10 µm; Lane 4: 20 µm; Lane 5: 50 µm) (left panel).

Others (Niu et al., 1998) have found that MEK1/2 inhibition by PD98059 prevents the reduction of BCL6 protein expression caused by anti-IgM (Niu et al., 1998) but surprisingly U0126, which is also a MEK1/2 inhibitor and would be anticipated to have the same effects as PD98059, caused only a minor reversal of the effect of IgM (Figure 4.1.b), and that increase in U0126 concentration from 10 to 75 $\mu$ M (whilst abolishing ERK1/2 phosphorylation) reduced BCL6 expression to that found with anti-IgM alone.

In order to pursue the cause of this discrepancy we directly compared U0126 and PD98059 (Figure 4.2). As anticipated both inhibitors abrogated ERK1/2 phosphorylation, but they differed in their effects on p38: whereas U0126 abolished p38 phosphorylation, PD98059 had no effect. U0126 is a potent MEK1/2 inhibitor but, at higher concentrations, inhibits other pathways including p38 MAPK (Duncia et al., 1998). A differential effect on p38 phosphorylation is, therefore, the cause of the observed difference in the effects of U0126 and PD98059. BCL6 expression, therefore, integrates positive and negative factors. ERK1/2 is a powerful negative regulator of BCL6 and, in this system, appears to overwhelm the positive effects of p38, which we identify as a positive regulator.

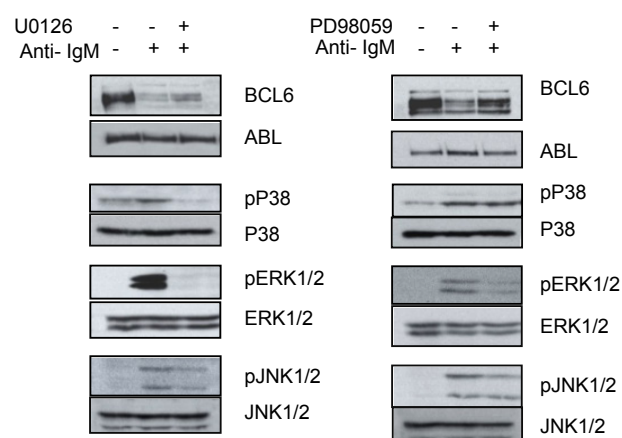


**Figure 4.2. Regulation of BCL6 by different MAPK.** Ramos cells were either treated (+) or not (-) with 10  $\mu$ M of U0126 (Left panel) or 50  $\mu$ M of PD98059 (Right panel). 30 minutes later 10  $\mu$ g/ml of anti-IgM were added in some cases (+) to the cell culture for 6 additional hours. Whole lysates were analyzed with the indicated antibodies.

Activated ERK1/2 regulates BCL6 at a post-translational level, and the inhibitor PD98059 has been shown not to affect BCL6 transcription (Niu et al., 1998). We measured changes in BCL6 mRNA in order to investigate the mechanism of action of p38 MAPK (Figure 4.3). SB202190 and U0126 reduced BCL6 mRNA expression in the absence of anti-IgM; a situation in which ERK1/2 are not activated and U0126 is predicted to be exerting its effects through p38. SB202190 produced a similar decrease in mRNA in the presence of BCR cross-linking (when ERK1/2 are activated), demonstrating that although BCL6 protein expression is reduced the major effect of p38 is to induce BCL6 mRNA transcription.

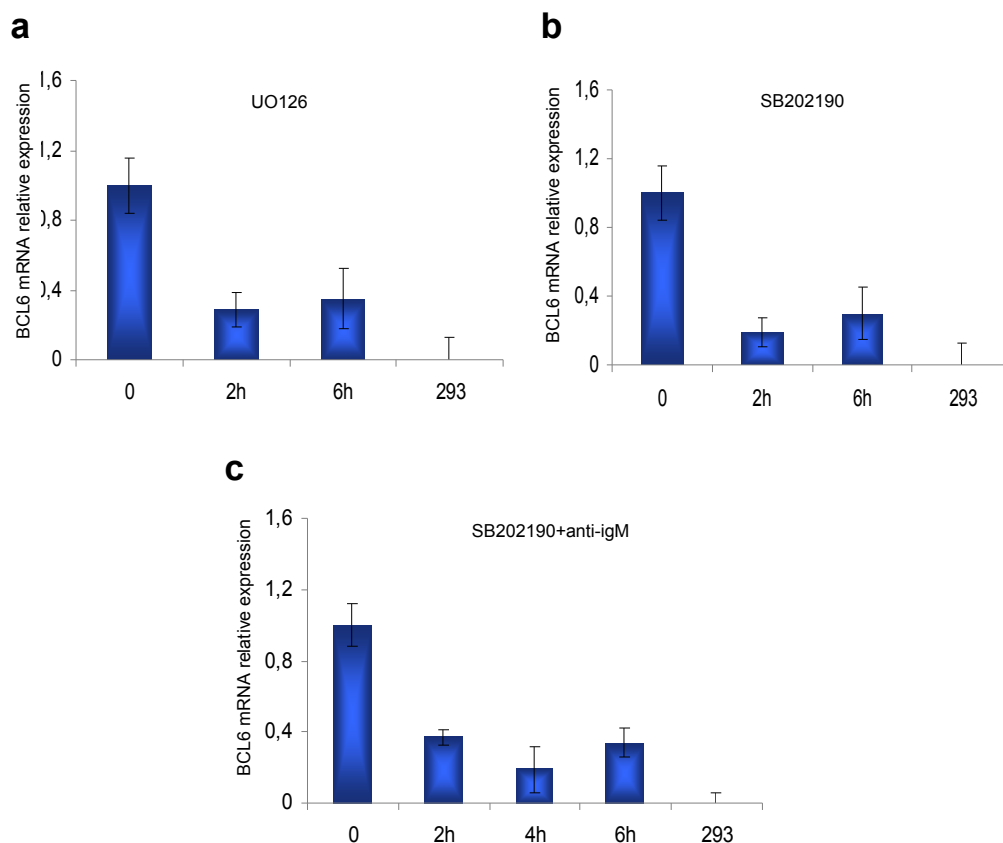


**Figure 4.3. p38 induces BCL6 expression.** Expression of BCL6 mRNA in Ramos cells after treatment time with (a) 75  $\mu$ M of the ERK inhibitor U0126 or with (b) 50  $\mu$ M of the P38 Inhibitor SB202190 for different lengths of time (c) or after preincubation with 50  $\mu$ M of the P38 inhibitor SB202190 for 30 minutes and then treatment with IgM for different lengths of time. BCL6 mRNA expression was analyzed by RT-qPCR and normalized to the level of HPRT

#### ***4.2.2. Soluble CD40 ligand induces BCL6 through inducing p38***

CD40/CD40L interaction is important for GC formation and B-cell survival, (Foy et al., 1994; Nonoyama et al., 1993). These two processes would imply BCL6 upregulated expression and contrarily previous reports showed that CD40 receptor stimulation with membrane bound CD40 ligand (mCD40) ligand leads to downregulation of BCL6 (Saito et al., 2007). Different groups using CD40 ligands from a variety of sources obtained either stimulation or inhibition of the NF $\kappa$ B pathway in Ramos cells (Wang et al., 2008; Zarnegar et al., 2004). Also, stimulation of specific Cd40 epitopes resulted in the activation of different signalling pathways and in diverse B cell responses (Sakata et al., 2000). Thus, it might be possible that different CD40 stimulus lead to produce different signalling outcomes. For this reason we investigated the effects of CD40 stimulation on BCL6 expression using CD40 ligands from two different sources. Ramos cells were treated with a commercial soluble CD40 ligand (Biovision; proteolytically cleaved 18 kDa soluble protein containing 149 aminoacid residues comprising the receptor binding TNF-like domain of CDE40L) or cultured on a monolayer mouse fibroblast cell line stable transfected with human CD40 ligand (mCD40L) (Wang et al., 2008; Zarnegar et al., 2004).

CD40 stimulation by mCD40L has been reported to reduce BCL6 protein expression due to NF- $\kappa$ B induced IRF4 activity (Saito et al., 2007). mCD40L on MAPK proteins was investigated and showed that ERK1/2, p38 and JNK1/2 MAPK were all phosphorylated (Figure 4.4.e). The strong inhibitory effects of phosphorylated ERK1/2 might, therefore, contribute to the effects of IRF4. Surprisingly, on using the alternative soluble CD40L (sCD40L) we found a strong induction of BCL6 protein (Figure 4.4.a), rather than the repression seen with mCD40L. This effect was observed over a concentration range of 0.1 to 20  $\mu$ g/ml of sCD40L. sCD40L caused phosphorylation of p38 without activating ERK1/2, JNK1/2 or inducing IRF4. We conclude that p38 is an important inducer of BCL6, but as with cross-linking of the BCR by anti-IgM, its effects are strongly counter-acted by repressors – ERK1/2 and IRF4. In confirmation of this, the

addition of the p38 inhibitor, SB202190, to sCD40L completely abolished induction of BCL6 (Figure 4.4.b).

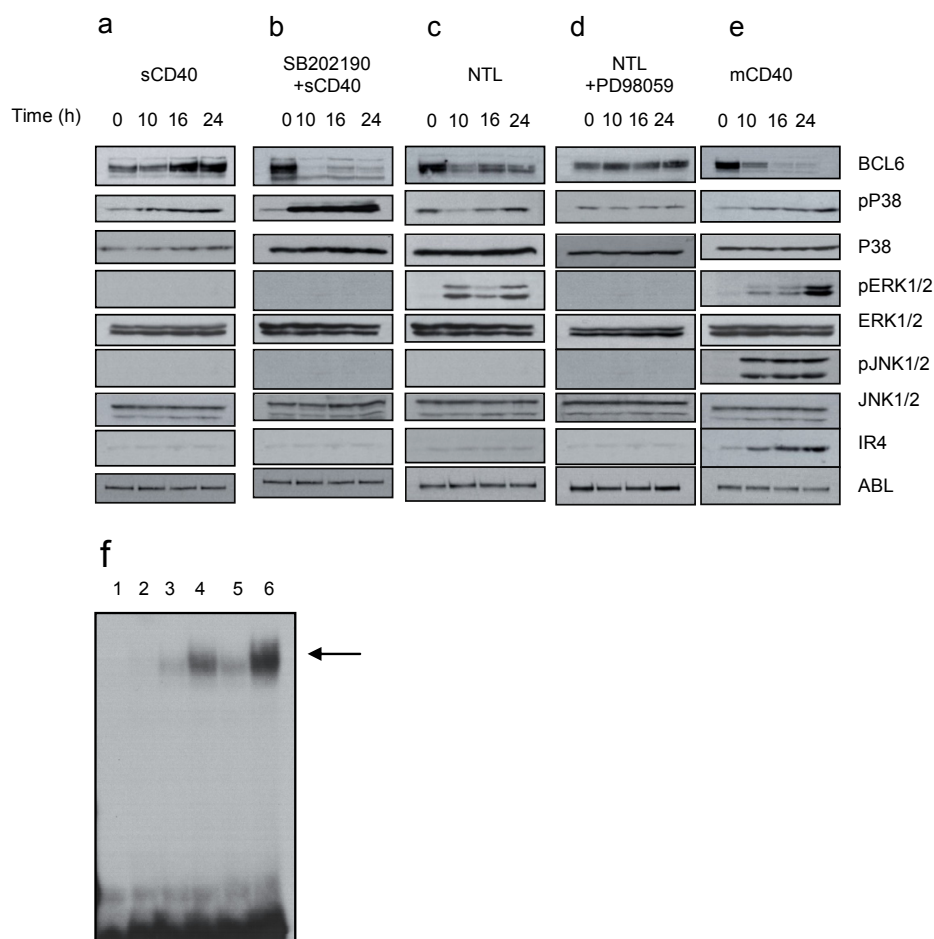


**Figure 4.4. Soluble CD40 ligand induces BCL6 through inducing p38.** Soluble CD40 ligand and membrane bound CD40 ligand differ in their effects on BCL6 expression. Ramos cells were treated with soluble CD40 (sCD40) (a) or with Soluble CD40 after 30 minutes of preincubation with 10 μM of the P38 inhibitor SB 202190 (b) or cocultured with normal non transfected fibroblasts (NTL) (c) or with non transfected fibroblast after 30 minutes of preincubation with the ERK inhibitor PD98059 (d) or with fibroblasts expressing CD40 ligand (Mcd40)(e) for the indicated lengths of time. Whole cell lysates were analyzed by western blot using the indicated antibodies. ABL was used as loading control for BCL6 and IR4. (f). EMSA to show the effect of sCD40L and mCD40L on NF-κB activation. A commercial probe obtained from Promega, known to bind the NF-κB subunit p50, a processed product of the p105 precursor, that forms a transcriptionally active heterodimer with the NF-κB subunit p65. Lane 1: no cell lysate. Lane 2: unstimulated Ramos cells; Lane 3: 4h with sCD40; Lane 4: 8h with sCD40; Lane 5: 4h with mCD40L; Lane 6: 8h with mCD40. The shifted band is indicated by the arrow.

mCD40L is expressed on the surface of the mouse fibroblast, L-cells. I reasoned that interactions of the B-cell line with adhesion molecules on the surface of fibroblasts may be important and, therefore, sought effects of this line alone on BCL6 (Figure 4.4.c). There was a transient decrease in p38 phosphorylation (lane 2; figure 4.4.c), with induction of ERK1/2 phosphorylation, without either JNK1/2 or IRF4 induction. Overall there was a reduction in BCL6 expression, which was likely to be due to the effects of ERK1/2 as BCL6 expression was restored by the ERK1/2 inhibitor, PD98059 (Figure 4.4.d). ERK1/2 phosphorylation is, therefore, caused, not only by IgM cross-linking (as previously reported (Niu et al., 1998), but also by interactions with fibroblast surface proteins. However, interactions between the B-cell line and the fibroblast does not induce IRF4, which appears to be a specific mCD40L effect.

IRF4 is activated by the NF- $\kappa$ B pathway (Saito et al., 2007). In order to distinguish effects of mCD40L and sCD40L on NF- $\kappa$ B, NF- $\kappa$ B activation was measured following incubation with sCD40L or mCD40L using gel shift assays (Figure 4.4.f). Whilst both ligands increased NF- $\kappa$ B activation, this was greater with the mCD40L. Taken together, these results suggest that IRF4 is induced when NF- $\kappa$ B activity is above a certain threshold level.

Next to find out if the effects of sCD40L were transcriptional BCL6 mRNA was measured. sCD40L causes an increase in BCL6 mRNA expression (Figure 4.5.a). Ramos expresses BCL6 at high level under basal conditions and, therefore, a 2-fold induction represents a significant increase in mRNA. The fibroblast layer alone produced no significant change in BCL6 mRNA (Figure 4.5.b) and mCD40L reduced mRNA expression as anticipated (Lossos, 2007) from the induction of IRF4 (Figure 4.5.c). Therefore, the effect of sCD40L is transcriptional and largely operates through phosphorylation of p38.

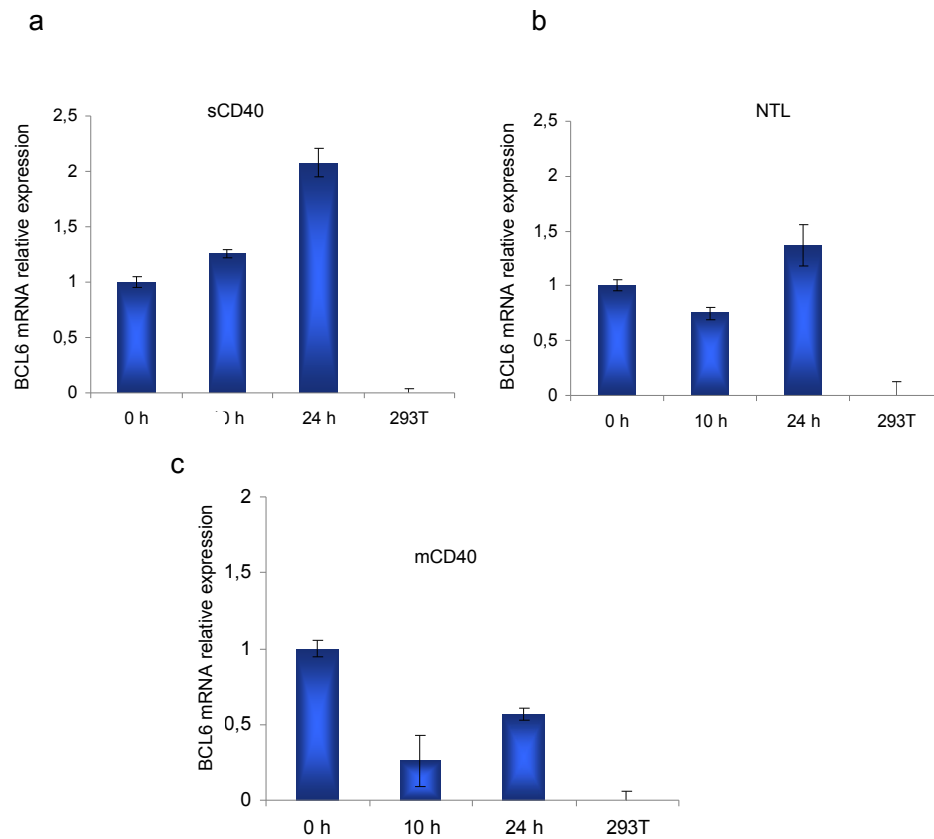


**Figure 4.5. CD40L effects on BCL6 transcription.** Ramos cells were treated with (a) soluble CD40 (b) or cocultured with normal non transfected fibroblasts or (c) with fibroblasts expressing CD40 ligand for different lengths of time. BCL6/HPRT mRNA relative expression was analyzed by RT-qPCR. The fourth column in all panels represents the BCL6/HPRT mRNA ratio of the BCL6 non-expressing cell line 293T.

#### 4.2.3. Expression of phosphorylated p38 in tonsil

In order to find out the relationship between BCL6 and phosphorylated p38 in primary germinal centres immunocytochemistry of human tonsil (Figure 4.6) was performed with the help of the Department of Histopathology at the Hammersmith Hospital.

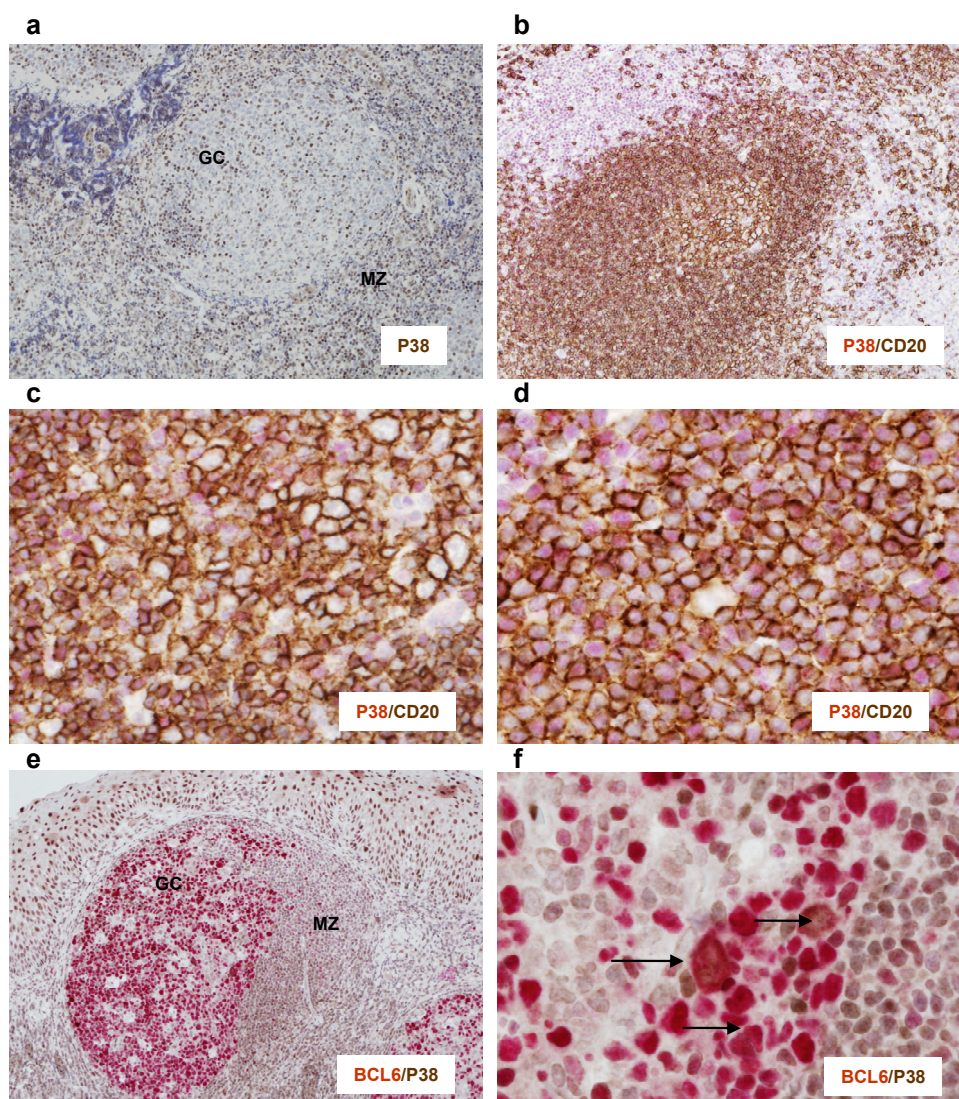


**Figure 4.6. Immunocytochemistry showing phosphorylated p38 and BCL6 expression in GC.** (a) Staining with anti-phospho p38(x10). Expression occurs mainly in the centrocytes. MZ stands for mantle zone and GC for germinal centre. (b) Double staining with anti-phospho p38 (red) and anti-CD20 (brown) (x10) (c) Higher power view of germinal centres stained with anti-phospho p38 (red) and surface CD20, a pan B marker, (brown) (x60). (d) Staining of the mantle zone with nuclear phospho p38 (red) and surface CD20 (brown). (e) double staining with anti-BCL6 (red) and anti phospho p38 (brown)(x10) (f) further high power view (x100) from the germinal centre/mantle border stained with anti-phospho p38 (brown) and anti-BCL6 (red). Cells staining for both proteins are illustrated by black arrowheads. Photographs were taken by Professor Kikkeri Naresh

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There are scattered phosphorylated p38 expressing cells present within germinal centres (Figure 4.6.a, 4.6.b and 4.6.c). In contrast, the majority of the cells of the mantle zone, a corone of variable thickness which surrounds the germinal centre and composed of small recirculating B lymphocytes, cells express phospho-38 MAPK protein. Cells that are morphologically centroblasts are negative and there are increased numbers of phosphorylated p38 expressing cells within the light zone of the germinal centre. Double staining with anti-BCL6 and anti-phosphorylated p38 (Figure 4.6.e, and 4.6.f) shows that a minor population of BCL6 expressing cells (~10%) express phosphorylated p38. It is therefore possible to suggest that activation of the p38MAPK pathway in B lymphocytes within the mantle zone might be responsible for turning on BCL6 expression.

#### 4.2.4. Site of action of p38 target genes at the BCL6 promoter

The p38MAPK signalling pathway is known to phosphorylate and activate a number of transcription factors. In order to determine which regions within the BCL6 promoter might be influenced due to the p38MAPK luciferase reporter assays were carried out. Assays for transcriptional activity of the *BCL6* promoter in BCL6 expressing cell lines are associated with specific difficulties. It has been recognised that transcriptional activity of the *BCL6* promoter is lower in BCL6 expressing cell lines than in non-expressing cell lines (Arguni et al., 2006) due to the presence of the negative autoregulatory BCL6 binding site in exon 1 (Wang et al., 2002). Therefore, both BCL6 non-expressing HEK293T and the BCL6 expressing Burkitt's lymphoma cell line, DG75 were used.

