The role of transcription factor MAFB in Multiple Myeloma

Esther van Stralen

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De rol van transcriptie factor MAFB in Multiple Myeloma

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 21 oktober 2008 des ochtends te 10.30 uur.

door

Esther van Stralen

geboren op 29 mei 1974, te Hoorn

Promotor: Prof.dr. H.C. Clevers
Co-promotor: Dr. E.J.E.G. Bast

Voor mijn gezin

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Chapter 1

General Introduction

General introduction

1   Multiple Myeloma

Multiple Myeloma (MM) is a progressive hematologic disease. It is a cancer of the post-germinal center long-lived bone marrow (BM) plasma blasts/plasma cells (PC) derived at the end of the B-cell development. These cells have undergone extensive somatic hyper mutation of the immunoglobulin (Ig) heavy chain (IgH) and Ig light chain (IgL) genes, antigen selection and productive IgH switch recombination. MM is an incurable disease despite advances in systemic and supportive therapies and accounts for 1% of all cancers and approximately 10% of the haematological malignancies. The median age of diagnosis is 68 years. Patients younger than this age who are treated with intensive chemotherapy have a median survival of 5 years with a 20% chance of living longer than 10 years [1].

The neoplastic cells of MM have a low proliferative index and produce a monoclonal Ig in serum and urine, called M-protein. In most of the cases the serum M-protein is of the IgG class, whereas the IgA class is involved frequently as well. IgM, IgE and IgD are rarely found. The amount of M-protein in the serum is a reliable measure of tumor burden and is therefore used as a tumor marker in the diagnosis of MM.

MM is usually preceded by an age dependent premalignant tumor called monoclonal gammopathy of undetermined significance (MGUS). MGUS is characterized by low concentrations of M-protein: in IgG < 30 g/L with no more than 10% of tumor cells in the BM [2]. MGUS usually remains stable for many years but can sporadically progress to malignant MM expressing the same M-protein at a rate of 0.6-3% per year [3]. Smouldering MM is distinguished from MGUS by a stable BM tumor cell content of 10%-30% and a higher rate of progression. Further progression of MM is associated with secondary pathologies, including osteolytic lesions, anaemia, immunodeficiency, decreased kidney function and may coincide with the development from intramedullary MM to extramedullary MM. Immortal human MM cell lines (HMCL) have been generated mainly from extramedullary MM. These HMCLs presumably include most oncogenic events involved in tumor initiation and progression of the corresponding tumor [4].

MGUS and MM have a strong dependence on the BM microenvironment for survival and growth [4] like normal long-lived PCs and non IgM MGUS and MM tumors have an extremely low rate of proliferation, usually with only a few percent of cycling cells until advanced stages of MM [5].

2   B-cell malignancies and remodeling of the Ig genes.

2.1   *B-cell development*

B-cell development is a highly regulated process of ordered events. During the early differentiation, a B-cell is generated from a hematopoietic stem cell differentiating into a B-cell expressing a unique Ig on its membrane. This process is antigen-independent and takes place in the BM. During this process a pool of B-cells is generated, in which each B-cell expresses one specific Ig with an appropriate affinity to a particular antigen. The B-cell repertoire has to be as diverse as possible to protect the individual against a large variety of pathogens. During a second antigen-dependent process, the resting B-cells can differentiate into Ig-secreting plasma cells or into memory B-cells by recognition of a specific antigen. To express functional Igs, B-cells undergo extensive genomic rearrangements within their Ig loci [6-8]: V(D)J recombination, somatic hyper mutation (SHM) and class switch recombination (CSR).