In order to mimic p38 activity the compound anisomycin which is known to be a p38 activator but is also a protein synthesis inhibitor (Geiger et al., 2005; Hong et al., 2007; Torocsik and Szeberenyi, 2000) was employed. HEK293T cells were a suitable

cell line for these experiments because anisomycin increased the levels of phosphorylated p38 MAPK (Figure 4.7.a).

Luciferase reporter assays examining 5 kb of the *BCL6* promoter region from the DNase I hypersensitive site 4.4 kb upstream of the transcription start site (HSS-4.4) to exon 1, which is about 500 bp downstream of the transcription start site were carried out. The activity due to the whole promoter was tested and the effects of deletions of either HSS-4.4 or exon 1 or both together were also examined.

Constructs with exon 1 had 3-fold greater transcriptional activity than those lacking this region. Addition of anisomycin, a p38 activator (Figure 4.7.a), induced a 30% increase (\*\*Paired tests;  $p=0.0012$ ) in luciferase in those constructs containing both the DNase I hypersensitive site and exon 1 (Figure 4.7.c).and, as anisomycin acts as well as a protein synthesis inhibitor, we concluded that this effect was mediated through p38 activity and not due to a general reduction in protein synthesis. Although it was not statistically significant, inhibition of p38 with the inhibitor, SB202190, decreased the luciferase activity below basal levels, suggesting that there may be a small amount of resting p38 activity. Despite maintaining a high basal transcriptional activity, those constructs without the DNase I hypersensitive site, did not show a significant enhancement after addition of anisomycin. On the other hand, deletion of exon 1 sequence from the 3' portion of the promoter construct (-4904 to +239) caused transcription to fall by >60%, making direct comparison with constructs retaining exon 1 difficult. It was however possible to see that anisomycin had little effect on the transcriptional activity of those constructs lacking exon1. Finally constructs containing only the exon1 region had no detectable transcriptional activity.

These experiments are difficult to interpret for the reasons given, but one conclusion is that the p38 effect on *BCL6* transcription seems to require both the DNase I hypersensitive site and the 5' region of the *BCL6* exon1. A binding site for a known target of p38, C/EBP $\beta$ , within exon 1 very close to the known negative autoregulatory binding site for *BCL6* was noted (Pasqualucci et al., 2003; Wang et al., 2002) (Figure

4.7.b). Binding sites for PU.1 and CTCF, two other putative p38 targets, are also located within the exon 1 region. As PU.1 and C/EBP $\beta$  are not expressed in 293T cells, we speculated that p38 may operate through an effect on CTCF, but this requires further work to substantiate.

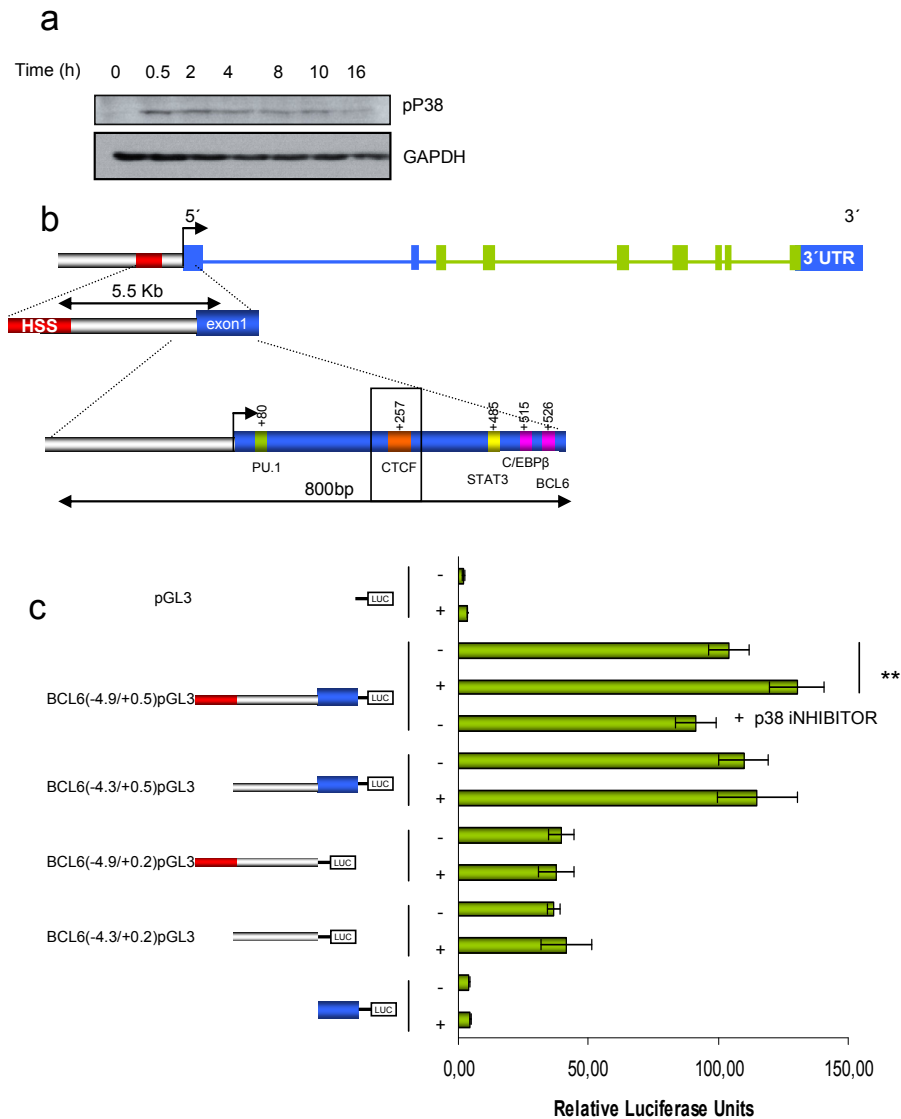


**Figure 4.7. p38 effect on *BCL6* promoter activity.** (a) 293T cells were treated with 1 $\mu$ g/ml of anisomycin for different lengths of time. Whole cell lysates were analyzed for pP38 expression. GAPDH was used as a loading control. (b) Schematic representation of the *BCL6* gene and regulatory region (c) Luciferase assays to show the effects of anisomycin and a p38inhibitor on *BCL6* transcription. 293T cells were transiently transfected with 4 $\mu$ g of different *BCL6*pGL3 reporter constructs (the constructs used are indicated on the left) and treated the day after with anisomycin for 2 hours (+ indicates treated with anisomycin whereas – indicates untreated). Means  $\pm$  SEM are shown. Each experiment was carried out 6 to 10 times with the exception of the p38 inhibitor+full length construct (2 times) and the exon-1 construct  $\pm$  anisomycin (2 times).\*\* Indicates paired-test; p=0,0012.HSS: Hipersensitivity site.

### 4.3. DISCUSSION

In this study the effects of BCR and CD40 stimulation on the B-cell line Ramos, a germinal centre representative cell line were examined. Cross linking of the BCR receptor or stimulation with mCD40L activated various MAPKs, some of which repressed (ERK1/2) whilst others induced (p38) BCL6. In most experimental situations the repressive signals were stronger than the inductive. By contrast, sCD40L activated p38 but not ERK1/2 or JNK1/2, and this caused an increase in BCL6 expression. One explanation is that sCD40L and mCD40L may be causing different amounts of CD40 cross-linking. Also the possibility that mCD40L and sCD40L have different kinetics of ligand-receptor association cannot be discounted. These hypotheses are supported by previous reports that have shown that different degrees of CD40 signalling can result in different biological outcomes. In macrophages strong CD40 signalling resulted in activation of p38 whilst weak signals led to ERK1/2 activation, and both have antagonistic effects (Mathur et al., 2004).

Activation of the ERK1/2 pathway is not only produced by IgM cross linking, but also by culture with untransfected mouse fibroblasts cells, suggesting that the ERK1/2 phosphorylation is not a specific BCR mediated effect as had been previously thought.

Thus, the major B cell stimuli, BCR cross linking and CD40 stimulation, could both activate p38. However, both also strongly activated pathways repressing BCL6: BCR activated ERK1/2 and mCD40L activated IRF4. The overall effect of BCR signalling and mCD40L was to reduce BCL6 expression, whereas sCD40L activated p38, without inducing signals repressing BCL6. It is very likely that these different signalling pathways are therefore integrated to regulate BCL6 through the different phases of the germinal centre reaction.

p38 was shown in this study to be expressed in almost all mantle zone B-cells. Although it is very likely that p38 is not sufficient for BCL6 expression, and might co-

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operate with other factors e.g. STAT5 (Scheeren et al., 2005), activation of the p38MAPK pathway might be essential for inducing BCL6 expression in B cells within the follicle. Interestingly, it has been shown that p38 expression is detected in DLBCL arising from follicular lymphomas (Elenitoba-Johnson et al., 2003), suggesting that an abnormal activation of this pathway might contribute to the transformation of these lymphomas. If this were to be the case, p38 might have a role in the treatment of patients with this type of lymphomas.

Further work will elucidate which p38 targets are directly responsible for the effect on BCL6 expression. Preliminary results suggest, that p38 might require both the *BCL6* distal promoter, which contains a recent identified hypersensitivity site (Papadopoulou et al., 2010) and the proximal region of the first non coding exon 1 to perform, its effect. It should be noticed that within the studied *BCL6* exon 1 region, binding sites for several p38 putative targets were identified, including a binding site for the multivalent regulator CTCF.

## **CHAPTER 5**

### ***CTCF involvement in BCL6 regulation***



## CHAPTER 5. CTCF involvement in BCL6 regulation

### 5.1.- INTRODUCTION.

Chapters 3 and 4 of this thesis suggested that there is a relatively short region on the *BCL6* first non-coding exon 1, spanning from +1 to +533, which is associated with both high level transcriptional activity in reporter assays and high transcription factors binding sites density, making it a likely region to be involved in the BCL6 regulation.

Using a bioinformatic approach, a putative binding site for the multivalent CTCF regulator was found at this *BCL6* gene region. CTCF is a highly conserved protein, which can potentially bind to thousands of sites in the genome. It is capable of interacting with various proteins that are known to be essential for genome regulation and organization (see general introduction). Besides its insulator function, CTCF has also been shown to act as both a transcriptional repressor and an activator. In the present study we aimed to investigate a potential role for CTCF as a BCL6 regulator and to establish the functional relevance of that regulation.

### 5.2.- RESULTS

#### 5.2.1. A putative CTCF binding site on the BCL6 exon 1

Whilst investigating potential p38 targets binding sites at the exon 1 of the *BCL6* gene, a putative binding site for the CTCF transcriptional regulator was found. A “CTCF binding sites database” (<http://insulatordb.utmem.edu>) (Bao et al., 2008) constructed from CTCF binding sites identified by genome-wide screens, has been widely used to estimate the probability of CTCF binding to putative sites (Bao et al.,

2008). We, therefore, analysed 5,6 kb of the *BCL6* gene resulting in the prediction of a CTCF binding site in the *BCL6* gene exon1 regulatory region, with a very high score (Matrix1: 18.0907; Matrix 2: 8.47517; Matrix3: 14.3321; Matrix 4 10.0761. Average: 12.75) (Figure 5.1).

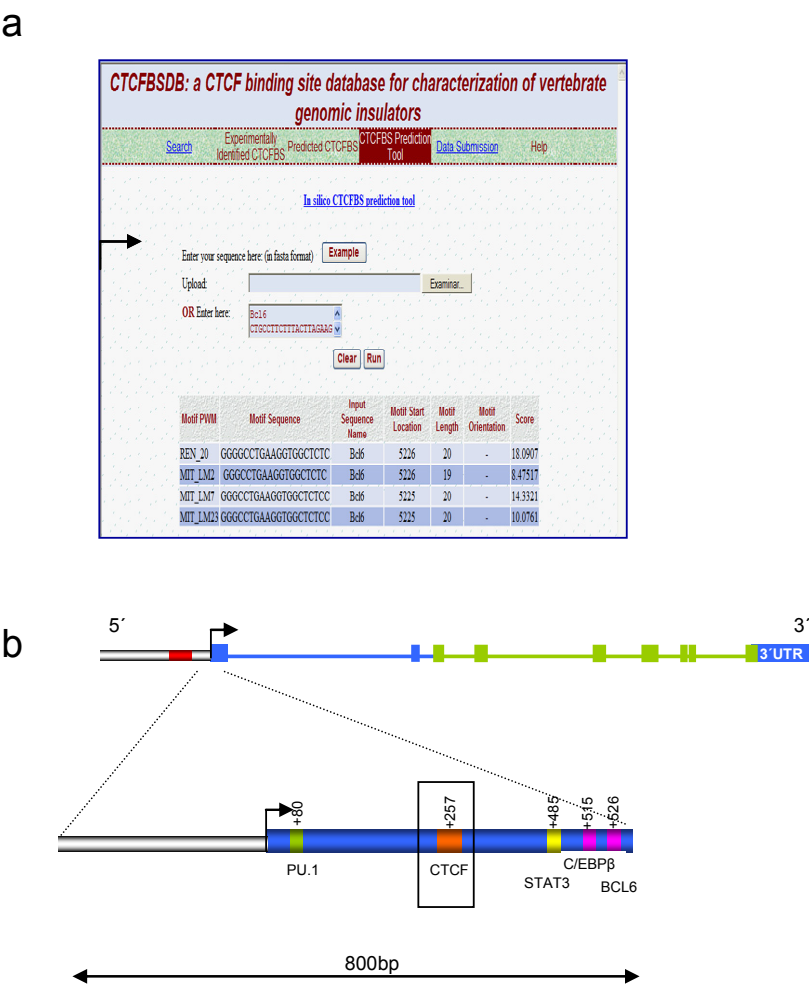


**Figure 5.1. Putative CTCF binding site on the *BCL6* exon 1.** (a) Analysis of putative CTCF binding sites on the *BCL6* gene was carried out using a “CTCF binding site database” for characterization of vertebrate genomic insulators (Bao et al, 2008), which uses four different matrices to perform the analysis (b) Schematic view of the *BCL6* regulatory region on exon 1, where the predicted CTCF binding site is located. The human *BCL6* transcription start site is indicted as +1 (Bernardin et al, 1997).

### 5.2.2. CTCF binds *in vitro* to the *BCL6* exon1 regulatory region

In order to investigate CTCF binding to the *BCL6* exon-1 regulatory region, electrophoretic mobility shift assays (EMSA) were carried out. A 130 bp radio-labelled probe, containing the CTCF BS (Figure 5.2.a), was generated by PCR amplification.

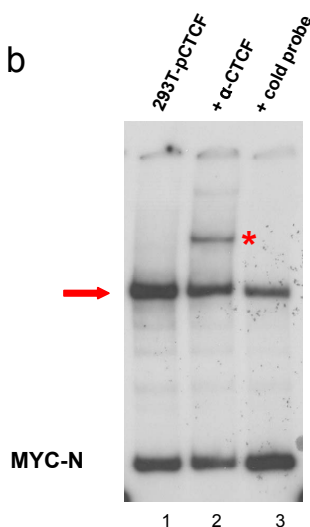
a

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TCAAAATACAGAAACCAAAATCCAAAGCCACCACACAGAAAGAAAAAACCCAGAACACAGCAAAAACCCCTG
TCCTAAATAAAATAAAGCAATGAACCCACCGAAAACTGCTTGCAAATATTTTCTCGTGGTGCCATATTCTAG
TTGGAAGAGAGCTGTGATGTTTATTTATTTTATTTTCTCTTACTCGCCTCTCTAACCCCTACTATATATAACACTT
TTCCAGTGTTTCAAACCTCTCGCTCCCTTTGTGCATTAGCTCGATCTGCTGAGTTT TTGGTAAAGAAAGG
TTTACCCCTGAG CCCGGAAAGCAAAGCGCACTCCCTCTTATGTCA CGAATAGCAAATTAGTTCTCAGAATT
CCAGAGGCCCGAGCTTTGCTACAGCGAAGGCCGCGACGTCACAGAGGAGGAGCCCACTGATGGTGGCGAGCA
GGCCATACCATCGTCTTGGGGCCCGGGAGGGAGAGCCACCTTCAGGCCCGT CGAGCCTCGAACCGGAACCTCCA
AATCGAGACGCTCTGCTTATGAGGACCTCGAAATATGCCGGCCAGTGAAAAATCTTGTGGCTTTGAGGGCTTTT
GTTTGGCCAGGGGCGTAAATCTCGGAGAGCTGACACCAAGTCTCCCTGCCACGTAGCAGTGGTAAAGTCC
GAAGCTCAAAATCGAGAATTGAGCTCTGTTGATCTTAGAACTGGGGTTCTTAGAAAGTGGTG ATGCAAGAGT TTG
TAGGAAGGCCCGGACACCAGGTGATTATTGCTGTTGCT
  
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Labels in the sequence: PU.1 (green), CTCF (orange), STAT3 (yellow), C/EBPβ (pink), BCL6 (magenta).

b



c

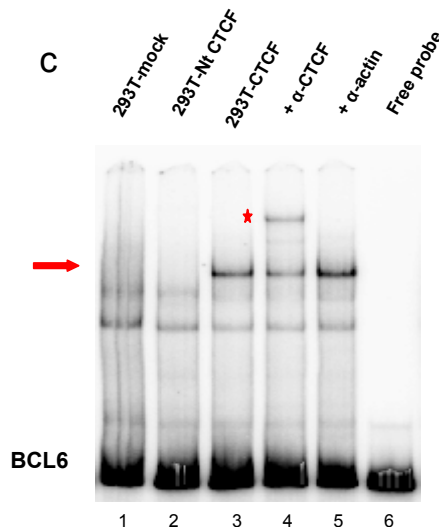


**Figure 5.2. EMSA to analyze *in vitro* binding of CTCF to *BCL6* exon 1** (a) *BCL6* exon 1 sequence that includes the CTCF binding site (orange) and the primers used for the EMSA (underlined) (b, c)  $^{32}$ P labelled PCR fragments of MYC-N (positive control, b) and *BCL6* exon 1 (c) were used as probes. Nuclear extracts were obtained from mock transfected 293T or from 293T cells transfected with either a pEGFP-CTCF N terminal or a pEGFP-CTCF full length expression vectors. CTCF shift bands are shown by red arrows. For supershift experiments an anti-CTCF monoclonal antibody was added. The supershift bands are indicated by a red star. To assess the specificity of the supershift, an anti-actin antibody was used.

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This method has been previously used to generate DNA probes when investigating CTCF binding capacity (Chau et al., 2006; Filippova et al., 1996; Fitzpatrick et al., 2007; Renaud et al., 2005; Renaud et al., 2007; Vatolin et al., 2005; Vetchinova et al., 2006; Yoon et al., 2005). The resulting radio-labeled probe was then incubated with nuclear extracts obtained from HEK293T cells that had been previously transfected, either with a CTCF expressing vector (pEGFP-CTCF), or with the empty vector. Band-shifts were optimized using a known CTCF binding site (MYC-N) (Ohlsson et al., 2001) as a positive control (Figure 5.2.b). Nuclear extracts obtained from cells transfected with CTCF, showed binding to the *BCL6* probe compared to extract from mock-transfected cells or cells transfected with the CTCF N-terminal vector (Figure 5.2.c lanes 1 to 3). The binding specificity was shown by incubating the nuclear extracts with a specific anti-CTCF antibody. A clear supershifted band was observed when this antibody was used (Figure 5.2.c lane 4) while no supershift was observed with an anti-actin antibody (Figure 5.2.c lane 5). CTCF, therefore, interacts in vitro with the predicted binding site at the *BCL6* exon 1.

### **5.2.3. Methylation of *BCL6* exon 1 does not alter *BCL6* binding.**

Although CTCF binding to the DNA has been shown to be methylation dependent in many cases (Fitzpatrick et al., 2007; Hark et al., 2000; Kanduri et al., 2000; Kim et al., 2006; Renaud et al., 2007; Yoon et al., 2005), DNA methylation did not alter CTCF binding in several other examples (Hong et al., 2005; Kim et al., 2009; Renaud et al., 2007; Vatolin et al., 2005). Could the CTCF binding to the *BCL6* gene be methylation sensitive or not? To address this question, additional electrophoretic mobility shift assays were performed using probes that were fully methylated due to the enzyme SssI methyl transferase, or unmethylated. (Figure 5.3.a) To verify that the SssI-treated-probe was completely methylated several digestions employing CpG-methylation sensitive restriction enzymes were carried out. Those enzymes are known to cut when the restriction site is non methylated, and consistently, only the non

methyated probe was cut by *Sma*I, *Hin*P1I, (*Hin*6I) and *Pml*I (*Eco*71I) methylation-sensitive enzymes (Figure 5.3.b).

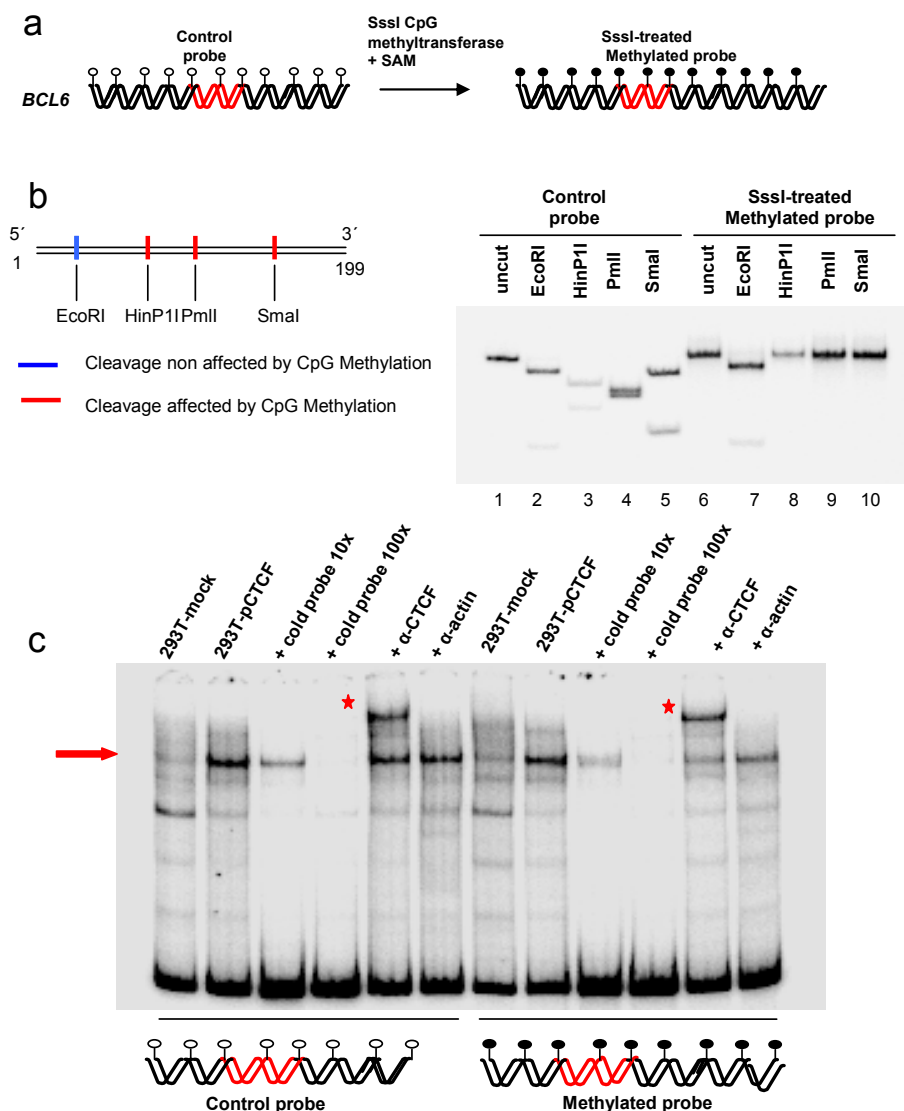


**Figure 5.3. CTCF binds *BCL6* exon1 in a methylation insensitive manner.** (a) Schematic illustration of the SssI-untreated (left) and treated (right) probes. CTCF BS is indicated in red. (b) Illustration of the probe showing the relative positions of several methylation sensitive (red) and non sensitive (blue) restriction enzymes. To assess complete methylation control probe (lanes 1-5) and SssI treated probe (6-10), were uncut or digested with the indicated restriction enzymes and run on an 8% polyacrilamide gel. (c) EMSA experiment using a control probe and a SssI-treated probe. Nuclear extracts were obtained from mock transfected 293T cells or from 293T cells transfected with the expression vector pEGFP-CTCF (lanes 2 to 6 and 8 to 12). The CTCF shift bands are shown by a red arrow. Unlabelled probe (cold) was used as a competitor. For supershift experiments an anti-CTCF monoclonal antibody was added. The supershift band is indicated by a red star. To assess the specificity of the supershift, an anti-actin antibody was used.

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On the contrary, both probes were efficiently cut with the non-methylation dependent restriction enzyme Eco-RI. These results show that methylation of the probe after SssI treatment was close to 100%.

We then made a comparison of CTCF binding capacity to both the control and the methylated probe. The methylation of the probe neither impeded nor reduced CTCF binding (Figure 5.3.c). Therefore, CTCF showed methylation insensitive binding to the *BCL6* exon1 site.

#### 5.2.4. *BCL6* and CTCF expression in different lymphoma cell lines

In order to investigate a correlation between *BCL6* and CTCF expression, western blots were carried out on a number of different lymphoma derived cell lines. *BCL6* protein expression was clearly different among the different cell lines and was consistent with the expression pattern observed previously by other authors. No detectable differences were observed in CTCF protein expression in the different B cell lines (Figure 5.4.a). This was not surprising, since CTCF normally shows ubiquitous expression.

If CTCF is regulating *BCL6* expression, this effect might depend on CTCF binding to *BCL6* gene in a cell type specific manner. Therefore, it might be possible to observe different patterns of CTCF occupancy in lymphoma cell lines with different *BCL6* expression pattern. The *BCL6* gene is very often disrupted by chromosomal translocations involving the *BCL6* exon1 region (see introduction). Thus, in order to find a possible correlation between *BCL6* protein expression profile and CTCF occupancy at the *BCL6* locus, *BCL6* gene rearrangements were investigated in cell lines and also in patients included in this study by fluorescence in situ hybridization (FISH), utilizing a commercial probe widely used for diagnostic purposes (Figure 5.4.b).

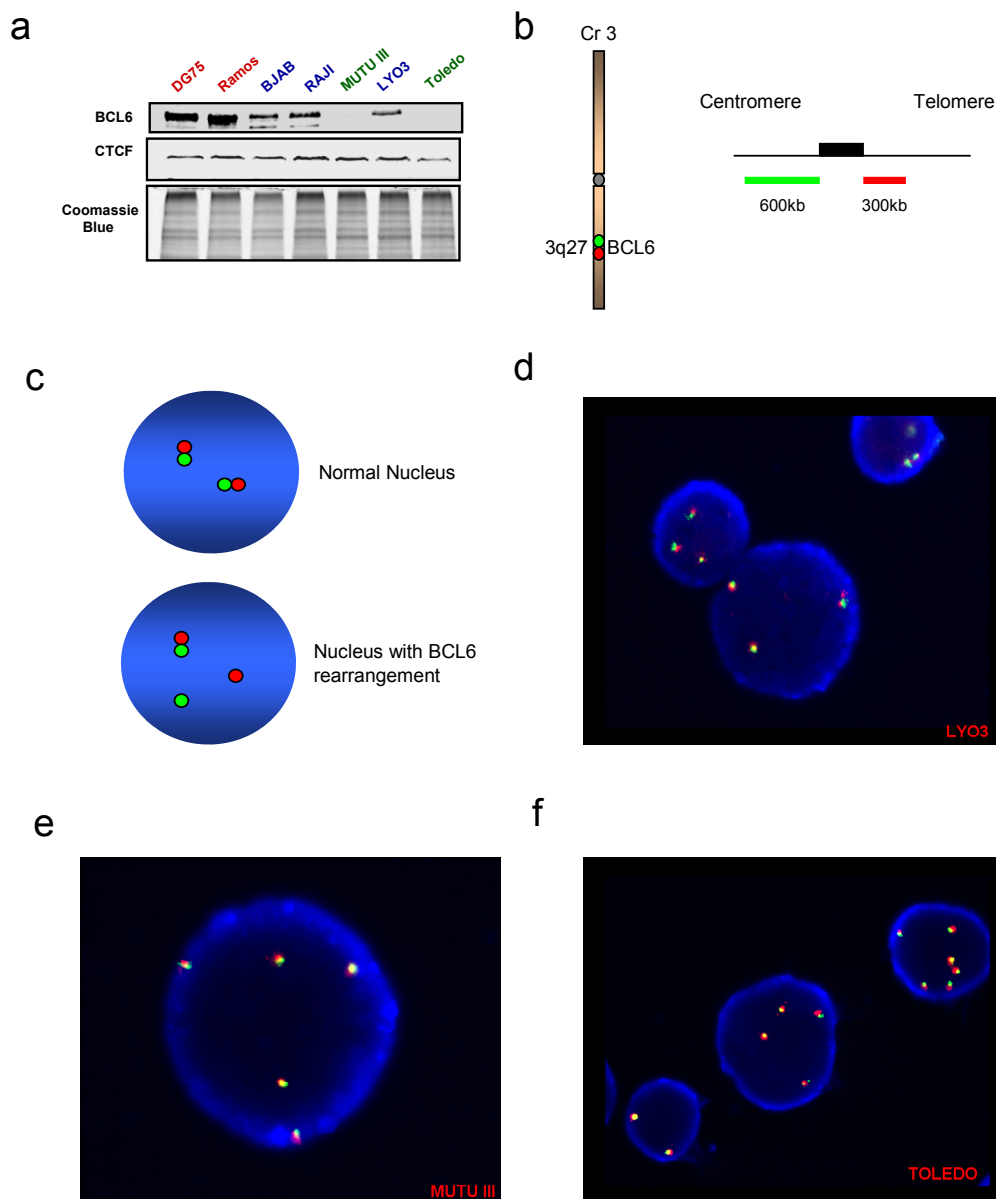


**Figure 5.4. BCL6 and CTCF expression in lymphoma cell lines.** (a) Western blot showing CTCF and BCL6 protein expression in several lymphoma cell lines. Coomassie blue stain of the gel was used as loading control. Cell lines showed high (red), intermediate (blue) and undetectable (green) BCL6 protein expression. (b) A schematic illustration of the LSI BCL6 break apart probe used for FISH analysis. (c) Hybridising with the LSI BCL6 in a normal nucleus results in two fusion signals (red-green or yellow) (top panel). The same probe hybridised on a nucleus with BCL6 locus rearrangement results in one fusion signal (representing the normal allele), one green signal and one red signal. (d, e and f) FISH using the LSI BCL6 break apart probe of LY03, Toledo and MUTU III lymphoma cell lines.

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When *BCL6* is not rearranged two yellow fusion signals are observed on a single nucleus, whilst those cells with a *BCL6* translocation will normally show one red signal, one green and one yellow signal (Figures 5.4.b-c). None of the cell lines utilized on this study showed rearrangement of the *BCL6* gene. It is noteworthy that several of the cell lines showed more than three yellow fusion signals, sometimes up to five (Figure 5.4.d-f), suggesting hyperploidy or amplification of the *BCL6* gene. Lymphomas often show hyperploidy, but this result is intriguing because amplification of the *BCL6* gene was found in cell lines, such as MUTUIII or Toledo, where BCL6 protein expression was undetectable (see western in figure 5.4.a).

#### 5.2.5. CTCF interacts *in vivo* with the *BCL6* exon 1 regulatory region

Chromatin immunoprecipitation (ChIP) experiments were performed in order firstly to investigate the CTCF *in vivo* occupancy of the *BCL6* exon 1 region by CTCF, and secondly to study whether this occupancy was modified in cell lines with different levels of BCL6 expression.

ChIP assays were initially carried out in two cell lines: K562, a myeloid cell line which does not express BCL6, and Ramos, a germinal centre derived cell line expressing high levels of BCL6. The immunoprecipitation experiments were performed with and without CTCF-specific antibodies. In all experiments the well established -5.9 Kb upstream region (H1 site) on the c-MYC gene (Gombert et al., 2003) was used as a negative control. To determine the reproducibility and efficiency of the technique the MYC-N CTCF BS was amplified simultaneously with the BCL6 target sequence and used as a positive control (Figure 5.5.a). ChIP analysis revealed occupancy of CTCF at the *BCL6* exon 1 region. Strong *in vivo* binding was demonstrated in Ramos whilst the CTCF enrichment was much lower in K562 cells (Figure 5.5.a), suggesting that CTCF binds to the *BCL6* exon-1 regulatory region preferentially in BCL6 expressing cells.

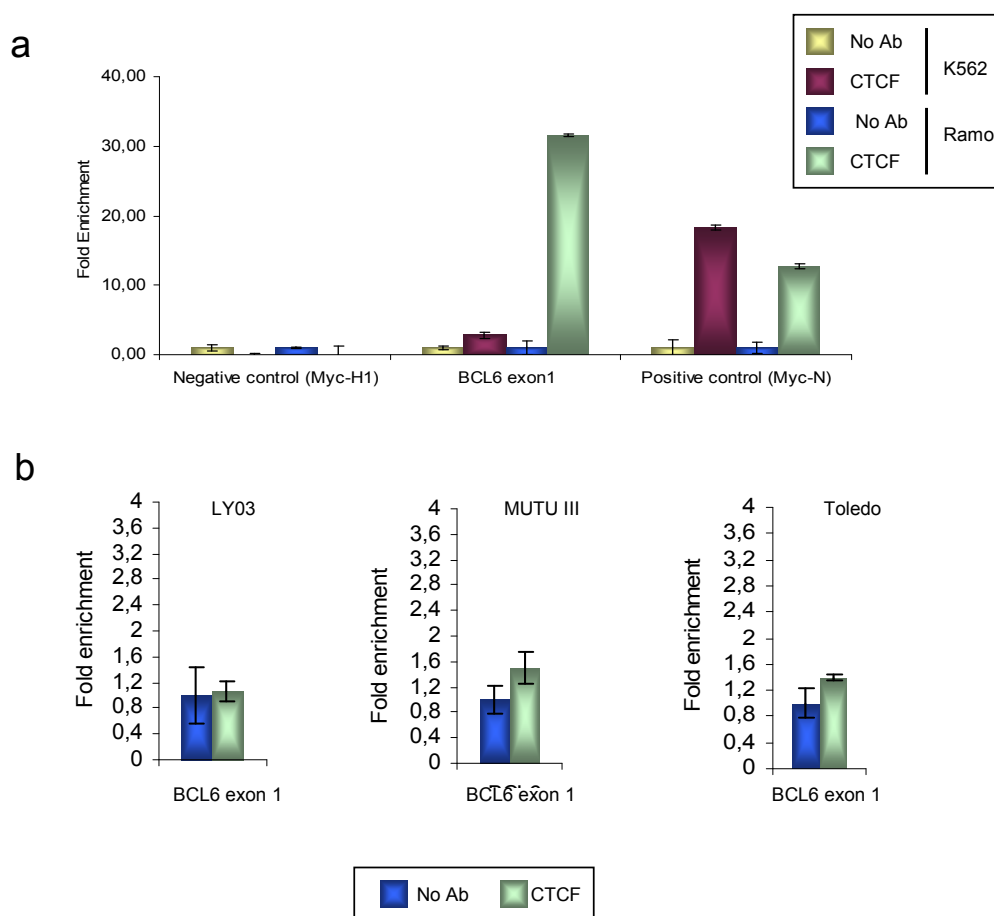


**Figure 5.5. CTCF interacts in vivo with BCL6 (a)** ChIP analysis on the BCL6 gene. Chromatin was prepared from K562 cells (undetectable BCL6 expression) and Ramos cells (high BCL6 expression). Chromatin was immunoprecipitated with a mixture of different anti-CTCF antibodies and analyzed by real time PCR with the indicated primer sets. Fold enrichment was calculated as described in Material and Methods section. CTCF Myc-N and Myc-H1 target sites were used as positive and negative controls, respectively. Values are means and SD of duplicate determinations from three independent ChIP experiments. **(b)** ChIP examining the relative fold enrichment of the CTCF binding site on the BCL6 exon1 in LY03, Toledo and MUTU III lymphoma cell lines.

Additional ChIP assays were then carried out, utilizing a variety of cell lines with different levels of BCL6 expression. As anticipated from the results described above, CTCF binding in the BCL6 low expressing cell lines, was almost undetectable

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(Figure 5.5.b). These results suggest that CTCF is preferentially bound to *BCL6* exon1 in cells where BCL6 is highly expressed.

To further investigate whether CTCF binding to the *BCL6* gene was specific to the exon1 site several amplicons at the *BCL6* regulatory region were analyzed by ChIP in Ramos cells (Figure 5.6.a). A region spanning ≈1500 bp was explored using three different sets of primers. As shown in figure 5.6.b. high CTCF enrichment was observed only with the primers covering the predicted CTCF BS in exon 1.

The variant histone H2A.Z has been reported to be a CTCF cofactor (Yusufzai et al., 2004) and to colocalize with CTCF genome wide (Barski et al., 2007). H2A.Z incorporation alters the stability of nucleosomes and is frequently detected in promoters of active genes or genes poised for activation (Sharma et al., 2010). We therefore asked whether H2A.Z binding was detected in the *BCL6* exon 1 regulatory region in Ramos cells. ChIP analysis revealed specific accumulation of H2A.Z at CTCF binding site of *BCL6* (Figure 5.6.c).

All together these results show specific interaction of CTCF with the predicted site on BCL6 gene exon 1 regulatory region.

#### **5.2.6. CTCF effects on *BCL6* promoter activity**

What could the function of CTCF at the *BCL6* promoter be? Luciferase assays were carried out to determine whether CTCF had any effect on the activity of the *BCL6* promoter. The effects of enforced and reduced CTCF expression on the *BCL6* promoter-exon-1 activity were examined in both DG75, a BCL6 expressing cell line, and in K562, a BCL6 negative cell line. These cell lines were selected because they can be very efficiently transfected (see Figure 2.2 in Material and Methods section). CTCF was efficiently silenced using plamid encoding for a shRNA against CTCF (see below figure 5.8 b and c). Overexpression of CTCF was achieved by transfecting both cell lines with a GFP-CTCF plamid (see figure 5.8.d).

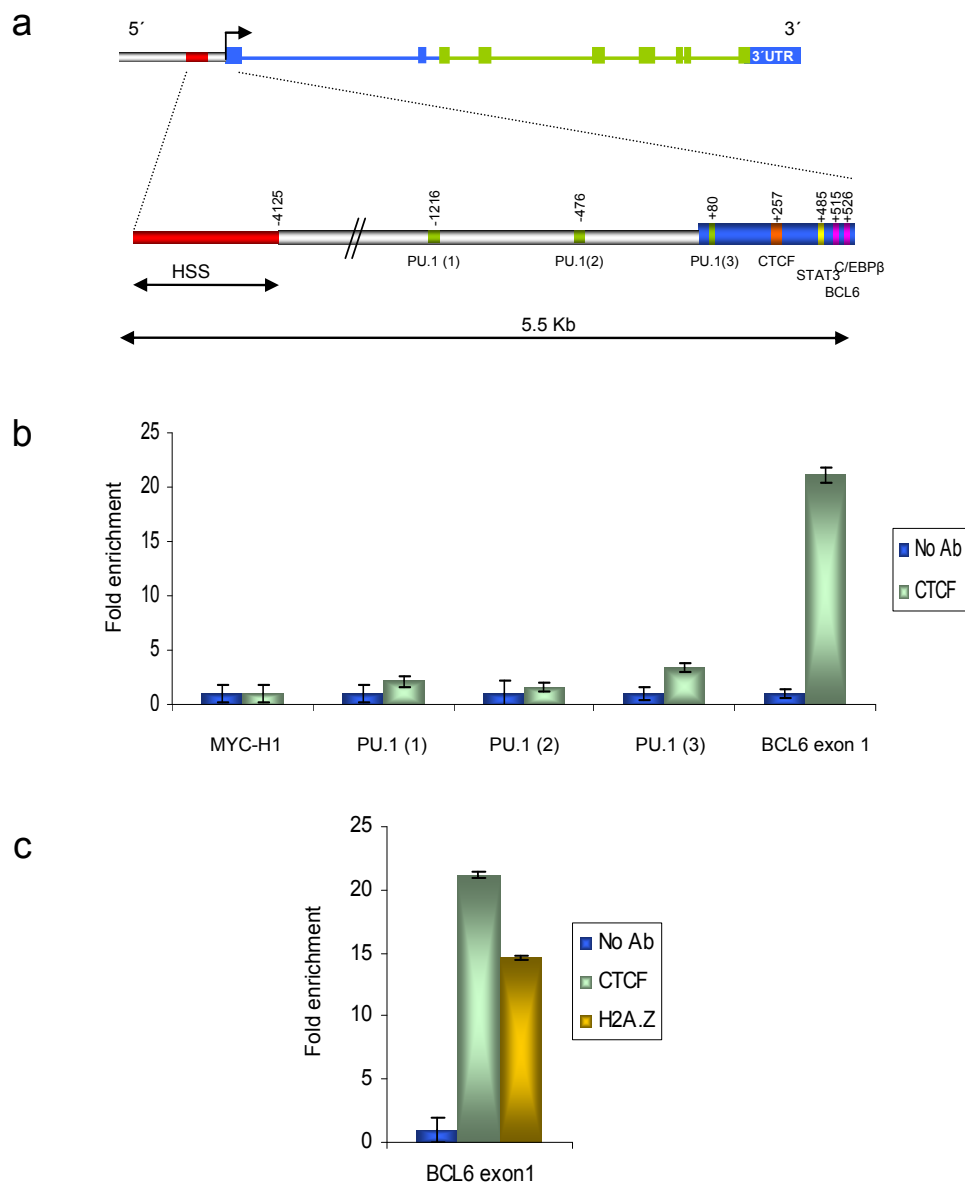


**Figure 5.6. Occupancy of the *BCL6* exon1 by CTCF and H2A.Z** (a) Scheme of the *BCL6* regulatory region, showing the relative position of several binding sites for different transcription factors that have been implicated in the *BCL6* regulation (b) ChIP assays were performed on the *BCL6* regulatory region in Ramos cells. Chromatin was immunoprecipitated with or without CTCF antibodies and subjected to quantitative PCR for the indicated amplicons. The fold enrichment was calculated as described in the Material and Methods section. Values are the mean and SD of duplicate determinations from two independent experiments. (c) Binding of CTCF and histone variant H2A.Z to *BCL6* exon1. ChIP analysis was performed in Ramos cells as in b.

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To perform these reporter assays, four different pGL3 reporter constructs containing various *BCL6* gene fragments were used (Figure 5.7). To investigate whether a recently discovered 5'upstream DNase I hypersensitive site (Papadopoulou et al., 2010) was influencing the CTCF effect two further constructs containing this hypersensitive site, were employed.

Reporter constructs were co-transfected with either a vector (pRS-CTCF) containing a CTCF-shRNA, which resulted in a significantly CTCF reduced expression or with CTCF expression vectors (pcDNA-CTCF or pEGFP-CTCF). Consistent with our earlier studies on non-expressing *BCL6* cells, the basal activity of the reporter constructs that contained the proximal exon1 region, was significantly higher when compared to the ones that lacked that region in K562 cells. Reduced expression of CTCF resulted in a dramatic increase in the reporter activity of the exon 1-*BCL6* constructs (figure 5.7.b). The rise in the reporter activity was not observed in those constructs that did not contain the CTCF BS. The DNase I hypersensitive site (HSS-4.4) seems to have little impact on the CTCF effects (figure 5.7.b).

Transfections were also carried out in the *BCL6* expressing cell line DG75. Compared to the K562 cells the plasmids containing the exon 1 region showed little basal reporter activity on the DG75 cells. This was not surprising, since the exon-1 region also contains an important autoregulatory *BCL6* binding site, that has previously been shown to reduce expression from the gene (Pasqualucci et al., 2003). Interestingly, CTCF silencing did not significantly modify the reporter activity in DG75 cells, suggesting, that *BCL6* binding to its own promoter has a stronger effect than the induced by the CTCF silencing (Figure 5.7.c). On the contrary, the reporter activity on the DG75 cells rose as a result of overexpressing CTCF using two different expressing vectors (Figure 5.7.d,e). This effect required the full length-CTCF protein, as cotransfections with individual CTCF domains (ZF or C-term domains) had no significant effect on the reporter gene activity.

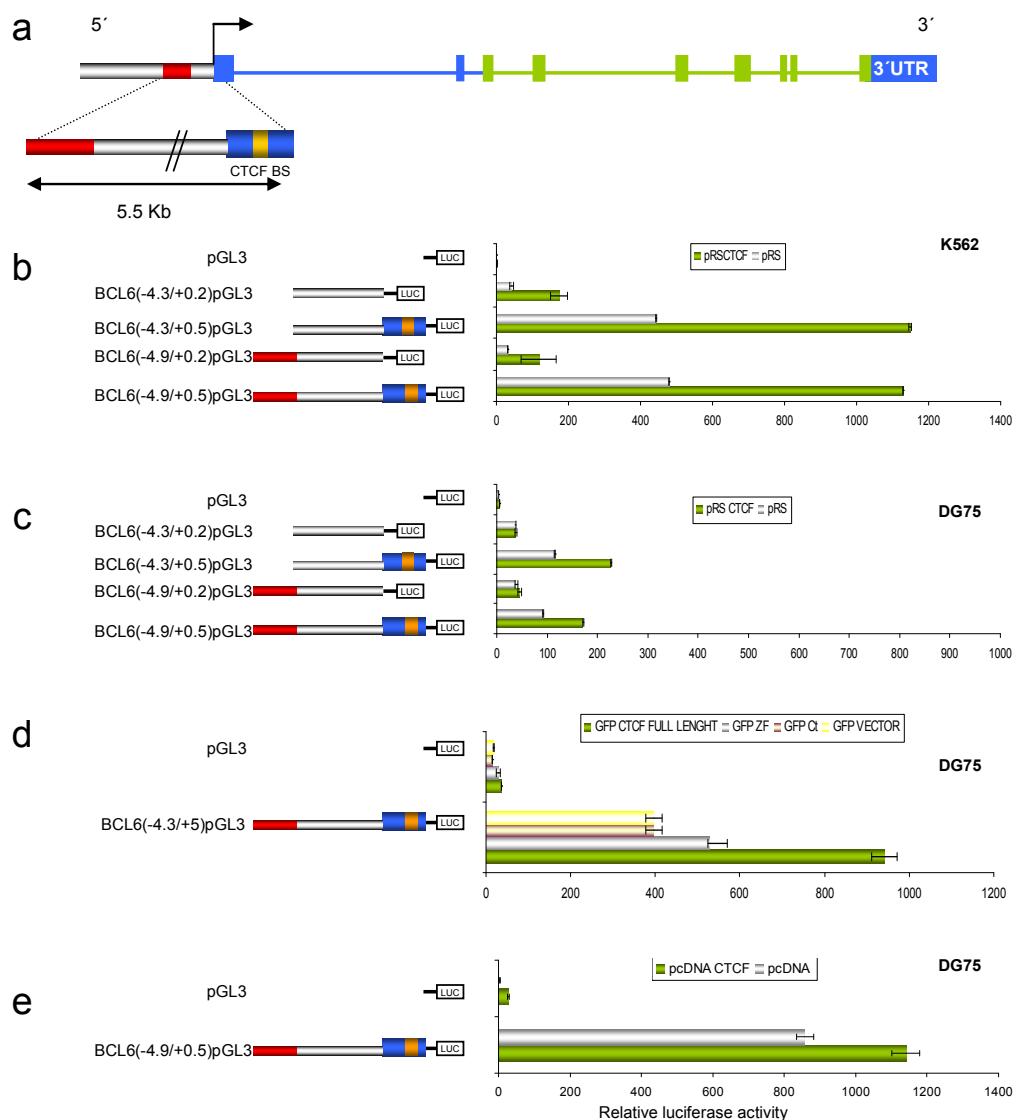


**Figure 5.7. CTCF effect on the transcriptional activity of the *BCL6* promoter** (a) Schematic representation of the *BCL6* gene and regulatory region indicating the 5' hypersensitivity site (red box) and the CTCF binding site (orange) (b, c) Luciferase assays showing the effects of silencing CTCF expression on *BCL6* promoter activity. K562 cells (b) or DG75 cells (c) were transiently cotransfected with either a pRS empty vector or a pRS-CTCF vector, and with different PGL3*BCL6* reporter constructs (indicated on the left) and with a pRL null plasmid. SD values of duplicates from 2 to 5 independent experiments are indicated. (d, e) Luciferase assays showing the effects of CTCF ectopic expression on *BCL6* promoter activity. DG75 cells were transiently cotransfected with either a pGL3 empty vector or with the pGL3/*BCL6* indicated constructs and with a pRLnull plasmid and with (d) pEGFP empty vector, pEGFP-ZF, pEGFP-Ct, or pEGFP-CTCF full length constructs or (e) pcDNA or pcDNACTCF vectors. SD values from two different experiments are indicated. pRL-null was used to normalize for transfection efficiency. Luciferase activity is shown as the increase in activation relative to the activity of the pGL3 vector alone.