2.2   *V(D)J recombination*

During B-cell development in the bone marrow, B-cell precursors form intact exons for the variable region of antibody molecules by recombining individual Ig gene segments. For the variable region exon of the Ig heavy chain (IgH), V (variable), D (diversity) and J (joining) gene segments are joined, whereas the V region exons of κ and λ light chains are each composed only of V and J segments [9,10]. This gene rearrangement process is called V(D)J recombination and occurs in an ordered fashion (Figure 1). Firstly, a DH segment is rearranged to a JH segment and followed by a VH to DHJH joining. V(D)J recombination is initiated when two lymphocyte-specific endonucleases, the recombination activating gene (RAG) 1 and 2 proteins, cut the rearranging gene segments at specific recombination signal sequences (RSS). These RSS flank the coding sequences of the V, D and J segments (reviewed in [11]). Each RSS is composed of a conserved heptamer sequence and a conserved nonamer, which is separated by a 12 or 23 bp long spacer. RAG1 and 2 cleave precisely between the RSS and the coding sequences of the two rearranging gene segments, yielding two blunt RSS signal ends and two covalently sealed hairpin coding ends (Figure2A). The signal ends are precisely joined, releasing the intervening DNA between the rearranging gene segments as a DNA circle. The hairpin structures of the coding ends are opened and the two ends are joined. During

this reaction, nucleotides may be removed from the coding ends by exonucleolytic digestion. Non-germ line nucleotides (N sequences) may be added by the lymphocyte-specific terminal nucleotidyltransferase (TdT) further increasing the diversity of V region genes (Figure 2A). This phase of the reaction is catalyzed, in addition to the RAG proteins, by enzymes that are also involved in non-homologous double-strand DNA break repair, including DNA-PK, DNA ligase IV and XRCC4 [11].
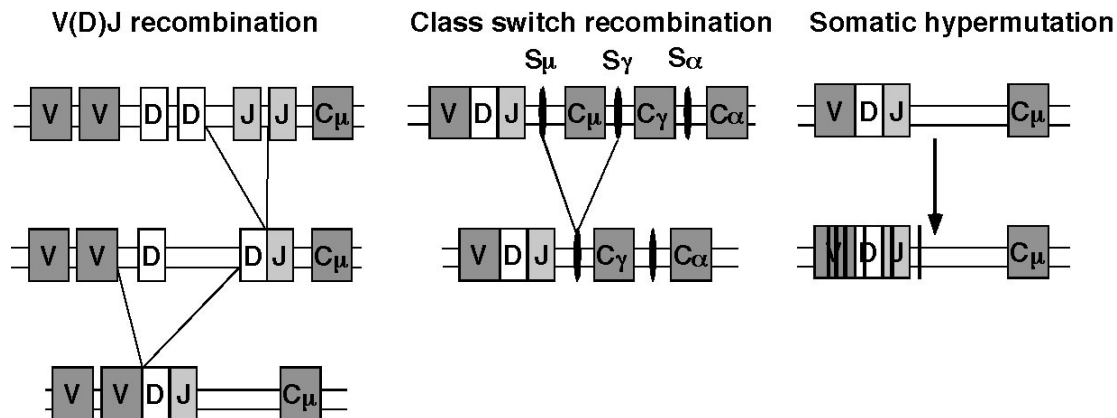

Figure 1: Molecular processes modifying immunoglobulin genes. See text for details.

2.3   Somatic hyper mutation

When mature B-cells are activated by binding a specific antigen with their antigen receptors and interaction with T helper cells, they migrate into B-cell follicles of secondary lymphoid organs (like lymph nodes and spleen) and establish histological structures called germinal centers (GC) [12]. In the GC, B-cells proliferate and activate a mechanism called somatic hyper mutation. This mechanism specifically introduces mutations into the V regions of Ig genes [13-15] (Figure 1) which is responsible for further antibody variety. Cells expressing a B-cell receptor (BCR) with increased affinity to the respective antigen are positively selected. GC B-cells undergo several rounds of proliferation, mutation and selection before they finally differentiate into memory B-cells or plasma cells.