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Unfortunately, despite repeated efforts, it was not possible to over-express CTCF in K562 cells, since the cells rapidly stopped growing, presumably due to the CTCF negative effect on the cell proliferation as previously reported (Rasko et al., 2001; Torrano et al., 2005).

#### **5.2.7. CTCF effect on BCL6 expression**

The effects of transient CTCF reduction and enforced expression on BCL6 mRNA and protein levels (figure 5.8.) were examined to determine further the CTCF regulatory effect on BCL6 expression,

CTCF silencing was achieved 48 hours after transfection with the pRS-CTCF vector and this effect was maintained until, at least, 96 hours (Figures 5.8.a to c). CTCF silencing in DG75 cells resulted in a small but reproducible down-regulation on both BCL6 mRNA (figure 5.8.a) and protein expression (Figure 5.8. b). As expected, after effectively silencing CTCF there was no effect on BCL6 protein expression in K562 cells (Figure 5.8.c).

Ectopic expression of CTCF was observed as soon as 48 hours after transfection (Figure 5.8.d). The CTCF exogenous protein was easily differentiated from the endogenous one on a western blot, because the exogenous CTCF was fused to the EGFP protein, and therefore migrated more slowly than the endogenous one. Over-expression of CTCF in DG75 cells showed no impact on BCL6 expression (Figure 5.8.d).

In summary, the effects of CTCF differ depending on whether BCL6 is expressed. It appears to restrain BCL6 transcriptional activity in cell lines that lack BCL6 protein expression. However, in BCL6 expressing cell lines reduction in CTCF caused a small reduction in BCL6 mRNA expression.

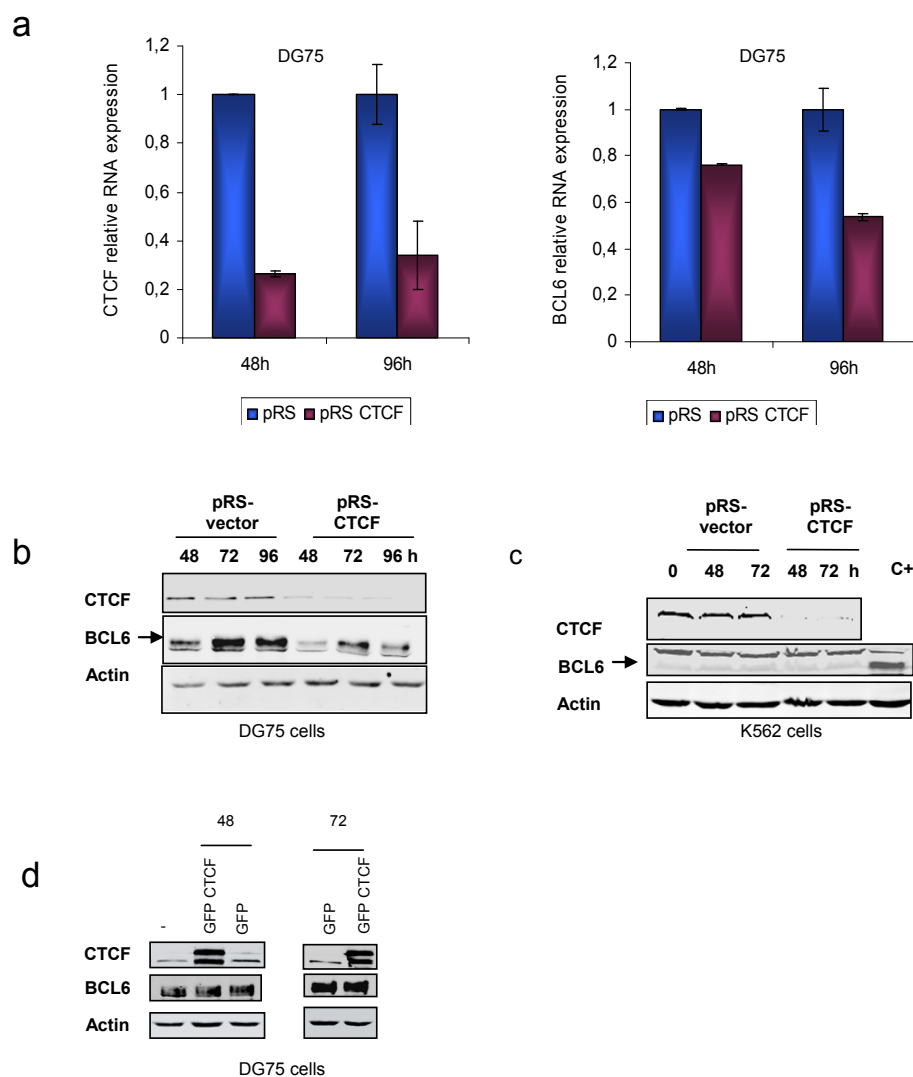


**Figure 5.8. Silencing CTCF effects on BCL6 expression** (a) RT-PCR showing silencing of CTCF and decreased BCL6 mRNA expression. DG75 cells were transfected with either an empty vector or pRS-CTCF plasmid. CTCF (left panel) and BCL6 (right panel) relative mRNA expression were analyzed at the indicated times. (b,c) Western showing CTCF and BCL6 expression on DG75 (b) and K562 (c) cells after silencing CTCF. Arrow indicates BCL6 protein as shown by the BCL6 positive control (Ramos cell lysate). The upper band in c is a non specific band (d) BCL6 and CTCF expression in DG75 cells transfected with a pEGFP empty vector or with a pEGFP-CTCF plasmid.

### 5.2.8. CTCF effects on the cell cycle of BCL6 positive and negative cell lines

CTCF has important roles on cell cycle regulation as well as apoptosis. Interestingly, regulation of cell proliferation and apoptosis is crucial in germinal centre

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cells, although not completely understood. We therefore wished to determine whether CTCF might be involved in cell cycle regulation of germinal centre cells. For this reason we transiently transfected either a CTCF expression vector or a pRS-CTCF vector into DG75 and MUTU III cell lines which are, respectively, BCL6 expressing and non-expressing lymphoma cell lines (Figure 5.9.). In both cell lines silencing of CTCF had very little effect on the cell cycle (Figure 5.9.a.). Conversely, enforced expression of CTCF induced significant cell cycle arrest and this effect was stronger in the BCL6 negative cells (MUTU III) than in the positive ones (DG75) (Figure 5.9.b). It is not clear from these experiments whether the *BCL6* exon 1 CTCF binding site is required for these effects and further experiments are required to elucidate its exact role.

### 5.3. DISCUSSION

Based on the previous reporter assays results, a 300 bp region located on the 5' region of exon-1 was shown to have important basal transcriptional activity and has a high concentration of transcription factor binding sites. Thus, this small region is probably required for an adequate BCL6 regulation. Upon investigation of potential transcriptional regulator binding sites in this area, a binding site for CTCF, a multivalent DNA binding factor was identified.

EMSA experiments revealed that *in vitro* CTCF binding to the *BCL6* exon 1 region was specific and not methylation dependent. The latter result was expected as the predicted CTCF binding site had no CpG dinucleotides. However, since the CTCF BS was identified inside a CpG island (see chapter 6), the analysis of its possible methylation sensitivity binding was considered necessary. ChIP assays confirmed that CTCF interacts *in vivo* with the *BCL6* regulatory region. Moreover, CTCF occupancy was associated with high BCL6 protein expression.

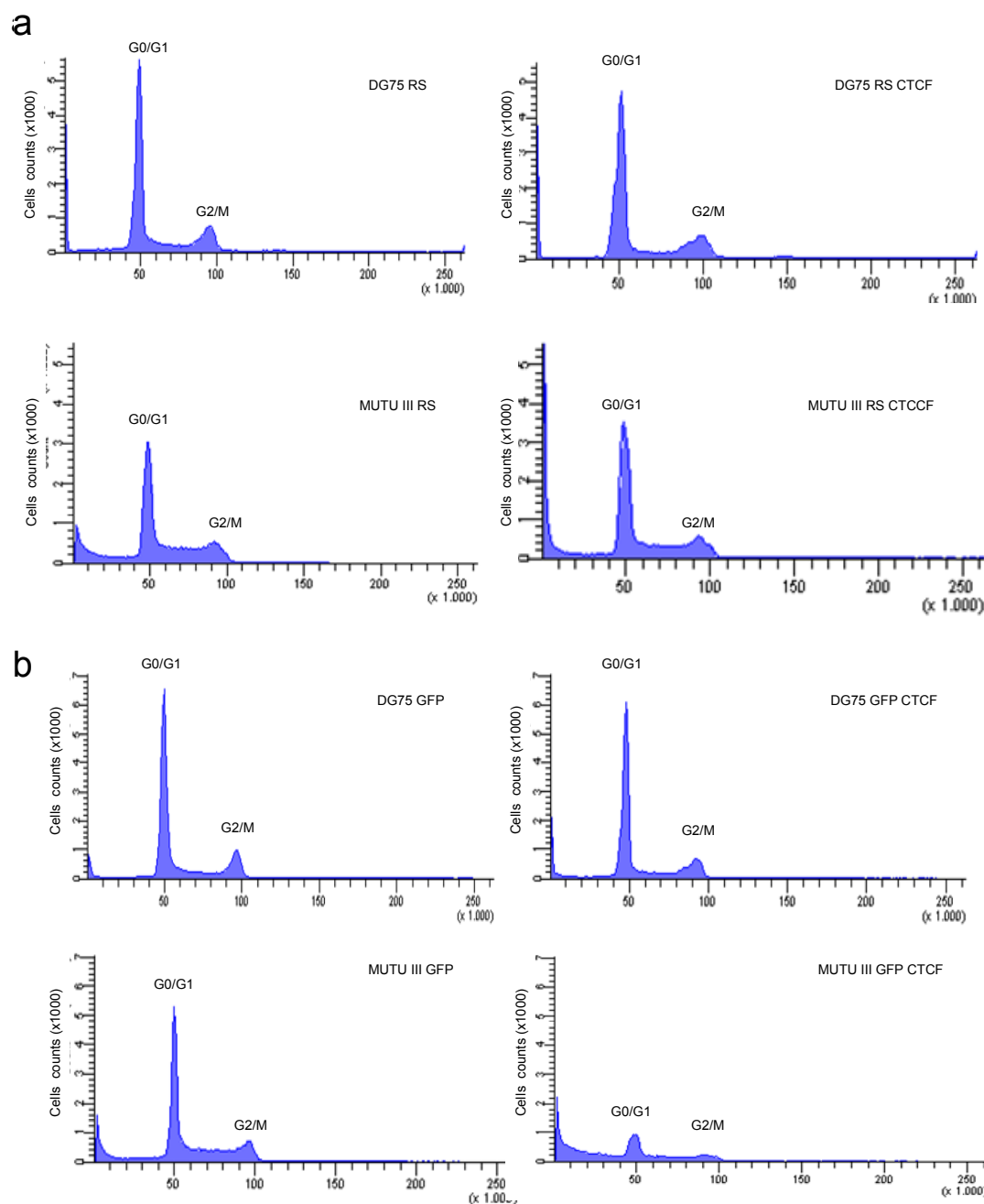


**Figure 5.9. CTCF effect on the cell cycle in lymphoma cell lines. (a)** CTCF knockdown. DG75 (upper panels) and MUTU III cells (lower panels) were cotransfected with GFPmax vector and with either a pRS vector (left panel) and a pRS CTCF plasmid (right panels). After 48 hours of transfection cells were separated using a FACS-SORTER ARIA II based on the GFP fluorescence expression. GFP positive cells were then allowed to recover for 24 hours and stained with propidium iodide for cell cycle analysis. **(b)** CTCF overexpression. DG75 (upper panels) and MUTU III cells (lower panels) were transfected with either a pEGFP empty vector (left panels) or with a EGFP-CTCF plasmid (right panels). After 48 hours cells were separated using a FACS-SORTER ARIA II based on the GFP fluorescence expression. GFP positive cells were then allowed to recover for 24 hours and stained with propidium iodide for cell cycle analysis

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To provide insights into how CTCF was regulating BCL6 expression, we transiently transfected reporter gene constructs containing the CTCF binding site, with either CTCF expressing vectors or vectors encoding for a shRNA against CTCF into BCL6 non expressing K562 cells, or expressing DG75 cells. In line with my previous results, basal activity of those constructs composed of the 5' extreme of the *BCL6* exon 1 was significantly higher in BCL6 non-expressing cells than in BCL6 positive ones probably because of the exon1 BCL6 binding site that is known to serve as a potent negative autoregulatory domain. Surprisingly, CTCF silencing on K562 cells significantly increased the reporter activity of those constructs containing the CTCF BS. Although these results seem to contradict our ChIP results, the discrepancy might be explained by the existence of CTCF indirect effects that might be contributing to the inhibition of BCL6 expression on those cells. Indeed, CTCF binding was almost undetectable in cell lines with BCL6 low expression supporting the idea that the BCL6 potential downregulation in these cell lines is probable mediated by CTCF indirect effects. It is also noteworthy that overcoming CTCF inhibitory effect is not sufficient for inducing BCL6 expression in K562 cells.

In contrast to K562 cells, silencing of CTCF in the BCL6 positive cell line, DG75, did not produce a significant change on the reporter activity. As discussed above, the BCL6 autoregulatory domain might have an overall effect on the reporter activity stronger than the induced by the CTCF silencing. Thus, the BCL6 interfering effect on the reporter assays make these two models, BCL6 positive and negative cell lines, not suitable for comparison. It is interesting that enforced expression of CTCF, using different CTCF expressing vectors, led to a reproducible increase in the reporter activity, suggesting that in BCL6 expressing cells CTCF might be acting as a transcriptional activator. In support to this conclusion, CTCF silencing led to a moderate but consistent reduction in both BCL6 mRNA and protein levels and also, ChIP assays showed that BCL6 expressing cells had considerable higher CTCF occupancy rates compared to those BCL6 negative cell lines. On the contrary, CTCF over-expression did not induce a

measurable change on BCL6 expression. This latter effect might be due to the strength of the endogenous CTCF effect, since these cells were shown to have high basal levels of CTCF protein. Indeed it has been suggested that CTCF alone has minimal effect on reporter gene expression (Phillips and Corces, 2009).

Several additional hypotheses emerge from these experiments. CTCF may have a role in the regulation of BCL6 but whether the CTCF effect on BCL6 regulation differs in BCL6 positive and negative cell lines, as suggested by our preliminary reporter assays, requires further investigation. Another prediction is that CTCF could be recruiting different protein complexes in a cell-type specific manner that could potentially have opposing functions. Another possibility would be that CTCF induces different epigenetic modifications at the BCL6 regulatory region or/and maybe CTCF could be responsible for long- distance interactions inducing the formation of loops or hubs (see general introduction). Some of these possibilities will be addressed in the next chapter.



## **CHAPTER 6**

### ***Epigenetic modifications and the regulation of BCL6 gene***



## CHAPTER 6. Epigenetic modifications and the regulation of *BCL6* gene

### 6.1. INTRODUCTION

In normal conditions BCL6 is highly expressed in B-cell within the germinal centre, suggesting that BCL6 expression relies on an exquisite and tight regulatory mechanism. In lymphomas BCL6 expression is variable. Constitutive over-expression of BCL6 can be accomplished by chromosomal translocations and promoter substitution whereas in other cases point mutations at the BCL6 binding site in exon 1 are responsible. However, there are cases in which abnormal expression of BCL6 cannot be explained by any of these mechanisms. This uncertainty opens a question, is there a role for the epigenetic modifications in the BCL6 regulation?. CTCF has been involved in the epigenetic regulation of several genes (Filippova, 2008; Klenova and Ohlsson, 2005; Recillas-Targa et al., 2006) and in the previous chapter a potential role of CTCF in the regulation of BCL6 was demonstrated, and the involvement of CTCF in such regulation of BCL6 was tested.

Epigenetic modifications include DNA-methylation, histone post-translational modifications and chromatin remodelling (Esteller, 2008; Jones and Baylin, 2007). These modifications play crucial roles in the control of gene expression and nuclear architecture. Epigenetic modifications and their potential role in diagnosis and treatment of cancer are active areas of research (Esteller, 2008; Sharma et al., 2010). Since the epigenetic status of the *BCL6* gene has not been investigated previously, in this study the epigenetic influence on BCL6 expression in lymphoma cells lines and in primary lymphoma cells was explored.

## 6.2. RESULTS

### 6.2.1. BCL6 expression is modulated by epigenetic modifications

Could BCL6 expression be modulated through epigenetic mechanisms such as DNA methylation and histone deacetylation? To assess this possibility a variety of different BCL6 positive and negative cell lines were treated with 5-aza-2'-deoxycytidine (5-azadC), a methyltransferase inhibitor that impedes *de novo* methylation, as well as with Trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor. As TSA treatment alone has been previously been shown not to be sufficient to up-regulate genes with methylated promoters (Rodriguez et al., 2008 and references therein), we decided to combine TSA with 5-aza-dC. BCL6 relative mRNA levels were measured by RT-PCR to study the effect of these drugs on BCL6 expression (Figure 6.1).

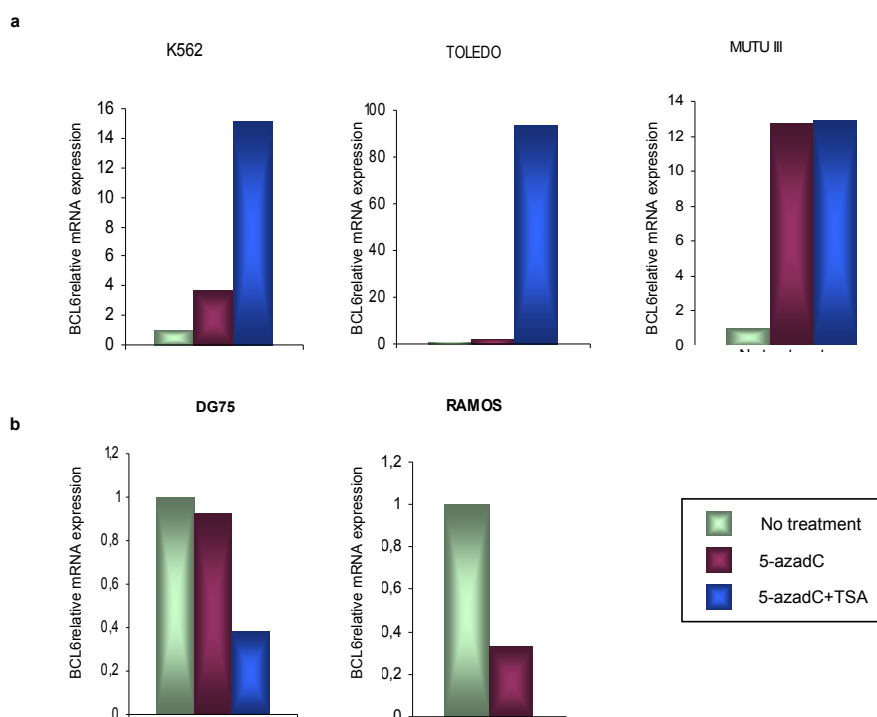


**Figure 6.1. Treatment with demethylating agents and HDAC inhibitor agents induces BCL6 expression.** RT-PCR analysis showing relative mRNA levels of BCL6 in cells untreated or treated with 5-azadC for three days or with 5-azadC for three days and TSA for three additional days as indicated. The experiments were performed in K562, Toledo, MUTU III cell lines with undetectable BCL6 expression (a), and in DG75 and Ramos BCL6 expressing cell lines (b). Values are means of duplicate determinations.

Treatment of different BCL6 negative cell lines with 5-azadC for three days resulted in a significantly increase on the BCL6 mRNA levels. Furthermore, BCL6 mRNA expression was dramatically increased when TSA was added to the medium for three additional days (Figure 6.1.a). In contrast, the BCL6 mRNA levels after treatment with 5-azadC showed either non significant difference or even a decrease, on the two BCL6 positive cell lines evaluated (Figure 6.1.b). In conclusion, results with these different chromatin modifying agents indicates a role of DNA methylation and histone modification in BCL6 gene regulation.

### 6.2.2. Methylation status of the BCL6 regulatory region

The above results suggest a role for epigenetics in the regulation of *BCL6* gene expression. A search was carried out for possible CpG islands within the *BCL6* exon 1 region because the 5' regulatory regions of genes are usually rich in CpG dinucleotides. The *BCL6* gene sequence NG\_007149.1. (NCBI website) was used. It should be noticed that the transcription start site was defined attending to the Bernardin et al work (Bernardin et al., 1997). 20 kb of the *BCL6* regulatory region were searched in the Methyl Primer Express software (Applied Biosystems) and the parameters used to find CpG islands were set as follows: minimum length of the island: 300bp; maximum length of island: 2000bp; C+Gs/Total bases > 50%; CpG observed/CpG expected >0.6.

Several intermediate-small CpG islands were found in the sequence, and interestingly, the *BCL6* exon 1 region containing the CTCF BS, is located within the first predicted CpG island (Figure 6.2.a). We therefore speculated that CTCF might be regulating the methylation pattern of *BCL6* gene regulation maybe controlling and impeding methylation progression, as it has been shown previously in other genes (De La Rosa-Velazquez et al., 2007; Renaud et al., 2005; Soto-Reyes and Recillas-Targa, 2010).

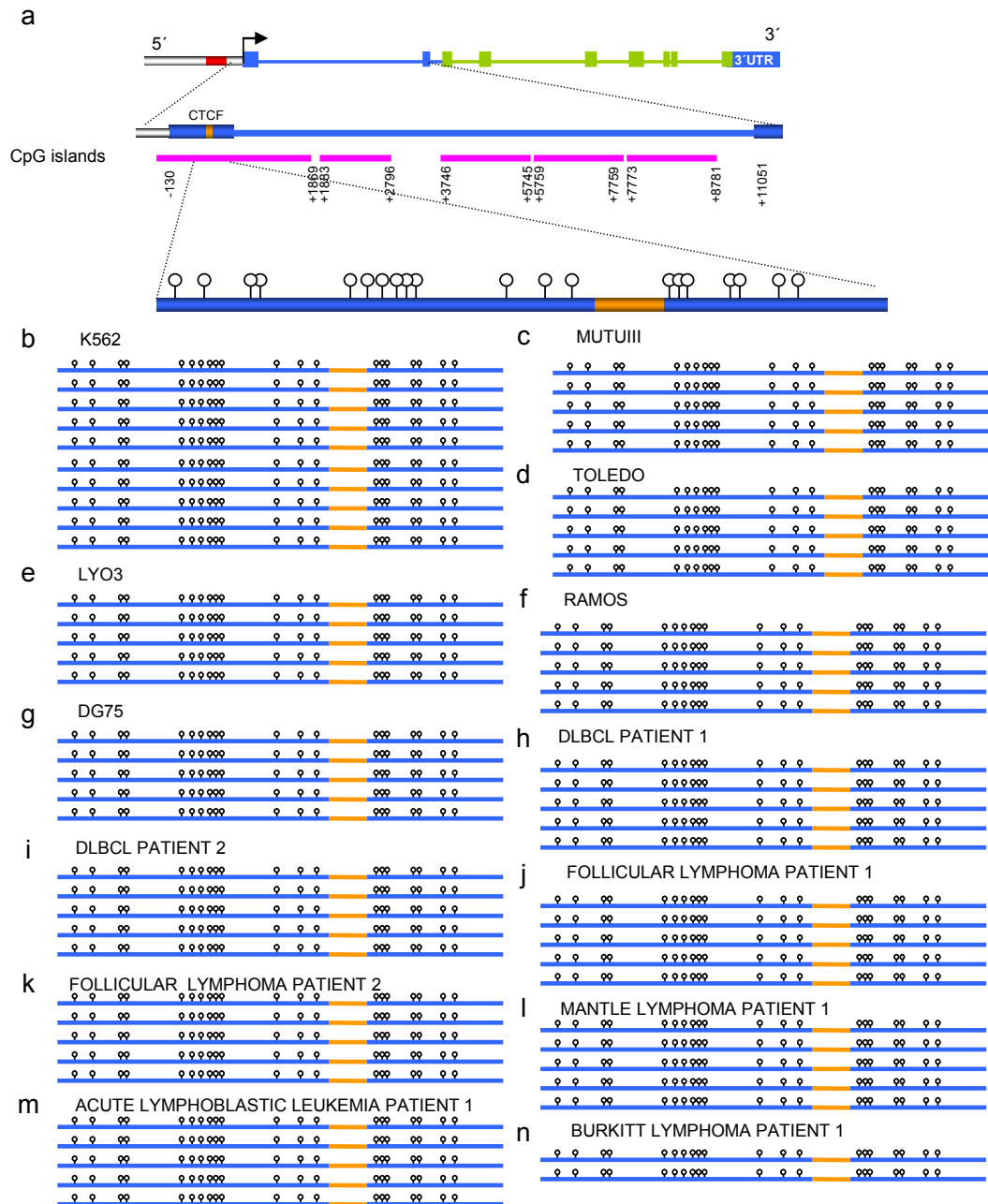


**Figure 6.2. Methylation status of BCL6 exon1 regulatory region** (a) Schematic view of the BCL6 gene, BCL6 proximal regulatory region and CpG islands. The CTCF binding site on the BCL6 exon1 is indicated. (b) DNA methylation status of the BCL6 gene was investigated by sequencing of bisulfite-modified genomic DNA of three cell lines with undetectable expression of BCL6 protein (b) K562 (c) MUTU III (d) Toledo, of three BCL6 expressing cell lines (e) LY03 (f) Ramos (g) DG75 and of (h-i) two diffuse large B cell lymphoma patient (DLBCL) samples (j-k) two follicular lymphoma lymphoma patientes (l) one mantle lymphoma (m) one acute lymphoblastic leukemia patient sample. (n) one Burkitt lymphoma patient. Unmethylated CpG dinucleotides are indicated as open circles.

For this purpose “Bisulfite modification Primers” (BSP) were designed using the Methyl Primer Express program, so that we could investigate the methylation status of the 10 CpG dinucleotides located upstream and the 7 downstream, of the CTCF binding site (Figure 6.2.a). Sodium bisulfite treated genomic DNA from three BCL6 expressing and three non-expressing cell lines were employed for PCR utilizing the BSP specific primers. The PCR product was then directly sequenced. Since in some cases the results of the direct sequencing were not clear enough, presumably due to the coexistence of methylated and non methylated sequences within a PCR product, we cloned the PCR products into the pGEM-T easy vector. Five to ten different clones of each cell line were thereafter sequenced. In all the cell lines evaluated the 17 CpG dinucleotides analyzed were unmethylated (Figure 6.2.b to f).

To further investigate the methylation status of that region, sodium bisulfite treated genomic DNA from seven patients suffering from a variety of B neoplasms (2 diffuse large B cell lymphoma (DLBCL); 2 follicular lymphoma (FL); 1 mantle lymphoma (ML); 1 Burkitt lymphoma (BL); 1 acute lymphoblastic leukaemia (ALL), were PCR amplified, cloned and sequenced. Again, none of the CpG dinucleotides studied were methylated in any of the patients (Figure 6.2.h to n). All together, these results indicate that differential methylation of the *BCL6* exon1 regulatory region including the CTCF BS, is not associated with regulation of BCL6 expression.

### 6.2.3. Histone marks present at *BCL6* exon 1 region

Histone functions are modulated through a variety of post-translational covalent modifications. The histone marks are truly epigenetic events that work by modifying the accessibility of chromatin or by recruiting different protein complexes involved in the regulation of the transcription (Sharma et al., 2010). The HDAC inhibitor TSA altered BCL6 expression (see Figure 6.1) suggesting a potential role for histone acetylation in regulating BCL6. For this reason, the presence of modified histones at exon1 in the

BCL6 positive cell line, Ramos, and in the non expressing BCL6 cell line, K562 was tested. (Figure 6.3.).

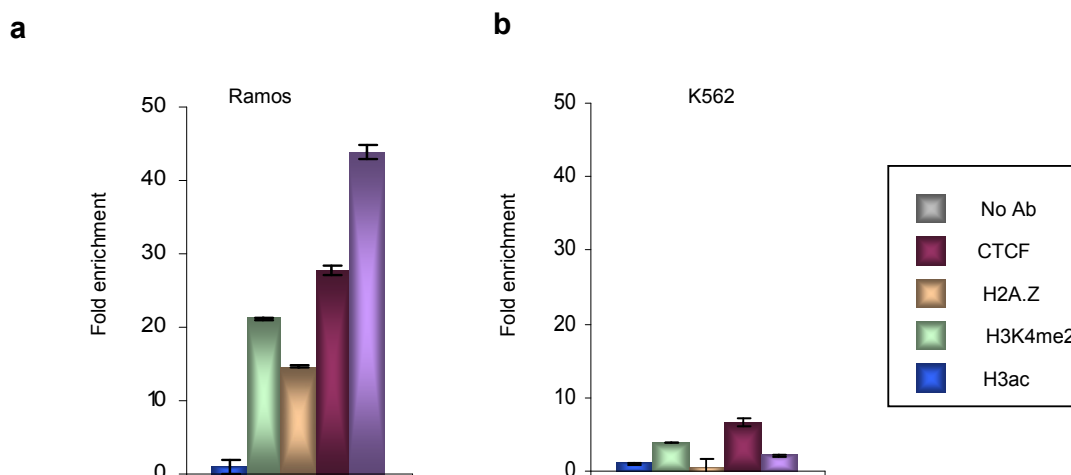


**Figure.6.3. Binding of modified and variant histones to the *BCL6* exon1 regulatory region.** Chromatin was prepared from Ramos (a) and K562 cells (b). Protein-DNA complexes were immunoprecipitated with antibodies against CTCF, H2A.Z, H3K4me2 and H3ac, as indicated. ChIP analysis shows specific binding to the *BCL6* exon1 region.

Ramos cells showed a prominent enrichment of active chromatin marks, such as histone H3 lysine 4 dimethylated (H3K4me2) or histone H3 acetylated (H3ac) as well as variant histone H2A.Z, and those levels were much higher than those observed in the K562 cells (Figure 6.3.). Collectively these results show a clear association between CTCF binding, presence of active histone marks at *BCL6* exon 1 regulatory region and *BCL6* expression. It is therefore, tempting to speculate that the *BCL6* exon 1 region, which seems to be an important regulatory region, might be modulated through changes in chromatin conformation. CTCF may be playing a crucial role in such *BCL6* gene regulation.

### 6.3. DISCUSSION

BCL6 regulation is crucial for a normal adaptive immune response. Deregulated expression of BCL6 occurs in many lymphoproliferative diseases, but the exact mechanisms of BCL6 regulation are unknown.

The aim of this section of the thesis was to determine whether BCL6 expression was regulated by epigenetic modifications, such as DNA methylation and histone modifications. The rearrangement status of the *BCL6* gene in several BCL6 positive and negative cell lines was investigated, because those cell lines without translocations involving the *BCL6* gene could be used for further studies. It was very interesting to discover that several copies of the *BCL6* gene with no sign of rearrangement were found even in BCL6 non expressing cell lines. This result suggested that other regulating mechanism such as epigenetic modifications might be crucial for the regulation of this gene.

It has been previously demonstrated that HDAC inhibitors are only capable of activating those genes with unmethylated promoters and that combination of both HDAC inhibitors and de-methylating agents act in a synergistic manner (Cameron et al., 1999; Meng et al., 2007; Saito et al., 2006) and maybe required for a complete gene reactivation. Therefore, as an initial approach we studied the effects of the de-methylating agent 5-azadC and of the HDAC inhibitor TSA on BCL6 expression. An impressive increase in BCL6 mRNA levels was observed in different cell lines with undetectable BCL6 expression. This finding suggested that epigenetic modifications might be crucial for the regulation of this oncogene although the effects of 5-azadC on BCL6 expression could be indirect. The epigenetic pattern of the *BCL6* gene has not previously been investigated but the methylation status of the *BCL6* gene might be responsible for BCL6 regulation in normal and malignant germinal centre cells.

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Moreover, controlling the methylation status of the promoter and regulatory regions of a gene appears to be a very appealing way of tightly regulating gene expression. It is known that CTCF is involved in the regulation of DNA methylation (Filippova, 2008; Recillas-Targa et al., 2006). This multivalent transcriptional regulator binds to the *BCL6* exon 1 region, therefore, and to find out whether CTCF may have a role in the methylation status of the *BCL6* gene further experiments were carried out. Thus, the first hypothesis was that CTCF might be acting as an insulator, blocking propagation of the methylation front. Although it is probably necessary to examine the DNA methylation status in a larger genomic area, we found lack of methylation of the 17 CpG dinucleotides around the CTCF binding site (that is itself within a CpG island) regardless of the pattern of *BCL6* expression. This result was not completely unexpected since, with some exceptions such as CpG islands within the promoters of imprinted genes or in some somatic tissue-specific genes, most CpG islands remain unmethylated during differentiation (Sharma et al., 2010).

Also, islands with low even intermediate content of CpG dinucleotides, often found in the control regions of tissue specific genes, are not usually regulated through DNA methylation but by covalent histone modifications (Kondo et al., 2008; Meissner et al., 2008; Soto-Reyes and Recillas-Targa, 2010; Weber et al., 2007). Indeed, ChIP assays support this idea, since the enrichment of H3K4me2 and of H3Ac observed in the *BCL6* expressing cell line Ramos was significantly higher than in the *BCL6* negative cell line, K562. Along with these active chromatin marks we found a notable enrichment of CTCF in Ramos, together with the H2A.Z, a histone variant often co-immunoprecipitated with CTCF and frequently detected in promoters of active genes (Sharma et al., 2010). In comparison, K562 enrichment of the H2A.Z was considerably lower. It is tempting to speculate that CTCF might be responsible for protecting the *BCL6* exon 1 regulatory region against repressive covalent histone modifications. Such a mechanism has been recently described for the CTCF regulation of the *p53* promoter (Soto-Reyes and Recillas-Targa, 2010). In addition, a similar mechanism related to the

loss of repressive histone modifications has also been proposed for the activation of the cancer-promoting gene *CLDN3* in ovarian cancer (Kwon et al., 2010). In order to confirm this hypothesis ChIP assays in BCL6 expressing cells after effectively silencing CTCF should be performed.



## **CHAPTER 7**

### ***General discussion***



## CHAPTER 7 GENERAL DISCUSSION

### 7.1. *Biological importance of BCL6*

BCL6 is an important transcriptional repressor that is essential for the development and regulation of the immune system. It has well-established functions in B-cells (Dent et al., 1997; Ye et al., 1997) and has more recently been shown to have essential roles in follicular helper T-cell development (Yu et al.; Nurieva et al.; Johnston et al.). BCL6 expression, therefore, has to be tightly regulated in B- and T-cell lineages. In addition aberrant expression is associated with the development of B-cell lymphomas (Cattoretti et al., 2005)

BCL6 expression is regulated at several levels. It is post-translationally regulated by phosphorylation and acetylation, and important cis-acting control mechanisms have been discovered. There is a negative autoregulatory mechanism that involves a BCL6 binding site in exon 1. Binding of BCL6 to this site in association with the co-repressor CtBP (Mendez et al., 2008) reduces BCL6 transcription. However, to allow full expression of BCL6 this mechanism must be suppressed. Our laboratory has recently added to this story by suggesting that elements at a DNase I hypersensitive site 4.4 kb upstream of the transcription start site (HSS-4.4) bind a protein complex also involving CtBP (Papadopoulou et al., 2010). A speculative model suggests that the transcription factor ZEB1 (binding at HSS-4.4) forms a repressive complex with BCL6 (binding at exon 1) and CtBP to shut down BCL6 transcription and when this complex is disrupted (by reduction in ZEB1 expression) BCL6 transcription takes place. Other cis-acting control sequences have also been identified: The repressive transcription factor IRF4 binds in intron 1 (Saito et al., 2007) and STAT5, which is an activating factor in primary cells (Scheeren et al., 2005), binds in exon 1. Other factors have also been demonstrated to bind at the *BCL6* promoter e.g. FoxO3a and PU.1, but their roles in lymphocytes have not been defined in detail.

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Much remains to be discovered about the lineage and temporal control of BCL6 expression. Following the discovery by others that BCL6 is induced in CML lymphoid blast crisis cell lines (Deininger et al., 2000a; Fernandez de Mattos et al., 2004), these cells were chosen to study the effects of BCR-ABL signalling pathways on BCL6 expression.

Dissection of three major BCR-ABL pathways the Ras/Raf/MEK, the PI3K/AKT/mTOR and the JAK/STAT5 as described here, showed that BCL6 regulation is not due to a single transcription factor but to the cooperation of many different signalling pathways. In this study and in agreement with Scheeren et al (Scheeren et al., 2005) STAT5, was also shown to upregulate BCL6 in BCR-ABL+ cells. However, STAT5 can not be responsible for the upregulation of BCL6 due to Imatinib since STAT5 becomes inactive in the presence of this drug. In this thesis it is shown that p38 MAPK, was responsible for driving BCL6 expression upon treatment with Imatinib.

The fact that BCL6 is not expressed in BCR-ABL cells in basal conditions despite the constitutive activation of p38 MAPK and STAT5 shows that additional strong repressive pathways must be inhibiting BCL6 expression in these cells. We demonstrated that mTOR, is one of the repressive pathways in the Z119 cell line, since its repression by Rapamycin led to a transient but significant increase in BCL6 expression. However, since we failed to achieve the same effect in other cell lines, the conclusion is that there must be other repressive pathways implicated in the BCL6 repression, which remain to be defined. A model for BCL6 regulation by BCR-ABL is depicted in [Figure 7.1](#).

### **7.2. BCL6 regulation by Imatinib**

The biological relevance of BCL6 upregulation in lymphoid BCR-ABL+ cells when treated with Imatinib is not clear. It has been shown that BCL6 upregulation in BV173 cells leads to downregulation of cyclin D2 (Deininger et al., 2001; Fernandez de

Mattos et al., 2004), which is known to be essential for proliferation. Therefore it is possible that BCR-ABL inhibits BCL6 expression to allow uncontrolled proliferation. BCL6 could also be involved in the apoptotic process induced by Imatinib in these cells. Accordingly, BCL6 has been reported to induce apoptosis in some cell types including a pro-B cell line (Albagli-Curiel, 2003).

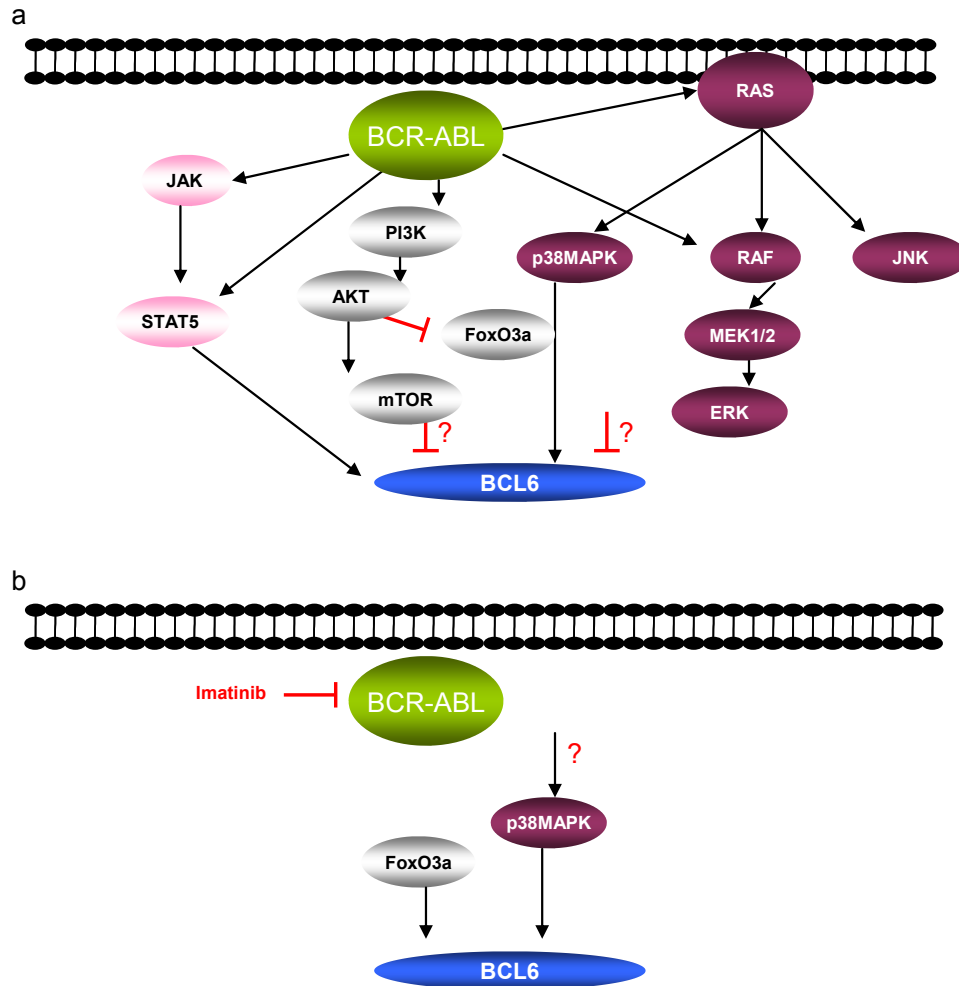


**Figure 7.1. A proposed model for BCL6 regulation by BCR-ABL in lymphoid cells.** (a) BCR-ABL can activate many signalling pathways including Stat5, Akt and P38MAPK. In basal conditions the BCR-ABL downstream signalling pathways JAK/STAT5 and p38MAPK induce BCL6 expression, and despite these strong positive signals, no BCL6 protein expression is detected, suggesting that additional strong repressive pathways must be inhibiting BCL6 expression in these cells (upper panel). Upon treatment with Imatinib (lower panel), BCR-ABL tyrosin kinase activity is inhibited and as a result the signalling pathways downstream BCR-ABL become inactive. P38MAPK remains active in these cells, supporting the idea of p38 being regulated by BCR-ABL dependent and independent mechanisms (Sanchez Arevalo Lobo et al, 2005). In BCR-ABL inactive cells (b) both the p38 MAPK and FoxO3a pathways induce BCL6 expression.

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Also, BCR-ABL could be repressing BCL6 expression in order to block the differentiation process (Duy et al., 2007). In this regard it has been shown that pre-B cells require BCL6 expression to differentiate and for the immunoglobulin (Ig) light chain gene recombination (Duy et al., 2010). Moreover, abnormal low expression of BCL6 combined with an increased expression of the protooncogene c-MYC was associated with a differentiation arrest in a number of acute lymphoblastic leukaemia cells, including the Ph<sup>+</sup> lymphoid leukaemia (Duy et al., 2007). This, however, does not explain why mice lacking BCL6 have normal B cell development at the bone marrow stage. One possible explanation is that different redundant pathways are regulating the same processes.

### **7.3. BCL6 and lymphomagenesis**

BCL6 is known to be essential for the generation of germinal centres (Cao et al., 1997). Germinal centres are areas within lymphoid nodes that are crucial for the generation of mature T cell dependent responses. Intense B proliferation together with somatic hypermutation are processes required for the production of high affinity antibodies, but these features also make a good environment for the generation of malignancy, and, in fact, over 50% of lymphomas have a germinal centre derivation.

Deregulated expression of BCL6 due to rearrangements, mutations of the negative autoregulatory BCL6 binding site in exon 1, and other aberrations, have been detected in a number of human lymphoproliferative processes with a germinal centre origin.

#### ***7.4. Biological Roles of p38 MAPK***

Here it has been shown for the first time that p38 MAPK induced BCL6 expression. The p38 MAPK pathway, can be activated by genotoxic and environmental stresses or cytokines, and has been involved in a wide range of processes. Thus it is known to mediate cellular stress responses inducing apoptosis and cell growth arrest, but it also plays key roles in the proliferation, differentiation and even cell survival processes in a number of cell types (Dong et al., 2002; Nebreda and Porras, 2000; Raman et al., 2007; Wagner and Nebreda, 2009). It is also interesting that constitutive activation of p38 can promote tumorigenesis (Greenberg et al., 2002) and even favour metastatic spread of cancers (Ito et al., 2006).

Interestingly there are some previous reports suggesting that p38 might also be regulating BCL6 in other cell types. Both p38 and BCL6 were upregulated in differentiating C2C12 myoblasts, a cell line which undergoes differentiation when cultured in serum-free medium (Kumagai et al., 1999; Nebreda and Porras, 2000) and both proteins were required to induce growth arrest and differentiation of those cells. Similarly, high concentrations of calcium induces differentiation in keratinocytes and, after calcium stimulation both BCL6 and p38 are activated (Efimova et al., 2002; Yoshida et al., 1996).

#### ***7.5. p38 MAPK and germinal centres***

Transfection of a BCR-ABL p210 vector produced a significantly decrease in the BCL6 protein expression in Burkitt's lymphoma cell lines, which are representative of germinal centre B-cells. Thus, the same pathways that operate in CML lymphoid blast crisis cell lines might also be important for BCL6 regulation in germinal centre cells. Both STAT5 and p38 were shown to be enhancers of BCL6 in these cells. Although STAT5 was previously shown to upregulate BCL6 in primary B tonsillar cells (Scheeren et al., 2005), the p38 effect on the BCL6 regulation in germinal centre cells has not been

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reported before.

Interestingly, mice with homozygous disruptions of the locus of the MAPK kinase kinase, MEKK1, have a very similar phenotype to that of mice lacking BCL6. Thus, these animals have defects in germinal centre formation with abnormal T-dependent antibody responses and also have an increased production of Th2 cytokines. The normal effects of MEKK1 were shown to be dependent on the activation of the JNK and p38 pathways (Gallagher et al., 2007). Therefore we speculate that the B-cell phenotype of MEKK1 deficient mice is due to reduced or absent induction of BCL6 by p38MAPK, and this remains to be proved in future work.

#### **7.6. p38 MAPK, BCL6 and germinal centre derived lymphomas**

Burkitt's lymphoma is an example of a germinal centre derived non-Hodgkin's lymphoma. It is highly proliferative and shows surface IgM expression. The immunoglobulin genes show somatic hypermutations and this process is on-going in at least one Burkitt's lymphoma cell line, Ramos (Sale and Neuberger, 1998). One of the defining characteristics of Burkitt's lymphoma according to the WHO classification is the expression of BCL6, without chromosomal translocations involving the *BCL6* locus. It is worth mentioning that constitutive activation of p38 in several Burkitt's lymphoma cell lines has been previously demonstrated (Horie et al., 2007; Vockerodt et al., 2001). Work reported here suggested that p38 is important for the induction and/or maintenance of BCL6 expression in germinal centre cells. Although the over-expression of the c-MYC oncogene in Burkitt's lymphoma is required and of undoubted importance in terms of proliferation and cell survival, inhibition of BCL6 using specific peptides to inhibit function, was able to produce apoptosis and cell cycle arrest in these cells (Capelson and Corces, 2004). It has also been demonstrated that p38 inhibition in Burkitt's lymphoma cell lines is able to induce growth arrest (Horie et al., 2007), which may link to its role in BCL6 expression.