The process of somatic hyper mutation introduces mostly nucleotide substitutions into rearranged V genes; deletions and duplications account for about 5% of the mutation events [16,17]. Mutations are introduced at a very high rate in a region about 1-2 kb downstream of the transcriptional promoter of both productively as well as non-productively rearranged V genes. It appears that hyper mutation is dependent on the presence of the Ig enhancers and on transcription of the Ig genes,

and that in particular transcription initiation is involved [18-20]. The mechanism of somatic hypermutation is still unknown. However, it has been demonstrated that hyper mutation is associated with double-strand DNA breaks and that an error-prone DNA polymerase is involved in the introduction of mutations in proximity of the DNA breaks [21,22].
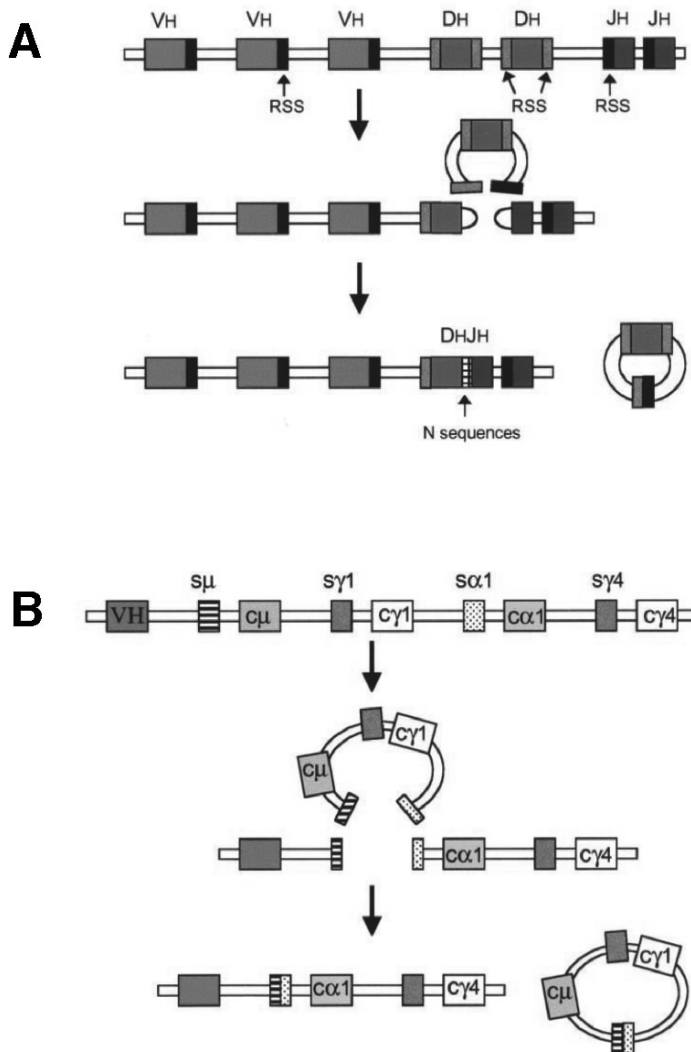

Figure 2: V(D)J recombination (a) and class switching (**b**). See text for details (adapted from [26])

2.4   Class switch recombination

A fraction of GC B-cells undergoes class switch recombination and thereby changes the isotype of the expressed BCR, resulting in altered effector functions of the antibody (Figure 1) [23,24]. Through class switching, the Cμ and Cδ genes that are originally expressed by a naive B-cell are subsequently replaced by the Cα, Cγ or Cε genes. In man, there are nine functional IgH constant region genes (1 Cμ, 1 Cδ, 4 Cγ, 1 Cε and 2 Cα), located downstream of the V, D and J gene cluster. Upstream of each CH gene, there are repetitive DNA sequences, the switch regions (Figure 2B). During class switch recombination, DNA strand breaks are introduced into both the switch μ (sμ) region and a switch region associated with one of the downstream CH genes. The DNA fragment between the switch regions is removed from the chromosome and the switch regions are joined, in such way that a new CH gene is placed downstream of the V region exon (Figure 2B). Although B-cells express only one functional VH gene rearrangement, class switching often takes place on both IgH alleles to identical classes [25]. It is suggested that the DNA-PK complex, which is involved in V(D)J recombination, and non-homologous double-strand DNA break repair is also needed for class switch recombination [24]. The molecular mechanisms of Ig remodeling is reviewed in reference [26].