Expression of phosphorylated p38 MAPK is detected in diffuse large B-cell lymphoma (DLBCL) and it has been implicated in the transformation of another type of non-Hodgkin's lymphoma, follicular lymphoma, into DLBCL. Inhibition of the p38 signalling pathway induced growth arrest and apoptosis of follicular transformed cells (Elenitoba-Johnson et al., 2003). Several Burkitt's lymphoma cell lines show constitutive activation of p38 and inhibition of p38 induces growth arrest. Expression of phosphorylated p38 MAPK is detected in DLBCL and it has been implicated in the transformation of follicular lymphoma into diffuse large cell lymphoma. Inhibition of the p38 signalling pathway induced growth arrest and apoptosis of follicular transformed cells (Elenitoba-Johnson et al., 2003).

As shown previously, abnormal regulation of BCL6 has also been implicated in the pathogenesis of DLBCL and although there is less evidence it might be implicated in the pathogenesis of follicular lymphoma (Gollub et al., 2009). Abrogation of BCL6 function leads to the death of Burkitt's lymphoma cell lines and, therefore, routes to inhibit p38 may critically reduce BCL6 expression contributing to lymphoma cell death.

p38 inhibitors are under investigation for the treatment of several diseases (Coulthard et al., 2009; Munoz and Ammit, ; Navas et al., 2006; Yong et al., 2009). Furthermore, specific p38 inhibitors have been employed as anti-inflammatory targets in clinical trials with promising results (Kumar et al., 2003). These drugs might have application in Burkitt's lymphomas and maybe in other BCL6 expressing lymphomas.

BCL6 expression defines a specific subtype of DLBCL (Alizadeh et al., 2000; Polo et al., 2007). Early studies (Offit et al., 1994) showed that DLBCL with BCL6 translocations had a better clinical outcome than those without. Relative BCL6 mRNA expression also proved to be a marker of good prognosis (Iqbal et al., 2007) and extension of this work to immunocytochemistry confirmed the usefulness of BCL6 for this purpose (Hans et al., 2004). The treatment of DLBCL has been dramatically improved recently by the addition of the anti-CD20 monoclonal antibody Rituximab to the CHOP (cyclophosphamide, daunorubicin, vincristine and prednisolone)

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chemotherapy regime. However, there is currently uncertainty about whether this new combination alters the status of *BCL6* translocations as a prognostic marker (Shustik et al., 2010). One large series of patients demonstrated that *BCL6* expressing cells are relatively resistant (Winter et al., 2006) to the effects of Rituximab, whereas another (Rimsza et al., 2008) showed that *BCL6* continues to be a marker of good prognosis when this antibody is included in the treatment.

Whilst lymphoma cells coated with antibody will be removed and killed by the reticulo-endothelial system, signalling through CD20 has also been reported. Rituximab inhibits several pro-survival and anti-apoptotic pathways - AKT, NF- $\kappa$ B, ERK1/2, p38 - (Bonavida, 2007). Pathways that are modified by Rituximab have been directly or indirectly implicated in the regulation of *BCL6* (Niu et al., 1998; Saito et al., 2007).

Taken together, these results might suggest that CD20 signalling modifies *BCL6* expression through p38 MAPK. Further work will investigate how CD20 signalling modifies *BCL6* expression through p38 MAPK in B-cell lines and primary lymphoma cells and will define the role of p38 inhibitors in synergising with anti-CD20 monoclonal antibodies in the treatment of DLBCL.

Rituximab is the first developed anti-CD20 antibody but more recently a group of antibodies binding to different CD20 epitopes has been developed. These class II antibodies, of which GA101 (Roche) is one, are believed to have enhanced ADCC and superior caspase-independent apoptosis induction in comparison to Rituximab. The signalling pathways involved in the GA101 effect have not yet been thoroughly analysed.

### ***7.7. BCR and CD40 signalling effects on BCL6 expression***

Both the B-cell antigen receptor (BCR) and CD40 play key roles in the regulation of germinal centre processes. The former is necessary for antigen/B cells specific interactions while the latter mediates interactions with the CD40 ligand expressed in T CD4<sup>+</sup> cells (Hollenbaugh et al., 1992). Both receptors have been shown

to negatively regulate BCL6 expression. The ERK1/2 pathway mediates IgM induced BCL6 protein degradation (Niu et al., 1998) and CD40 stimulation leads to BCL6 transcriptional down-regulation through the NF- $\kappa$ B/IRF4 pathway (Lossos, 2007). However, it is also known that both receptors significantly activate the p38 pathway (Sutherland et al., 1996). We ought to find out the role of p38 MAPK on the BCL6 regulation after stimulation of the BCR and CD40 receptors. In agreement with previous results we found that BCR cross-linking simultaneously activated pathways that repressed (ERK1/2) and induced (p38, STAT5) BCL6. However, we showed by utilizing inhibitors of single pathways that repressive signals were stronger than the inductive. BCL6 repressive pathways (ERK1/2 and IRF4) were also stronger than the activating pathways (p38 MAPK) when Burkitt's lymphoma cell lines were stimulated with membrane bound CD40 ligand (mCD40L). The only situation in which BCL6 was further up-regulated was on stimulation with sCD40 and this was mainly due to the activation of the p38 pathway since SB202190, a p38 MAPK inhibitor antagonised this effect. Remarkably, none of the known BCL6 inhibitory pathways including the NF- $\kappa$ B/IRF4 pathway were activated by sCD40.

Collectively, these results show that, despite p38 and ERK1/2 signalling pathways belonging to the same MAPK super-family, they have opposing effects on BCL6 expression. There are precedents for such dichotomy. In macrophages activation of p38 or ERK1/2 MAPK leads to antagonistic immune responses (Mathur et al., 2004).

CD40/CD40L interaction is required for B-cell survival, antibody isotype switching and memory B cell formation (Aruffo et al., 1993; Foy et al., 1994). CD40 stimulation is also important for germinal centre formation and more controversially prevents B cell terminal differentiation (Randall et al., 1998). An interesting aspect of this work is that qualitatively different CD40 stimuli defined by responses to sCD40L and mCD40L in my experiments, produce different signalling outcomes corresponding, speculatively, to the early and late phases of CD40 signalling (Basso et al., 2004). There are several previous findings that support this idea. First, it has been shown that strong

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and weak CD40 signalling in macrophages activates p38 or ERK1/2 signalling pathways respectively and the outcome is the production of different spectrum of cytokines (Mathur et al., 2004). Second, stimulation of specific CD40 epitopes resulted in the activation of different signalling pathways and in diverse B cell responses (Sakata et al., 2000). Third, different groups of researchers using CD40 ligands from a variety of sources obtained either stimulation or inhibition of the NF- $\kappa$ B pathway in Ramos cells (Cai et al., 2008; Zarnegar et al., 2004). Fourth, our hypothesis also has some bearing on observations by others that transient or prolonged B-cell/T-cell contact produce different effects on BCL6 expression, and as a result diverse cellular outcomes. Transient CD40 signalling may be compatible with continued residence within germinal centre whilst prolonged CD40 signalling could be associated with germinal centre exit (Cerchietti et al., 2008).

Whether the different effects on BCL6 expression produced by mCD40L and sCD40L are due to different amounts or quality of CD40 cross-linking or, to differences in the kinetics of the ligand-receptor interaction remain to be determined.

### ***7.8. Non-lymphoid cell/B-cell interactions may also regulate BCL6***

CD40 signalling activates many signalling pathways including p38 and ERK1/2 in different cell lines. Correspondingly interaction of CD40 with mCD40L not only induced p38 activation but also ERK1/2. However, we demonstrated that co-culture of Ramos with untransfected mouse fibroblasts cells also showed phosphorylation of this MAPK family member, and it cannot, therefore, be a specific CD40 mediated effect. Moreover, Ramos interaction with fibroblasts alone also produced a reduction of BCL6 and this effect was mediated by the ERK1/2 pathway since it was abrogated by the PD98059 Inhibitor. Consequently ERK1/2 may be activated, *in vivo*, in germinal centre cells by cell contact mediated by receptor- ligand pairs that are not unique to lymphoid cells. For instance, integrin signalling which lowers the threshold for early activation cells

may also have a role in the later stages of germinal centre reaction (Batista et al., 2007).

### **7.9. p38 targets and BCL6**

There are several p38 targets that could mediate the p38 transcriptional regulation of BCL6. The Ccaat enhancer box binding proteins (C/EBP) family members play key roles in terms of cell differentiation and proliferation and control genes involved in inflammatory and immune responses (Chen et al., 1997). My preliminary reporter assays results showed that a small region at the beginning of the first exon of *BCL6* might be important for the p38 transcriptional regulation. It is interesting that within the exon 1 region there is a binding site for the polyvalent transcriptional regulator CTCF .

### **7.10. CTCF regulation of BCL6**

One consequence of my p38 work was that specific regions of the *BCL6* promoter, including exon 1, will be the subject of further work. A previously unrecognised CTCF binding site was discovered and CTCF is a genomic regulator implicated in a number of functions including insulator activity, transcriptional activation and repression, and regulation of the cell cycle and cell proliferation.

CTCF functions are modulated through post-translational modifications. Phosphorylation of the CTCF C-terminal serine residues by CKII interferes with CTCF regulatory function (Klenova et al., 2001) and interestingly, this phosphorylation may be dependent on the p38 MAPK pathway (Sayed et al., 2000). Therefore, it is tempting to speculate that p38 might be regulating BCL6 expression by controlling CTCF regulatory function. It has been reported a CTCF functional role in insulin-stimulated cell proliferation through ERK and AKT signalling pathways (Gao et al., 2007). Furthermore, EGF induced activation of ERK cascades resulted in increased CTCF expression in corneal epithelial cells (Li and Lu, 2005). The NFkB pathway was involved in this CTCF

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upregulation (Lu et al., 2010). Thus CTCF seems to be a downstream target protein of growth factor-induced pathways. However there are no reports on the possible regulation of CTCF by the p38 MAPK pathway. We will address this issue in the future (see below future work)

In this study, CTCF was shown to bind *in vitro* to *BCL6* exon 1 and occupancy of this site *in vivo* was selectively detected in *BCL6* expressing cells. This suggested that CTCF might be involved in the transcriptional up-regulation of *BCL6*, and accordingly several reporter assays using constructs consisting of the CTCF BS and performed in *BCL6* expressing cells showed a consistent increase in the reporter activity when those cells were transfected with two different CTCF expressing vectors. Also, CTCF silencing in germinal centre *BCL6* expressing cell lines led to a moderate but very reproducible reduction on the *BCL6* protein expression. Although no significant effect was observed in the reporter activity of the constructs containing the CTCF BS after CTCF silencing in DG75 cells, the basal activity of this constructs is low probably due to the very potent negative autoregulatory *BCL6* domain. In fact, it is known that the *BCL6* positive expressing cell lines might not be adequate models for studying the reporter activity of this region.

CTCF enforced expression in the *BCL6* negative cell line K562 led to cell arrest within less than 24 hours, and thus, it was not possible to study the effect of the CTCF over-expression on the reporter activity of the *BCL6*. However, it was possible to silence effectively CTCF in K562 cells which caused a large increase in the reporter activity of these constructs. Although this result seems to contradict our previous findings, the discrepancy might be explained by CTCF having opposing effects in different cell types. CTCF might be acting through direct and indirect effects which might be regulated in a cell type dependent way. Speculatively CTCF could be recruiting different protein complexes in *BCL6* positive and negative cell lines. However, this latter possibility is less probable since ChIP assays revealed no significant occupancy by CTCF in *BCL6* negative cell lines. An alternative explanation could be that different post-translational

modifications might be responsible for the antagonistic functions and, in fact, there are precedents for such a dichotomy. Two different CTCF forms with opposite roles in breast normal and cancer cells can be identified attending to their poly(ADP-ribosyl)ation status (Docquier et al., 2009).

Finally, specific mutations of CTCF have been shown to interfere with CTCF function in certain tumors (see introduction). Point mutations of the CTCF binding site at the H19 ICR locus interfered with CTCF binding and function (Pant et al., 2004). Surprisingly, no other specific mutations on the other CTCF's targets binding sites have been reported yet. Our preliminary results suggest that such mechanism might be operating in the *BCL6* gene, since different point mutations on the CTCF BS were found in several BCL6 non-expressing cell lines. Further studies should be conducted to study the potential repercussions of such mutations in terms of interference with CTCF binding and with BCL6 expression.

#### **7.11. Epigenetics and BCL6**

Epigenetic changes are heritable variants in gene expression that are not due to any alteration in the DNA sequence. They include DNA methylation, histone modifications and chromatin remodelling (Esteller, 2008; Jones and Baylin, 2007). One of the most well known mechanisms of silencing DNA is through DNA methylation. The methylation pattern of the whole genome in certain tumours has been extensively investigated by several groups. Generally, in cancer, it is frequent to observe a predominant hypomethylation pattern of the genome with a few genes being aberrantly hypermethylated. Investigating the methylation status of specific genes can in some circumstances lead to their use as biomarkers (Nakayama et al., 2004). The methylation status of different tumor suppressor genes has been examined by a number of research groups. However, the potential role of the different epigenetic modifications of oncogenes in differentiation and in cancer is less well understood. In particular the

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epigenetic influence in the *BCL6* gene regulation in normal conditions and in lymphomagenesis has not been thoroughly investigated.

FISH studies revealed that some *BCL6*-non-expressing lymphoma cell lines showed three or more copies of the *BCL6* gene with no sign of rearrangement. It could be possible that, despite there being extra copies of the *BCL6* gene, the *BCL6* expression is silenced in those cases through epigenetic mechanisms. Moreover, different epigenetic modifications might explain the variability on the *BCL6* expression found in different lymphoma samples, regardless sharing a common genotype. In this study the epigenetic influence on *BCL6* expression was explored.

The *BCL6* gene contains several CpG islands in the region from the promoter to the first intron. and CTCF is essential for the regulation of CpG methylation of a number of genes. (see introduction). While this Thesis was in progress, several CTCF binding sites within the *BCL6* intron 1 were described (Lai et al., 2010). At these sites, CTCF binds in a methylation dependent manner, with higher methylation (and less CTCF binding) being found in cell lines in which the *BCL6* locus was transcriptionally active. Accordingly, administration of a methyl transferase inhibitor increased CTCF binding in *BCL6* the Burkitt cell line Raji, whereas silencing of CTCF caused an increase in *BCL6* mRNA in a plasma cell line (Lai et al., 2010).

CTCF binding to the described CTCF BS within the *BCL6* exon-1 is not dependent on methylation, despite being at a CpG island. Moreover, and in agreement with previous studies (Ramachandrareddy et al., 2010), no methylation was observed at that region, despite having different levels of *BCL6* protein expression. Also, in contrast to the intronic sites, CTCF binding at the exon1A site is highest in *BCL6* expressing cell lines. The exon1A CTCF site is close (250 bp) downstream of the major transcription start site in a region that has previously been implicated in the regulation of *BCL6* (Arguni et al., 2006; Papadopoulou et al., 2010). It is likely that a complex, repressing *BCL6* transcription, involving *BCL6* itself and CtBP assembles at exon1A based on a *BCL6* binding site. We speculated that the repressive complex involved the two-handed

zinc finger transcription factor ZEB1, which also requires CtBP as a co-repressor, binding to a DNase I hypersensitive site 4.4 kb upstream of the transcription start site. The location of the exon1A CTCF site suggests that it may be important in the regulation of BCL6, and data presented here demonstrates that CTCF binding is associated with high level expression of BCL6. Silencing of CTCF in BCL6 positive DG75 lymphoma cells (this work), but apparently not the plasma cell line NCI-H929 (Lai et al., 2010), is associated with reduction in BCL6. Speculatively dismissal of CTCF from its exon1A binding site may be one of the early events switching off the BCL6 locus and allowing assembly of the repressive complex. A similar model has been previously shown within the p53 locus (Soto-Reyes and Recillas-Targa, 2010). We have found association of CTCF with active histone marks in the BCL6 expressing cell line Ramos. Others have found RNAPIII enrichment near the CTCF binding site at exon1 (Lai et al., 2010). Lack of CTCF could lead to reduced binding of RNA polymerase II, active histone marks and of the variant histone H2A.Z near the BCL6 transcription start site eventually and to lead to the incorporation of repressive histone marks, We will address all these issues in the future (see next section). A proposed model for BCL6 regulation by CTCF at exon1 based in our current results is depicted in [\(Figure 7.2\)](#).

There are many other possible mechanisms whereby CTCF may cause epigenetic changes to alter BCL6 expression. Poly(ADP-ribosyl)ation modulates CTCF functions (Klenova and Ohlsson, 2005; Yu et al., 2004) and this post-translational modification might be interfering with CTCF binding to the *BCL6* exon 1, leading to an epigenetic silencing of BCL6. This mechanism has been previously described (Soto-Reyes and Recillas-Targa, 2010; Witcher and Emerson, 2009). Alternatively, CTCF binding to the *BCL6* regulatory region might be required to protect against heterochromatin spreading, and binding to DNA might be dependent on poly(ADP-ribosyl)ation as shown for the p16 (INK4a) gene (Witcher and Emerson, 2009). It is interesting that a PARP-1 binding site has been found by our group, relatively close to the CTCF binding site (Ambrose et al., 2007).

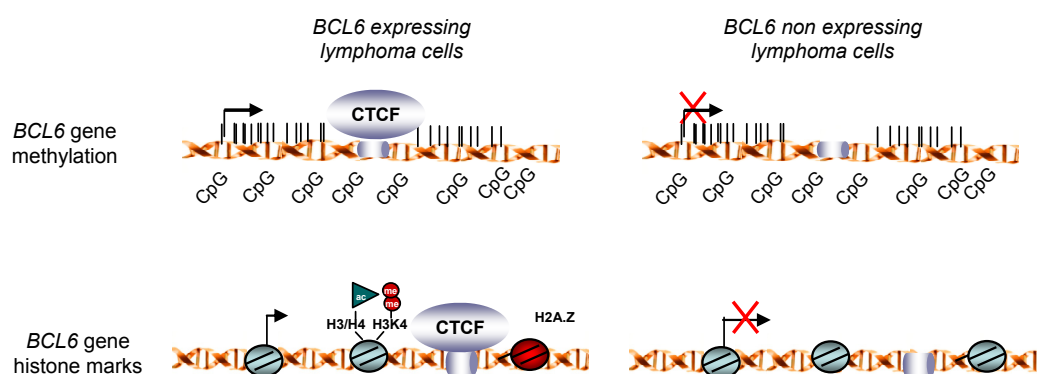


**Figure 7.2. A proposed model of BCL6 regulation by CTCF.** The CTCF binding site is included within a CpG island of the *BCL6* exon 1. However, DNA methylation of this region is not directly involved in BCL6 silencing in lymphoma cells (upper panels). CTCF and H2A.Z bind to BCL6 exon 1, favouring the incorporation of active histone modifications. BCL6 silencing is associated to lack of CTCF, H2A.Z and active histone marks binding (lower panels).

## 7.12. Summary and future work

1. BCL6 has been shown to be upregulated in lymphoid blast crisis cells upon treatment with Imatinib. Although it has been suggested that BCR-ABL represses BCL6 expression in order to block the differentiation process (Duy et al., 2007), the biological relevance of BCL6 upregulation in those cells after treatment with Imatinib is yet not clear and requires further investigation. Combination of Imatinib with specific peptide inhibitors against BCL6 might provide new insights in the biological relevance of BCL6 modulation in those cells.

2. It has been impossible using a variety of growth factors and other signals to induce BCL6 in cultured B cells and one reason for this may be that signals repressing and inducing BCL6 are produced by the same stimuli: the repressive ones being dominant. There might only be restricted circumstances *in vivo* in which positive signals predominate to cause BCL6 activation. A minority of germinal centre B-cells (~10%)

(Figure 5.7) expresses phosphorylated p38 MAPK and it may be possible that this population represents cells in which BCL6 expression is being switched on. Others (Basso et al., 2004) have shown that a minority of germinal centre cells express activated NF- $\kappa$ B, and a similar argument has been made that this is the population in which BCL6 is being down-regulated prior to exit from the germinal centre

Collectively these results reveal, for the first time, that the p38 pathway has an important role in the regulation of BCL6 in germinal centre cells. It was demonstrated that BCL6 regulation is due to cooperation between different signalling pathways and the findings suggest that the relative impact of the negative pathways is stronger than the positive ones. We have reported that sCD40L seems to activate only p38 and induces BCL6 expression.

p38 inhibitors are under investigation for the treatment of several diseases (Guo et al., 2000). Furthermore, specific p38 inhibitors have been employed as anti-inflammatory targets in clinical trials with promising results (Mihara et al., 2008). Our results suggest that these drugs might have applications in Burkitt's lymphomas and maybe in other BCL6 expressing lymphomas.

3. The important transcriptional regulator, CTCF was also shown to have a role in the regulation of BCL6, although a precise mechanism of action has not been defined. One appealing hypothesis is that CTCF might be locally organizing chromatin at the BCL6 exon1 and may be required to protect the BCL6 transcriptional start site against repressive histone marks. ChIP assays for repressive histone marks in basal conditions in Ramos cells as well as ChIP assays for active and repressive histone marks and for the RNAPol II in Ramos cells upon silencing of CTCF will be performed in order to verify this hypothesis. Also, as our major interest is to validate our findings utilising primary lymphoma cells. CTCF occupancy in primary cells from patients with BCL6 expressing lymphomas and myeloma (BCL6 negative) will be analyzed.

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TSA combined with 5-azadC led to increased BCL6 expression in the BCL6 non-expressing cell lines Toledo and K562. The effects of TSA alone will now be tested. If TSA is effective as a single agent, it is possible that TSA administration will cause increased acetylation of histone H3, leading to CTCF binding at BCL6 exon1. Altogether, these results might help to validate our hypothetical model of BCL6 regulation by CTCF shown in [Figure 7.2](#).

Finally, this work has shown that both p38 MAPK and CTCF modulate BCL6 expression through BCL6 exon-1. As CTCF function can be modulated through MAPK-mediated phosphorylation, it might be possible that CTCF regulation of BCL6 depends on the p38 MAPK signalling pathway. Future challenge will be to validate this hypothesis and to further investigate this possibility differential CTCF phosphorylation in a variety of B-cell lines after treatment with p38 inhibitors will be analyzed. Also CTCF occupancy of BCL6 exon 1 and the pattern of histone marks after treatment with p38 inhibitors will be explored.

## **CHAPTER 8**

### ***Bibliography***



## CHAPTER 8. Bibliography

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## CD40 and B-cell receptor signalling induce MAPK family members that can either induce or repress Bcl-6 expression

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### ARTICLE INFO

#### Article history:

Received 5 January 2009

Received in revised form 29 January 2009

Accepted 2 February 2009

Available online 6 March 2009

#### Keywords:

Germinal centre

Bcl-6

Transcription

MAPK

### ABSTRACT

Bcl-6 is essential for germinal centre development and normal antibody responses, and has major roles in controlling B-cell proliferation and differentiation. Bcl-6 expression is tightly controlled, but neither the nature of all the regulatory signals nor their interactions are known. Bcl-6 expression is induced in Bcr-Abl expressing lymphoid cell lines by the tyrosine kinase inhibitor, imatinib. We show that p38 MAPK mediates induction of Bcl-6 following inhibition of Bcr-Abl by imatinib. Next we analyze p38 function in a germinal centre B-cell line, Ramos. p38 is phosphorylated under basal conditions, and studies with p38 inhibitors show that it induces Bcl-6 expression. Membrane bound CD40 ligand activates p38 but also other MAPK pathways that strongly repress Bcl-6 and the overall effect is reduction in Bcl-6 expression. Surprisingly soluble CD40 ligand induces Bcl-6 by activating p38 without activating the repressive pathways. Hence different types of CD40 signalling are associated with varying effects on Bcl-6 expression. Transcription reporter assays demonstrate p38 responsive sequences at about 4.5 kb from the transcription start site. Immunocytochemistry of tonsil sections show phosphorylated p38 in a minor population of germinal centre B-cells. We demonstrate for the first time that p38 induces Bcl-6 transcription, but increased protein expression occurs only when the strong pathways repressing Bcl-6 are not activated.

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### 1. Introduction

Bcl-6 is a zinc finger transcription factor, whose expression is required for normal germinal centre formation (Dent et al., 1997; Ye et al., 1997) and, therefore, for normal high affinity antibody responses to T-dependent antigens. Bcl-6 protein expression is tightly regulated and it is not found in naïve B-cells or plasma cells (Cattoretti et al., 1995).

Bcl-6 is constitutively expressed through involvement in chromosomal translocations in 20–30% of human diffuse large B-cell lymphoma (Offit et al., 1994; Ye et al., 1995, 1993), and is expressed (largely without structural changes to the locus) in follicular lymphoma, Burkitt's lymphoma and lymphocyte-predominant Hodgkin's lymphoma. Bcl-6 allows B-cell proliferation (Reljic et al.,

2000; Shvarts et al., 2002) and prevents terminal differentiation to plasma cells (Diehl et al., 2008; Reljic et al., 2000; Tunyaplin et al., 2004).

Understanding the regulation of Bcl-6 expression has been the focus of work by several groups firstly, because this protein is fundamental to germinal centre development, and, secondly, because disruption of Bcl-6 function may be therapeutically useful in some non-Hodgkin's lymphomas and certain autoimmune diseases. A B-cell within the germinal centre receives signals from growth factors, from antigen presented by the follicular dendritic cells and from cognate interactions with T-cells. The Bcl-6 proximal promoter region contains several consensus STAT sites, and in B-cell lymphoma cell lines STAT5 has a repressive effect on Bcl-6 transcription (Walker et al., 2007), whereas in primary tonsillar B-cells its effect is to induce Bcl-6 transcription (Scheeren et al., 2005).

B-cell receptor (BCR) and CD40 signalling both activate mitogen activated protein kinases (MAPK). MAPK are serine/threonine protein kinases and comprise many families of which the best studied are extra-cellular signal related kinases 1 and 2 (Erk1/2), the Jun-N-terminal kinases (Jnks) and the p38 kinases. In turn the MAPKs are

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activated by MAP kinase kinases (MKKs), with Erk1/2, Jnk and p38 each being predominantly regulated by different MKKs (Raman et al., 2007). BCR cross-linking the of the germinal centre representative cell line, Ramos, causes activation of p44 Erk1 and p42 Erk2, which then phosphorylate Bcl-6, leading to its nuclear export and degradation by the ubiquitin/proteasome pathway (Niu et al., 1998).

More recently, CD40 stimulation (by the T-cell surface antigen CD40 ligand ectopically expressed on the surface of a fibroblast cell line) has also been shown to reduce Bcl-6 expression, in cell lines and purified tonsillar centroblasts, through NF- $\kappa$ B activation and induction of IRF4 (Saito et al., 2007). Thus, it appears that both BCR engagement and CD40 signalling cause a reduction in Bcl-6 expression, possibly associated with the production of a high affinity antibody at the end of the germinal centre reaction. Therefore, whilst several signals altering Bcl-6 expression are known, their relative importance is not understood.

Chronic myeloid leukaemia (CML) cells bear a reciprocal translocation between chromosomes 9 and 22 that creates a non-receptor tyrosine kinase fusion protein, Bcr-Abl. It has been previously shown that Bcl-6 is induced when Bcr-Abl is inhibited with the tyrosine kinase inhibitor, imatinib (Deininger et al., 2000) in lymphoid blast crisis cell lines. This occurs before apoptosis takes place, and is not likely to be secondary to cell death. The main signalling pathways controlled by Bcr-Abl are Jak/Stat, Raf/Mek/Erk and PI3K/Akt and, therefore, the CML cell lines are a conditional system for analyzing the regulation of Bcl-6 by these molecules (Steelman et al., 2004). Preliminary work suggests that Bcl-6 is a functionally important survival factor in Bcr-Abl driven acute lymphoblastic leukaemia crisis (Duy et al., 2008).

Here, we define the regulation of Bcl-6 by MAPK pathways firstly, utilising CML lymphoid blast crisis cell lines and secondly in the germinal centre representative cell line, Ramos. We show that p38 is an important inducer of Bcl-6 in both systems but that MAPK proteins can both induce and repress Bcl-6 with the overall effect depending on the precise stimulus used to activate this signalling pathway.

## 2. Materials and methods

### 2.1. Cell lines and culture

The Bcr-Abl-positive cell lines (BV173, Nalm-1, Z119, Z-33, TOM-1 and K562), acute myeloid leukaemia cell line (MV4;11) and the B-cell lines DG75 and Ramos were grown in RPMI-1640 (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (Lonza, Basel, Switzerland), 2% L-glutamine and 5% penicillin/streptomycin (Invitrogen, Paisley, UK).

Informed consent was obtained for the use of a sample of frozen mononuclear cells from a patient with lymphoid blast crisis of CML from the Hospital Marques de Valdecilla, Department of Haematology, Santander, Spain. Cell viability was assessed by trypan blue staining.  $10^7$  cells were cultured in 1 ml of FCS-free-medium (Iscove's Modified Dulbecco's medium (Sigma, St. Louis, MO, USA), 1% penicillin-streptomycin-L-glutamine and serum substitute (BIT: StemCell Technologies, Vancouver, BC, Canada) and kept in culture overnight (Hamilton et al., 2006).

As a source of CD40 stimulation either soluble CD40 ligand (sCD40L) (Biovision, Mountain View, CA, USA; 0.1–20 ng/ml) or co-culture with a mouse fibroblast cell line stably transfected with human CD40 ligand (mCD40L) were used. Non-transfected fibroblast cells (NTL) were used as controls. The cell layers were 80–90% confluent and irradiated (30 Gy) before use.  $10^7$  Ramos cells were cultured in 5 ml of complete medium when using sCD40L.

### 2.2. Treatment with inhibitors and stimulants

The Bcr-Abl-positive cell lines, Z119 and BV173, and the germinal centre B-cell lines, Ramos and DG75, and primary cells were incubated with inhibitors of the Erk1/2 pathway (using the Mek1/2 inhibitors U0126 or PD98059 (Calbiochem, Nottingham, UK)) or p38 pathway (SB 202190; Calbiochem, Nottingham, UK). Where indicated, imatinib (0.5–5  $\mu$ M; Novartis; Pharma, Basel, Switzerland) was included in the cell suspension. Stock solutions of the inhibitors were prepared with DMSO. The final concentration of DMSO in all the experiments was below 0.8%. For cross-linking surface IgM we used goat anti-human IgM (Southern Biotech, Birmingham, AL, USA; 2020-01).

### 2.3. Bcl-6 reporter gene assay

Fragments of Bcl-6 genomic DNA (base position as measured from the transcription start site (Bernardin et al., 1997): –4904 to +533; –4904 to +239; –4128 to +533; –4128 to +239 and +239 to +533) were obtained by PCR and subcloned into the pGL3 basic reporter vector (Promega, Madison, WI, USA). The pRL luciferase control vector was purchased from Promega, Madison, WI, USA.

HEK293T cells were transfected with the pRL transfection control vector (Promega, Madison, WI, USA) and with either the pGL3 basic vector or with equimolar concentrations of different Bcl6pGL3 constructs, using Lipofectamine2000 (Invitrogen, Paisley, UK). To assess the effect of phospho-p38 on the Bcl6 promoter, 1  $\mu$ g/ml of anisomycin (Sigma, St. Louis, MO, USA) was added to the medium 2 h before lysing the cells. Supernatants were assayed using the Dual-Glo Luciferase Reporter System following the manufacturer's instructions (Promega, Madison, WI, USA).

### 2.4. Western blot analysis and antibodies

Cells ( $2 \times 10^6$  cells) were washed once in cold PBS and resuspended in RIPA lysis buffer (1% nonidet P-40, 100 mM NaCl, 20 mM Tris-HCl (pH: 7.4) with 10 mM NaF and complete protease and phosphatase inhibitors (Sigma, St. Louis, MO, USA). Lysates were incubated on ice for 30 min and centrifuged for 20 min (12,000 rpm) at 4 °C. 40–100  $\mu$ g of protein were separated on 7.5–10% SDS-polyacrylamide gels followed by transfer onto a PVDF membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was blocked, either in 5% skimmed milk or 5% BSA, in Tris-buffered saline and then incubated sequentially with the primary and the secondary antibodies. Specific bands were visualized using the enhanced chemiluminescence system (ECL, GE Healthcare, Chalfont St. Giles, UK). To control for protein loading, the blots were restained with either anti-GAPDH (Cell Signalling Technology, Danvers, MA, USA, #2118, 1:2000) or anti-abl (Santa Cruz Biotechnology, Santa Cruz, CA, USA). We used commercial lysates: Jnk (sc-24769) and p38 (sc-2217) as controls for anti-phospho-Jnk and anti-phospho-p38.

The following antibodies were obtained from Cell Signalling Technology, Danvers, MA, USA: HRP-conjugated anti mouse IgG, HRP-conjugated anti-rabbit IgG, anti-phospho-c-Abl (Tyr412) #2865, 1:500; anti-phospho-STAT5 (Tyr694) #9351, 1:500; anti-phospho-p38 #9215, 1:1000; anti-p38, 1:1000; anti-phospho-JNK, #4668; 1:1000; anti-Jnk, 1:1000; anti-phospho-Erk1/2, #9101; 1:1000; anti-Erk1/2, #9102; 1:1000 and anti-IRF4, 1:1000. Other antibodies were anti-4G10 used as 1:1000 (Upstate Biotechnology, Lake Placid, NY, USA); anti-Bcl-6 (N-3) (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-858, 1:100) and anti-STAT5 (Santa Cruz Biotechnology, sc-835, 1:100).

### 2.5. Real-time PCR (Taqman)

Total RNA was isolated with a RNeasy kit (Qiagen, Crawley, UK) and treated with DNase I (Invitrogen, Paisley, UK). RNA (2 µg) was reverse transcribed using an Omniscript kit (Qiagen, Crawley, UK), and the cDNA was used in real-time semi-quantitative PCR analysis. Primer and probe mixtures were purchased from Applied Biosystems, Foster City, CA, USA: HPRT-1 (hs99999909\_m1) and Bcl-6 (Hs00153368\_m1). Amplifications were performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA, USA) for 10 min at 95 °C, 2 min at 50 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 min at 60 °C. All measurements were performed in triplicate. Each sample was analyzed for Bcl6 and HPRT expression and values were expressed as Bcl-6:HPRT-1 ratios.

### 2.6. Electrophoretic mobility shift assay

Nuclear proteins were extracted (Schreiber et al., 1989) from ten to twenty million cells. Cells were washed in PBS and resuspended in buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT and 5 µl of 10% NP40). After centrifuging the nuclei were resuspended in buffer C (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 M EDTA and 0.5 mM DTT). Protease and phos-

phatase inhibitors (Sigma, St. Louis, MO, USA) were included in all solutions. Each binding and binding competition assay mixture (10 µl) contained 0.05 pmol of the <sup>32</sup>P-labelled probe and unlabelled competitor DNA at either 1, 5 or 10 pmol, respectively, in 5 mM MgCl<sub>2</sub>, 50 mM Tris-HCl, 2.5 mM EDTA, 2.5 mM DTT, 20% glycerol and 250 mM NaCl. Cell lysate and radiolabelled probe were incubated at room temperature for 30 min. Mixtures were run on a 4% native acrylamide gel.

### 2.7. DNase hypersensitive site assays

Nuclei preparation, DNase I digestion, DNA extraction, and Southern blot analysis were performed as previously described (Vyas et al., 1992). Details of single-copy probes used for Southern blot analysis are available on request.

## 3. Results

### 3.1. p38 MAPK induces Bcl-6 in CML lymphoid blast crisis cell lines

One of the effects of the tyrosine kinase inhibitor imatinib is to increase expression of Bcl-6 mRNA in Bcr-Abl lymphoid blast crisis

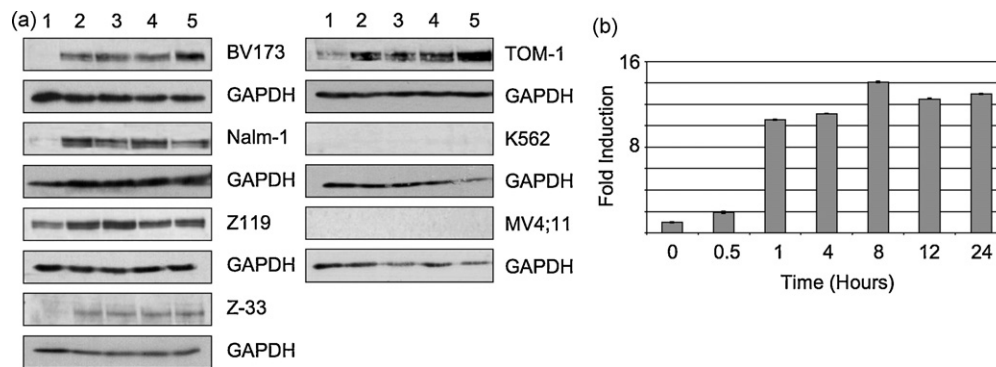


**Fig. 1.** Imatinib induces Bcl-6 transcription and protein expression in Bcr-Abl expressing lymphoid cell lines. (a) Westerns showing Bcl-6 expression after treatment with different concentrations of imatinib for 24 h in lymphoid Bcr-Abl cell lines (BV173, Nalm-1, Z119, Z-33 and TOM-1) and myeloid Bcr-Abl cell lines (K562 and MV4;11) by the tyrosine kinase inhibitor, imatinib (lane 1, 0 µM; lane 2, 0.5 µM; lane 3, 1 µM; lane 4, 2 µM and lane 5, 5 µM). Loading controls were carried out for each cell line. (b) Real-time PCR for Bcl-6 mRNA was carried out on BV173 cells after treatment with 1 µM of imatinib for different lengths of time.

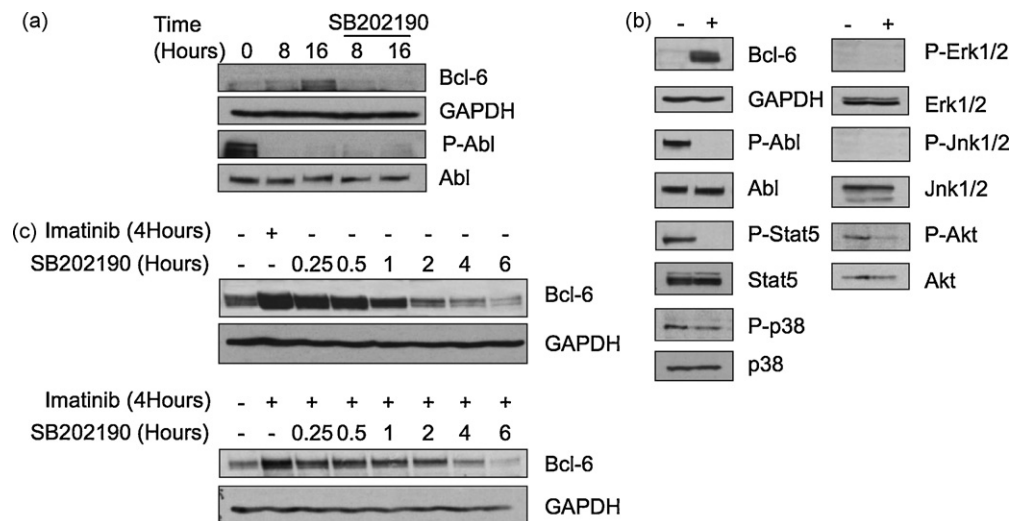


**Fig. 2.** Imatinib induces Bcl-6 in primary CML cells and cell lines through a p38 mechanism. (a) Primary lymphoid blast crisis cells were treated with imatinib alone, or imatinib plus a p38 inhibitor (SB202190) for 8 and 16 h. Loading control is GAPDH. To confirm the effectiveness of imatinib we also analyzed expression of phosphorylated Bcr-Abl. (b) Westerns of phosphorylated and total Bcr-Abl, Stat5, p38, Erk1/2, Jnk1/2 and Akt in BV173 cells in the absence of imatinib and after 6 h incubation with 1 µM imatinib. (c) Western of Bcl6 expression in BV173 cells after treatment with 10 µM of SB202190 for different lengths of time. The effect of 4 h culture with 1 µM imatinib is indicated in lane 2. (d) Western showing the effect of SB202190 on Bcl-6 expression in BV173 treated with 1 µM imatinib. Loading control is GAPDH.

cell lines (Deininger et al., 2000; Fernandez de Mattos et al., 2004). We carried out further testing of Bcr-Abl lymphoid cell lines: all showed induction of Bcl-6 protein expression on treatment with imatinib (Fig. 1a), and this was largely due to increases in Bcl-6 transcription (Fig. 1b). The Bcr-Abl myeloid cell lines tested showed no induction of Bcl-6. Primary cells from a patient with lymphoid blast crisis (without Abl kinase domain mutations in the leukaemic cells) were treated with imatinib and also demonstrated increased Bcl-6 expression (Fig. 2a).

Imatinib abolishes phosphorylation of Bcr-Abl and thereby prevents phosphorylation of target proteins. We found that imatinib reduced phosphorylation of STAT5 and AKT, but that p38 was relatively resistant to the effects of this drug (Fig. 2b). We reasoned that, because it was persistently phosphorylated in the presence of imatinib, p38 was a candidate protein inducing Bcl-6 expression. Utilising Z119 we found that a specific inhibitor of p38, SB202190, reduced Bcl-6 expression (Fig. 2c). When Z119 was treated with imatinib together with SB202190 for varying lengths of time, Bcl-6 expression was again reduced (Fig. 2d). This effect was also observed in BV173 (not shown) and primary lymphoid blast crisis cells. We conclude that p38 acts to induce Bcl-6 expression under both basal conditions and in the presence of imatinib.

### 3.2. Multiple MAPKs, including p38, are induced by anti-IgM in Ramos

Bcl-6 has important roles in normal germinal centre B-cells and the germinal centre derived non-Hodgkin's lymphomas. We sought to find out the effects of p38 on basal Bcl-6 expression in the human Burkitt lymphoma cell line, Ramos (Niu et al., 1998; Saito et al., 2007). We found that SB202190 reduces Bcl-6 expression (Fig. 3a). We also obtained this result with another Burkitt cell line, DG75 (not shown). (As previously reported (Oster et al., 2008; Wang et al., 2000) SB202190 increased p38 phosphorylation, probably because, whilst p38 activity is inhibited, phosphorylation by proteins in the MAPK signalling cascade continues.)

B-cell receptor signalling reduces Bcl-6 expression largely by post-translational mechanisms acting through Erk1/2 (Niu et al., 1998). However, we found that BCR signalling increased p38 phosphorylation as well as Erk1/2 phosphorylation (Fig. 3b), suggesting that this stimulus activates pathways that both induce and repress Bcl-6. When used together with anti-IgM, the specific p38 inhibitor, SB202190, reduced Bcl-6 expression further (Fig. 4a). Others have found that MEK1/2 inhibition by PD98059 prevents the reduction of Bcl-6 expression caused by anti-IgM (Niu et al., 1998), but surprisingly we found that U0126 causes only a minor reversal of the effect of IgM (Fig. 4b). Indeed, at the highest concentration of U0126 (75  $\mu$ M) Bcl-6 expression was reduced to that found with anti-IgM alone.

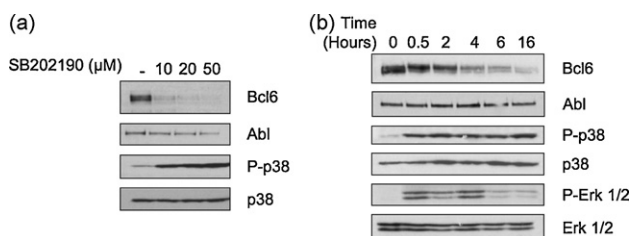


**Fig. 3.** Activated p38 induces Bcl-6 in Ramos cell line, but its effects are overcome by repressive Erk1/2. (a) Westerns for Bcl-6 expression in cells treated with varying concentrations of SB202190. SB202190 caused an increase in phosphorylated p38 (as previously reported (Oster et al., 2008; Wang et al., 2000)), but this form of the molecule is inactive. Loading control is Abl. (b) Westerns showing the effects of IgM cross-linking (10  $\mu$ g/ml) for different lengths of time on Bcl-6 expression. Loading control is Abl.

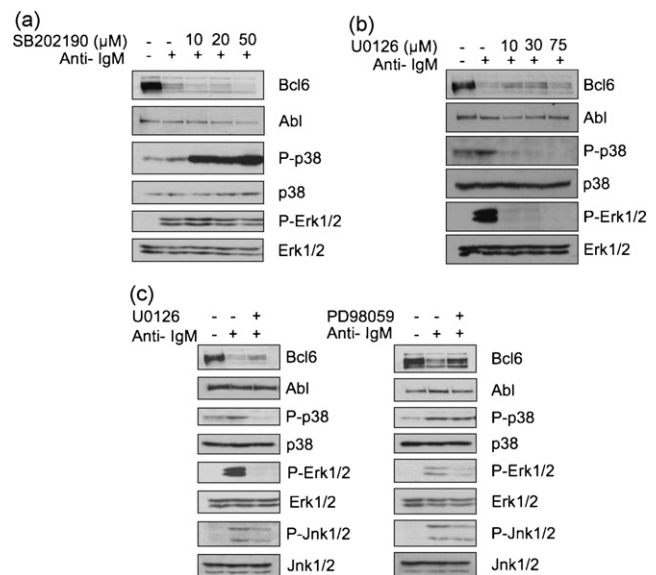


**Fig. 4.** Differential effects of Mek1/2 inhibitors, U0126 and PD98059, on p38 phosphorylation. (a) Westerns showing the combined effect of anti-IgM and increasing concentrations of SB202190. The effect of anti-IgM alone is shown in lane 2. Loading control is Abl. (b) Western showing the effect of anti-IgM and increasing concentrations of the Mek1/2 inhibitor U0126. The effects of anti-IgM alone are shown in lane 2. Loading control is Abl. (c) Ramos cells were either untreated, treated with anti-IgM alone or treated with U0126 10  $\mu$ M (left hand panel) or PD98059 50  $\mu$ M (right hand panel) for 30 min followed by the addition of anti-IgM (10  $\mu$ M) for 6 h. Whole cell lysates were then analyzed by westerns using antibodies as indicated to the right of the panels. Loading control is Abl.

In order to pursue the cause of this discrepancy we directly compared U0126 and PD98059 (Fig. 4c). As anticipated both inhibitors abrogated Erk1/2 phosphorylation, but they differed in their effects on p38: whereas U0126 abolished anti-IgM-induced p38 phosphorylation, PD98059 had no effects. U0126 is a potent Mek1/2 inhibitor but, at higher concentrations, inhibits other pathways including p38 (Favata et al., 1998). Therefore, Bcl-6 expression integrates the effects of positive and negative factors. Erk1/2 is a powerful negative regulator and, in this system, appears to overwhelm the effects of p38, which we identify as a positive regulator.

### 3.3. Expression of phosphorylated p38 in tonsil

In order to find out the relationship between Bcl-6 and phosphorylated p38 we carried out immunocytochemistry of human tonsil (Fig. 5). There were scattered phosphorylated p38 expressing cells within germinal centres (Fig. 3a) and the majority of mantle zone (MZ) cells expressed this protein. In order to confirm that germinal centre B-cells express phosphorylated p38 we carried out double staining with antibodies directed against the surface protein CD20 and anti-phosphorylated p38 (Fig. 5b–d). Double staining with anti-Bcl-6 and anti-phosphorylated p38 (Fig. 5e and f) shows that a minority of Bcl-6 expressing cells (5–10%) express phosphorylated p38.