2.5   Involvement of Ig remodelling in B-cell malignancies.

The above-mentioned Ig remodelling events involve double-strand DNA breaks in the Ig genes. These rearrangement processes, apart from occurring within the chromosome 14, occasionally lead to chromosomal translocations. Most of these translocations may be lethal, but some may confer a growth advantage to the cell, and may be seen after relevant diagnostic procedures. Two major forms of productive translocations may be discerned: In the first chromosomal translocation, two genes are fused together resulting in expression of a new fusion gene. A well defined example is t(9;22)(q34;q11) whereby *ABL* gene is fused with *BCR* gene. The fused gene can progress cell division, inhibits DNA repair and can cause genomic instability [27]. The t(9;22)(q34;q11) is associated with chronic myelogenous leukemia (CML). In the second one, chromosomal translocations have replaced the normal regulatory sequence of a gene with heterologous regulatory elements that drive inappropriate gene expression near the breakpoints. For example, in lymphomas mistakes in the V(D)J recombination are the translocations t(11;14)(q13;q32) *BCL-1*

/IgH translocation in mantle zone lymphoma and the t(14;18)(q32;q21) *BCL-2*/IgH translocation in follicular lymphoma [28,29]. These are translocations between the V(D)J region located on the IgH locus on chromosome 14 and the *BCL-1* or *BCL-2* gene on chromosome 11 or 18. Other examples include the t(1;14)(q21;q32) involving the *BCL-9* gene in an acute lymphoblastic lymphoma and the t(1;14)(p22;q32) involving the *BCL-10* gene in mucosa-associated lymphatic tissue (MALT)-type lymphoma [30-32].

V(D)J recombinase-associated translocations are not restricted to B-cell lymphomas but also occur in T-cell malignancies, involving the T-cell receptor loci [33]. This is because T-cells use the same enzymatic machinery for the assembly of T-cell receptor genes as B-cells. A few example are t(8;14)(q24;q11) and t(11;14)(q23;q11) in T-cell acute lymphoblastic leukemia.

SHM is probably also involved in t(8;14)(q24;q32)- *C-MYC*/IgH in Burkitt's lymphoma. *C-MYC* is often joined to the IgH locus in a rearranged and somatically mutated IgH variable (V) region [16,34,35]. SHM can also target non-immunoglobulin loci, such as *BCL-6* [36,37]. In diffuse large cell lymphoma (DLCL), several other genes like *pax-5* and *pim-1* are targeted by SHM [38]. Mutations were found 1-2 kb downstream of the promoters and their pattern indicates that the mutations result from SHM. These mutations were not found in a number of other GC-derived B-cell lymphomas neither in normal GC B-cells. This suggests a DLCL cells or their precursors -associated malfunction of the SHM machinery. A relative high frequency of mutated genes were found so it is likely that the number of loci targeted in DLCL is considerably higher compared to other B-cell lymphomas. Therefore, aberrant hyper mutation of coding or regulatory sequences of many genes may be the basis for DLCL pathogenesis and represent a novel type of genomic instability involved in tumor development.

Chromosomal translocations involving CSR have been detected mainly in MM. The breakpoints of these translocations are located in IgH switch regions [39]. Because of the location of the breakpoints in the switch regions, these translocations may happen as a mistake of the CSR. During this process, DNA double strand breaks are introduced into the switch regions of the recombining CH genes and switch-like regions on other chromosomes, sometimes resulting in the translocation of oncogenes to the der14. These oncogenes are juxtaposed to the strong IgH enhancers, resulting in their dysregulated expression. Some examples of these translocations causing over expression of specific genes are - t(4;14)(p16;q32):

MMSET/FGFR3; t(6;14)(p21;q32): *CYCLIN D3*; t(11;14)(q13;q32): *CYCLIN D1*; t(14;16)(q23;q32): *C-MAF* and t(14;20)(q32q12): *MAFB* in MM [40,41] and t(8;14)(q24;q32): *C-MYC* in sporadic Burkitt's lymphoma [42,43].