### 3.4. Soluble CD40 ligand induces Bcl-6 through inducing p38

CD40 stimulation by membrane bound CD40 ligand (mCD40L) has been reported to reduce Bcl-6 protein expression due to NF- $\kappa$ B induced IRF4 activity (Saito et al., 2007). We measured the effects of mCD40L on MAPK proteins and found that Erk1/2, p38 and Jnk1/2 were all phosphorylated (Fig. 6e). The strong inhibitory effects of phosphorylated Erk1/2 on Bcl-6 might, therefore, contribute to the effects of IRF4. We also tested soluble CD40 ligand (sCD40L) for its effects on Bcl-6 protein expression and surprisingly found a strong

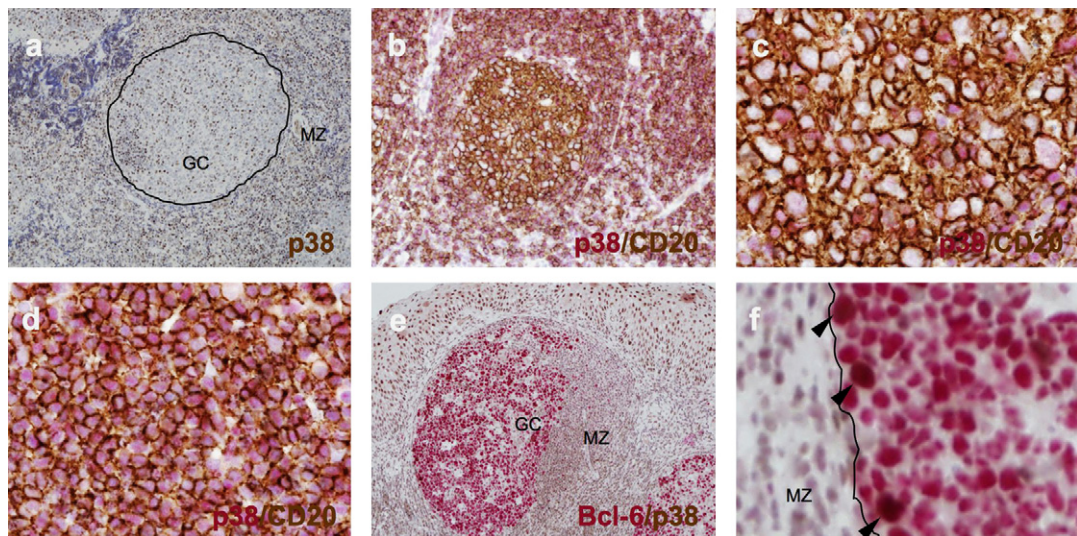


**Fig. 5.** Immunocytochemistry showing phosphorylated p38 expression in normal human tonsil germinal centres. (a) Staining with anti-phospho p38 ( $\times 10$ ). Expression occurs mainly in centrocytes and not centroblasts. MZ is mantle zone and GC is germinal centre. (b) Double staining with anti-phospho p38 (red) and anti-CD20 (brown) ( $\times 10$ ). (c) Higher power view of germinal centre cells stained with anti-phospho-p38 (red) and surface CD20 (brown) ( $\times 60$ ). (d) Staining of the mantle zone with nuclear phospho-p38 (red) and surface CD20 (brown) ( $\times 60$ ). (e) Double-staining with anti-Bcl-6 (red) and anti-phospho p38 (brown) ( $\times 10$ ). (f) Further high power view ( $\times 100$ ) from the germinal centre/mantle zone border stained with anti-phospho-p38 (brown) and anti-Bcl-6 (red). Cells staining for both proteins are indicated by black arrowheads. The majority of germinal centre cells are phospho-p38<sup>+</sup>-Bcl-6<sup>+</sup>. Cells positive for phospho-p38 only are seen in the mantle zone (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

induction (Fig. 6a). This effect was observed over a concentration range of 0.1–20  $\mu\text{g/ml}$  of sCD40L. sCD40L caused phosphorylation of p38 without activating Erk1/2, Jnk1/2 or inducing IRF4. We conclude that p38 is an important inducer of Bcl-6, but as with cross-linking of the BCR by anti-IgM, its effects are strongly counteracted by repressors—Erk1/2 and IRF4. In confirmation of this the addition of SB202190 to sCD40L completely abolished induction of Bcl-6 (Fig. 6b).

mCD40L is expressed on the surface of the mouse fibroblast line, L-cells. We reasoned that interactions of the B-cell line with adhesion molecules on the surface of fibroblasts may be important and we, therefore, sought effects of this line alone on Bcl-6 (Fig. 6c). There was a transient decrease in p38 phosphorylation (lane 2, Fig. 6c), with induction of Erk1/2 phosphorylation and without either Jnk1/2 or IRF4 induction. Overall there was a reduction in Bcl-6 expression, which was restored by PD98059 (Fig. 6d). We con-

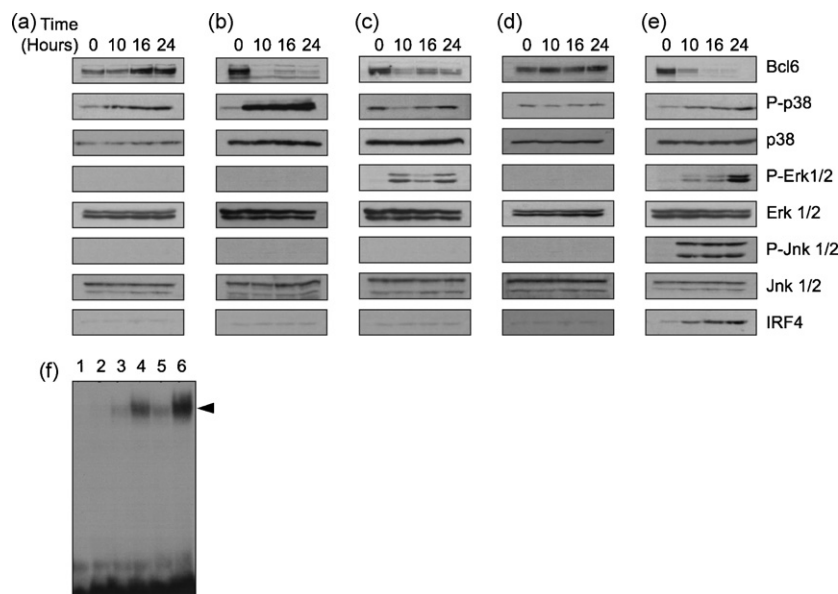


**Fig. 6.** Soluble CD40 ligand and membrane bound CD40 ligand differ in their effects on Bcl-6 expression. Ramos cells were treated for 0, 10, 16 and 24 h. Cell lysates were analyzed by Western blot using antibodies indicated to the right of the panels. (a) Cells treated with sCD40L. (b) Cells were co-cultured with the p38 inhibitor, SB202190. (c) Cells were co-cultured with the untransfected mouse fibroblast layer. (d) Ramos cells were treated for 30 min with the Erk1/2 inhibitor PD98059 and then co-cultured with untransfected mouse fibroblasts for different lengths of time as indicated. (e) Ramos cells were co-cultured with fibroblasts expressing mCD40L. (f) Gel shift assay to show the effect of sCD40L and mCD40L on NF- $\kappa$ B activation. Lane 1, no cell lysate; lane 2, unstimulated Ramos cells; lane 3, 4 h with sCD40L; lane 4, 8 h with sCD40L; lane 5, 4 h with mCD40L and lane 6, 8 h with mCD40L. The shifted band is indicated by the arrowhead to the right.

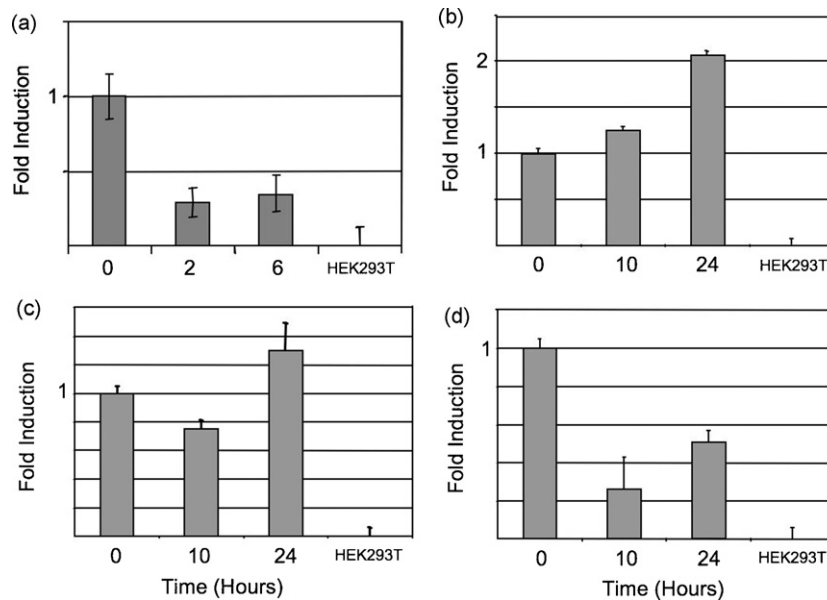


**Fig. 7.** Manipulation of p38 activity causes changes to Bcl-6 expression (a–d) are real-time PCR measurements of Bcl-6 mRNA in Ramos normalised to HPRT at different time points as indicated. Means  $\pm$  SEM are shown. The right-hand column of each graph represents the measurement of Bcl-6 mRNA in HEK293T cells. This was included as a negative control. (a) Effect of the p38 inhibitor, SB202190, on expression of Bcl-6 mRNA. (b) Effect of sCD40L. There is an increase of 2-fold over basal expression of Bcl-6. (c) Effect of BCR cross-linking by anti-IgM on Bcl-6 transcription. (d) Culture of Ramos with fibroblasts expressing CD40L (mCD40L).

clude that Erk1/2 phosphorylation is caused by interactions with fibroblast surface proteins but there is no induction of IRF4, which is specifically due to mCD40L (Fig. 6e). sCD40L, therefore, causes specific activation of p38 with no detectable phosphorylation of Erk1/2 or Jnk1/2.

IRF4 is activated by the NF- $\kappa$ B pathway (Saito et al., 2007). We measured NF- $\kappa$ B activation following incubation with sCD40L or mCD40L using gel shift assays (Fig. 4f). Whilst both ligands increased NF- $\kappa$ B activation this was greater with mCD40L demonstrating that under the experimental conditions IRF4 is induced when NF- $\kappa$ B activity is above a threshold level.

### 3.5. Effects on Bcl-6 transcription

Next we sought effects on Bcl-6 transcription. The p38 inhibitor SB202190 produced a reduction in Bcl-6 mRNA (Fig. 7a). sCD40L causes a 2-fold increase in Bcl-6 mRNA expression (Fig. 7b). The fibroblast layer alone produced no significant change in Bcl-6 mRNA (compatible with a post-translational effect of phosphorylated Erk1/2 (Niu et al., 1998) (Fig. 7c) and mCD40L reduced mRNA expression as anticipated from the induction of IRF4 (Saito et al., 2007) (Fig. 7d). We conclude that p38 acts at the level of Bcl-6 transcription.

### 3.6. Site of action of p38 target genes at the Bcl-6 promoter

p38 acts to phosphorylate and activate a number of transcription factors. In order to find the site of action of p38 we carried out luciferase reporter assays. Bcl-6 promoter assays in Bcl-6 expressing cell lines are complicated by a negative autoregulatory effect (Wang et al., 2002), which suppresses transcriptional activity, and we, therefore, followed others (Saito et al., 2007) in using 293T human embryonic kidney cells (HEK293T). HEK293T cells express low levels of phosphorylated p38, and this can be increased by anisomycin (Fig. 8a). We measured luciferase activity due to about 5.5 kb (positions –4904 to +533 relative to the transcription start site) of the Bcl-6 promoter comprising sequence 4.5 kb upstream of the transcription start site to exon 1 (Fig. 8b). There was strong transcriptional activity due to the full-length construct and this was

enhanced a further 25% by anisomycin (paired *t*-test;  $p=0.0012$ ). Inhibition of p38 by SB202190 reduced luciferase activity below basal levels (not statistically significant), suggesting that there may be a small amount of resting p38 activity. Deletion of 300 bp from the 5' portion of the construct (–4128 to +533) retained transcriptional activity, but the addition of anisomycin no longer produced significant enhancement. Deletion of exon 1 sequence from the 3' portion of the promoter construct (–4904 to +239) caused transcription to fall by >60%. Comparison with the constructs possessing

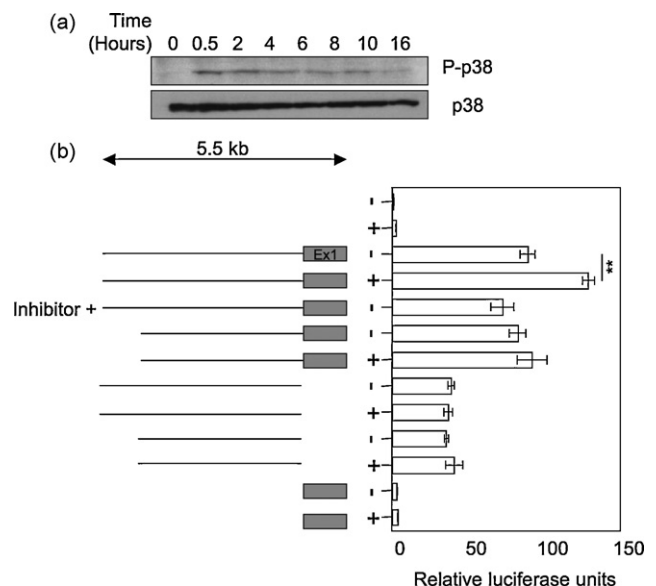


**Fig. 8.** p38 drives transcription through sequences in the Bcl-6 promoter region. (a) Western showing time course of p38 phosphorylation in response to anisomycin in HEK293T cells. (b) Luciferase assays to show the effects of anisomycin and a p38 inhibitor on transcription from a 5.5 kb section of the Bcl-6 promoter '+' indicates that the transfected cells were exposed to anisomycin and '-' indicates absence of anisomycin. The constructs used are indicated to the left. Means  $\pm$  SEM are shown. Each experiment was carried out 6 to 10 times with the exception of the p38 inhibitor + full-length construct (2 times) and the exon1 construct  $\pm$  anisomycin (2 times).

full transcriptional activity is, therefore, difficult and we cannot exclude a small anisomycin effect. Finally, exon 1 alone had virtually no transcriptional activity. We conclude that exon 1 with other sequences in this 5.5 kb region is required for full transcriptional activity, and that p38 responsiveness is abolished by deletion of the distal promoter region.

#### 4. Discussion

Germinal centres are sites of intense B-cell proliferation associated with somatic hypermutation and isotype-switching. These processes are necessary for the production of high affinity antibodies but may also contribute to lymphomagenesis. Absent or dysregulated germinal centre reactions can result in immunodeficiency (Aruffo et al., 1993; Revy et al., 2000) or autoimmunity. There is a wealth of evidence that Bcl-6 is required for normal germinal centre function (Dent et al., 1998; Ye et al., 1997) and its overexpression in mice is sufficient for the development of lymphomas (Cattoretti et al., 2005). Mutations of *cis*-acting Bcl-6 regulatory elements are associated with human DLBCL (Pasqualucci et al., 2003; Saito et al., 2007; Wang et al., 2002). Failure or over-activity of the signalling pathways regulating Bcl-6 can, therefore, have profound effects on germinal centre function and lymphomagenesis.

Bcl-6 is induced by imatinib in Bcr-Abl cell lines (Deininger et al., 2000). Utilising this as a model system, we showed that induction of Bcl-6 is a general effect in Bcr-Abl lymphoid cell lines (and primary lymphoid blast crisis cells) but not Bcr-Abl myeloid cell lines. In one Bcr-Abl lymphoid cell line, BV173, the transcription factor FoxO3a directly induces Bcl-6 (Fernandez de Mattos et al., 2004) but the MAPK pathways have not been analyzed. We showed that p38, like FoxO3a, is a mediator of the Bcl-6 response to Bcr-Abl inhibition by imatinib.

We next transferred our analysis to the B-cell line Ramos, which has been used before, as a germinal centre representative, to characterise Bcl-6 regulation (Niu et al., 1998; Saito et al., 2007) and somatic hypermutation (Sale and Neuberger, 1998). We investigated responses to important B-cell signalling pathways mediated by BCR cross-linking and CD40 stimulation. We found that BCR cross-linking and mCD40L activated MAPKs, some of which repressed (Erk1/2) whilst others induced (p38) Bcl-6, and that the repressive signals were stronger than the inductive (Fig. 9). By contrast sCD40L activated p38 MAPK but not Erk1/2 or Jnk.

CD40 signalling is required for germinal centre formation, specific antibody production and memory B-cell development (Aruffo et al., 1993; Foy et al., 1994; Nonoyama et al., 1993; Ochs et al., 1994) and activates p38 (Craxton et al., 1998; Sutherland et al., 1996). Recent work shows that NF- $\kappa$ B signalling, caused by CD40 stimulation, mediates down-regulation of Bcl-6 (Saito et al., 2007) and, by inference, exit from the germinal centre. Thus, apparently opposite roles can be assigned to CD40 signalling in the germinal centre. mCD40L, which repressed Bcl-6, clearly causes NF- $\kappa$ B activation but sCD40L, which increased Bcl-6 expression does not activate this pathway to the same degree in Ramos cells. Gene expression and immunofluorescence studies show that CD40 signalling does not occur in most germinal centre cells but is found in a subset of centrocytes (Basso et al., 2004). We found that phosphorylated p38 was expressed in a minority of tonsillar germinal centre B-cells. We speculate that signals, either repressing or inducing Bcl-6 are only active transiently and in small germinal centre populations. p38 is expressed in almost all mantle zone B-cells, and it is, therefore, not sufficient for Bcl-6 expression, but is likely to co-operate with other factors.

Mechanistically, sCD40L and mCD40L may cause different amounts of CD40 cross-linking or there may be differences in the kinetics of the ligand–receptor interaction that are crucial

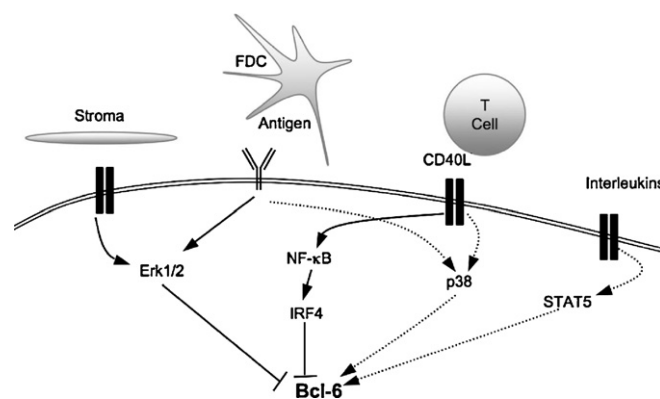


**Fig. 9.** Proposed model for the regulation of Bcl-6 expression as the outcome of weak positive and strong negative signals. Bcl-6 expression in germinal centre B-cells is regulated by BCR and CD40 signalling. The strong inhibitory pathways are shown as heavy lines and the weak activating signals as dotted lines. BCR cross-linking activates inhibitory Erk1/2 signalling (heavy lines) acting post-transcriptionally (Niu et al., 1998) as well as p38 (dotted lines) and the overall effect is Bcl-6 down-regulation. CD40 stimulation also drives pathways inhibiting Bcl-6 transcription by IRF4 (Saito et al., 2007) (heavy lines) and increasing transcription by p38 (dotted lines). The overall effect depends on the quality of the signal: physiological signals equivalent to membrane bound CD40 ligand inhibit Bcl-6 expression and lead to germinal centre exit, but signals similar to those delivered by soluble CD40 ligand cause increased Bcl-6 expression, and continued residence within the germinal centre. Stromal cell contacts may also down-regulate Bcl-6 through Erk1/2 activation. Interleukins cause STAT5 activation and increase Bcl-6 transcription (Scheeren et al., 2005). This scheme is not complete: neither the transcriptional effects of FoxO3a (Fernandez de Mattos et al., 2004) or the post-transcriptional effects of Bcl-6 acetylation (Bereschenko et al., 2002) are included.

for the outcome of signalling. Others have also found that different CD40 signalling strengths can produce different outcomes. In macrophages strong CD40 signalling produced p38 activation whereas weak signals caused Erk1/2 phosphorylation, and each produced unique and biologically opposing effects (Mathur et al., 2004). Soluble CD40 ligand has been suggested to be produced and have biological roles in primary mouse B-cell development (Kilmon et al., 2007; Wykes et al., 1998).

Using mCD40L we obtained activation of Erk1/2 but culture of Ramos with untransfected mouse fibroblast cells also produced phosphorylation of this MAPK family member. Phosphorylation of Erk1/2 cannot, therefore, be a specific CD40 effect. Previously, BCR cross-linking has been shown to activate Erk1/2, leading to ubiquitin-mediated Bcl-6 degradation (Niu et al., 1998). However, *in vivo* Erk1/2 may be activated in germinal centre B-cells by cell contact mediated by receptor–ligand pairs that are not unique to B-cells. For example, integrin signalling which lowers the threshold for early activation of B-cells (Batista et al., 2007; Carrasco et al., 2004) may also have a role in the later stages of a germinal centre reaction.

Finally mice bearing homozygous disruptions of the locus of the MAPK kinase kinase, Mekk1, have a B-cell autonomous defect in germinal centre formation and T-dependent antibody responses (Gallagher et al., 2007). These animals have defective activation of Jnk and p38. We speculate that the B-cell phenotype of Mekk1 deficient mice is due to reduced or absent induction of Bcl-6 by p38. It is interesting that Mekk1 deficient animals (Gallagher et al., 2007) have increased production of  $T_H2$  cytokines similar to Bcl-6 deficient mice (Dent et al., 1998, 1997; Ye et al., 1997).

The major B-cell stimuli – BCR cross-linking and CD40 ligand – both activate p38. However, both also strongly activate pathways repressing Bcl-6: BCR activates Erk1/2 and mCD40L activates IRF4. The overall effect of BCR signalling and mCD40L signalling is to reduce Bcl-6 expression and they are likely to have roles in B-cell exit from a germinal centre. sCD40 ligand activates p38, without inducing signals repressing Bcl-6, and may mimic signals required

for maintaining B-cell residence in a germinal centre. There is little data on p38 expression in primary lymphomas, but DLBCL arising from follicular lymphoma appear to express phosphorylated p38 (Elenitoba-Johnson et al., 2003).

p38 inhibitors have been employed as anti-inflammatory agents in clinical trials. Bcl-6 is a validated drug target (Chattopadhyay et al., 2006; Polo et al., 2004) in human diffuse large B-cell lymphoma and our work suggests that p38 inhibitors may have applications in this and other Bcl-6 expressing non-Hodgkin's lymphoma, such as Burkitt lymphoma.

In summary we show that p38 induces Bcl-6, probably acting in concert with STAT5 and other transcription factors *in vivo* (Scheeren et al., 2005) (Fig. 9). It has been impossible using a variety of growth factor and other signals to induce Bcl-6 in cultured B-cells and a contributory reason for this may be that signals repressing and inducing Bcl-6 are produced by the same stimuli. There may only be restricted circumstances *in vivo* in which positive signals predominate and cause Bcl-6 activation, as is suggested by immunocytochemistry of tonsil germinal centres in which a minority of Bcl-6 expressing cells co-express phosphorylated p38.

Future work will elucidate the *in vivo* signalling requirements that correspond to sCD40L in our experimental system, and also define the protein complexes assembled at the CD40 receptor in response to sCD40L and mCD40L.

## Acknowledgements

We are grateful to the Foundation Marques de Valdecilla for their support of A.B. and to Amgen and the Lymphoma Research Trust for their support of V.P. This work was also supported by a grant from the Kay Kendall Leukaemia Fund to S.D.W. We would like to thank Kay Elderfield for technical assistance with immunocytochemistry.

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