CSR is not only involved in reciprocal translocation events, but can also lead to oncogene dysregulation by insertion events. One example for this event is an insertion of a DNA sequence deleted in a switch recombination on chromosome 11 near the *Cyclin D1* gene in MM [44].

3   Cytogenetics and other genetic events in the pathogenesis of MM.

3.1   Primary Ig translocations are present in a majority of MM tumors.

As mentioned above translocations involving the IgH locus are a common event in MM. Conventional karyotyping has not been conclusive to detect these events (due to the low proliferative properties of the tumor cells within the highly proliferative normal BM, as well as the distal localisation of the IgH locus on the short 14q arm), but these IgH translocations are efficiently detected by fluorescence in situ hybridization (FISH) analysis. Large studies by several groups show that the prevalence of IgH translocations increase with the stage of the disease: about 50% in MGUS or SMM, 55-70% for intramedullary MM, 85% in PCL and >90% in HMCL's [4,45-48]. Using a sensitive combination of FISH and immunofluorescent staining, our group recently found IgH translocation to occur in 90% of intramedullary MM [49] Some studies indicate that translocations involving the IgL locus are present in about 10% in MGUS and SMM and about 15-20% in intramedullary MM and HMCLs [4,47].

Currently, there are seven well-defined recurrent chromosomal partners and oncogenes that are involved in IgH translocations in approximately 40% in MM tumors [50]: t(4;14)(p16;q32): *MMSET/FGFR3* (15%); t(6;14)(p21;q32): *CYCLIN D3* (2%); t(11;14)(q13;q32): *CYCLIN D1* (15%); t(12;14)(p13;q32): *CYCLIN D2* (<1%); t(14;16)(q32;q23): *C-MAF* (5%); t(14;20)(q32q12): *MAFB* (2%) and t(8;14)(q24.3;q32): *MAFA* (<1%).

These translocations appear to be simple reciprocal translocations mediated by errors in SHM and CSR mechanisms. Since there is no evidence that SHM and CSR mechanisms are active in normal PC or PC tumors, presumably these translocations represent primary or even initiating oncogenic events as normal B-cells pass through GC's.

3.2   Secondary Ig translocations.

Translocations involving the MYC gene are typical examples for secondary translocations in MM. These translocations are rare or absent in MGUS, but occur in 15% of MM tumors, 44% of advanced tumors and nearly 90% in HMCLs. Mostly, these rearrangements involve *C-MYC*, but about 2% of primary tumors ectopically express *N-MYC*. The translocations involving MYC appear to be a very late secondary event at a time when MM tumors are becoming less stromal cell dependent and/or more proliferative. These translocations do not involve B-cell specific recombination mechanisms and are usually complex rearrangements or insertions, sometimes involving three different chromosomes [51,52]. Also, Ig loci are not always involved in these translocations [51].

3.3   Aneuploidy is associated with two different pathogenic pathways in MM.

Karyotypes from MM cells are usually very complex but in general MM can be divided into two cytogenetic categories: hyperdiploid (HRD) (48 to 74 chromosomes) and non-hyperdiploid (NHRD) (fewer than 48 or more than 74 chromosomes). Patients with HRD MM have recurrent trisomies, particularly of chromosomes 3,5,7,9,11,15 and 19, but only infrequently (<10%) have one of the primary IgH translocations. NHRD tumors usually (~70%) have one of the primary IgH translocations [53-55]. Tumors that have a primary t(11;14) may represent a distinct category of NHRD tumors as they are often diploid or pseudodiploid, sometimes with this translocation as the only karyotypic abnormality detected by conventional cytogenetics.

Other genetic events in MM like 17p loss, p53 mutations, RAS mutations and secondary translocations often occur with a similar prevalence in HRD and NHRD tumors, this in contrast to the selective occurrence of recurrent IgH translocations in NHRD tumors. Extramedullary MM and HMCL's nearly always have an NHRD phenotype; leading to the hypothesis that HRD tumors are more stromal-cell dependent than NHRD tumors [45,56]. There is no information about the mechanisms, timing or molecular consequences of hyperdiploidy. Also, it is not known whether the extra chromosomes are accumulated one at a time, or as a single catastrophic event. For HRD tumors that have a primary translocation it is not known whether the hyperdiploidy occurred before or after the translocation. Patients with NHRD MM tumors are associated with a less favourable outcome than patients with a HRD MM

(with exception of patients with tumors that have a t(11;14) translocation, they appear to have a better survival) [53]. This maybe due to the high incidence of IgH translocations like t(14;16) and t(14;20), which is associated with a significantly shortened survival [40].

3.4   Loss of chromosome 13/13q14.

About 50 % of MM tumors [48,56-59] and 40-50% of MGUS tumors [47,56,60] have a deletion 13 in most tumor cells. This suggests that deletion 13 is often an early event in the pathogenesis of MM. In most cases the deletion 13 represents whole-chromosome monosomy but in a subset of tumors the common deleted region seems to be located at 13q14. The retinoblastoma (Rb) gene, a tumor suppressor gene involved in cell cycling, falls within the minimally deleted region. Inactivating mutations of the remaining allele are not commonly seen. There is a correlation of deletion 13 and Cyclin D expressing tumors. Deletion 13 occurs in 80-90% of tumors that have either a t(4;14) or t(14;16)/t(14;20) but has a much lower prevalence in other MM tumors [46,61]. The loss of chromosome 13 sequences occurs in about 70% of NHRD tumors but only in about 40% of HRD tumors.

3.5   Gain of chromosome 1q21.

By using a combination of new techniques, like FISH, comparative genomic hybridization array (aCGH) and gene expression profiling (GEP) a number of studies have identified a gain of sequences and corresponding increased gene expression at 1q21 in 30-40% of MM tumors. The gain of 1q21 can occur as isochromosomes, duplications or jumping translocations in MM [62,63] and are concentrated substantially in tumors that have a t(4;14) or t(14;16)/t(14;20) or have a high proliferation expression index [64-66]. Some studies show that between deletion of chromosome 13/13q14 and gain of 1q21 are highly associated and have an adverse prognostic outcome in patients with MM. Also, it has been demonstrated that gains of 1q21 increase the progression from SMM to extramedullary MM, suggesting that these regions contain critical genes for disease progression [67,68].

It has been implicated that increased expression of *CKS1B*, a candidate gene on 1q21, plays a role in the increased proliferation of these tumors [69]. Other identified candidate genes such as *COPA* and *ARF1*, play a role in vesicle-mediated transport

from the endoplasmic reticulum (ER) to the Golgi region [70-72]. The role of these genes in MM still remains unclear.

3.6   Activating Ras mutations.

Ras proteins (H-, K-, and N-Ras) have been shown to play a key role in signal transduction pathways leading to survival or growth, and in malignant transformation. Ras proteins cycle between an inactive guanosine 5'-dihosphate (GDP)-bound state and an active guanosine 5'-triphosphate (GTP)-bond state. Regulatory proteins that control the GDP/GTP cycling rate of Ras include GTPase-activating proteins (GAP's), which enhance the rate of GTP hydrolysis to GDP, and guanine nucleotide exchange factors (GEFs), which induce the dissociation of GDP to allow association of GTP [73]. Active GTP-bound Ras modulates the activity of effector proteins including Raf-1 and PI-3K. Point mutations at codons 12, 13 or 16 lead to a constitutive active GTP-bound state.

In MM, Ras plays an important role in the control of proliferation/apoptosis and drug resistance. In MM cells, IL-6 triggers the transition of Ras from the inactive GDP-bound form to the active GTP-bound form. This activates the MAPK pathway leading to proliferation [74,75]. Ras proteins transduce proliferation signals through the activation of Raf-MAPK pathwayand are also involved in the transduction of survival and growth through pathways such as PI-3K/Akt and NF-κB [76].

The prevalence of activating Ras mutations is about 30-40% in newly diagnosed MM tumors and about 45% in HMCL's [77], with only a small increase occurring during tumor progression [78,79]. Importantly, less than 5% of MGUS tumors have Ras mutations, suggesting that mutations of Ras represent a progression event rather than an initiating event. The presence of Ras mutations is associated with an adverse prognosis, a poor response to therapy, and a shorter survival [79,80].

3.7   Activation of the nuclear factor-κB pathway.

Nuclear factor-κB (NF-κB) is a transcription factor involved in the regulation of a variety of cellular processes including apoptosis, differentiation, and inflammatory responses [81]. In most cell types, NF-κB resides in the cytosol and is inactivated by its association with IκB family inhibitors. Activators of NF-κB such as TNF-α, activate IκB kinase (IKK), which phosphorylates IκB in a site-specific way. This leads to the

ubiquitination-dependent degradation of IκB by the 28S proteasome [82]. As a result, NF-κB translocate to the nucleus, where it binds to specific DNA sequences in the promoters of target genes leading to transcription of these genes.

Constitutive activity of NF-κB by activating mutations found by array studies have been identified in about 20% of patient samples and in 20/44 HMCLs [83,84]. The most common event is inactivation mutation of *TRAF3* in 13% of patients. In addition, inactivating mutations of *TRAF2*, *cIAP1/2* and *CYLD* were identified. Chromosome translocations and amplifications resulting in activation of NF-κB-inducing kinase (*NIK*) [83], *CD40*, *LTBR*, *TACI*, *NF-κB1* and *NF-κB2* were also reported [84].

50% of primary MM tumors have an expression signature of NF-κB target genes with activating mutations identified in less then half of these patients. Presumably other mutations or ligand-dependent interactions in de BM microenvironment are responsible for the NF-κB activation in the remaining patients.

4   Dysregulation of a Cyclin D gene and TC classification in MM

It has been shown that dysregulation of a Cyclin D gene provides a unifying, early oncogenic event in MGUS and MM [85]. About 25 % of MM tumors have an IgH translocation that directly dysregulates a Cyclin D gene (11q13-*CCND1* and 6p21-*CCND3*) or a MAF gene (16q23-*C-MAF* and 20q11-*MAFB*) encoding a transcription factor that directly up regulates *CCND2* [86]. Most MM tumors with a t(4;14) (*MMSET/FGFR3*) express high levels of *CCND2*, but usually at a level that is somewhat lower than tumors with translocations that target the MAF genes. The cause of increased *CCND2* in these t(4;14) tumors is still unknown. Comparing normal BM PCs, nearly 40% of MGUS and MM tumors which do not carry a t(11;14) but are hyperdiploid, have multiple trisomies of the eight odd chromosomes, and bi-allelically express *CCND1*. Most other tumors, about half of which are hyperdiploid and have multiple trisomies of the eight odd chromosomes, show increased expression of *CCND2* compared to normal BM PCs. Only a small part of MM tumors do not express increased levels of a Cyclin D gene compared to normal PC's. Summarized, these data suggests that increased Cyclin D expression is a unifying and early oncogenic event in the development of MM.

The critical role of Cyclin D dysregulation in MM highlights the importance of the Cyclin D/ Retinoblastoma (Rb) pathway (Figure 3). *CCND1*, *CCND2*, or *CCND3*

positively interact with cyclin-dependent kinase cdk4 or cdk6 to regulate phosphorylisation of Rb thereby facilitating the G_1/S cell-cycle transition [87,88]. Besides Cyclin D other components of the Rb pathway are also commonly dysregulated in MM. The *p16INK4A* and *p15INK4A* genes are methylated in about 20-30% of MGUS and MM tumors, and in most HMCLs [89] and the inactivation of p18INK4C, a critical gene for normal plasma cell development, is likely to contribute to increased proliferation. It is found that 30% of HMCLs and tumors with a high proliferation index have a biallelic deletion of p18INK4C [90].
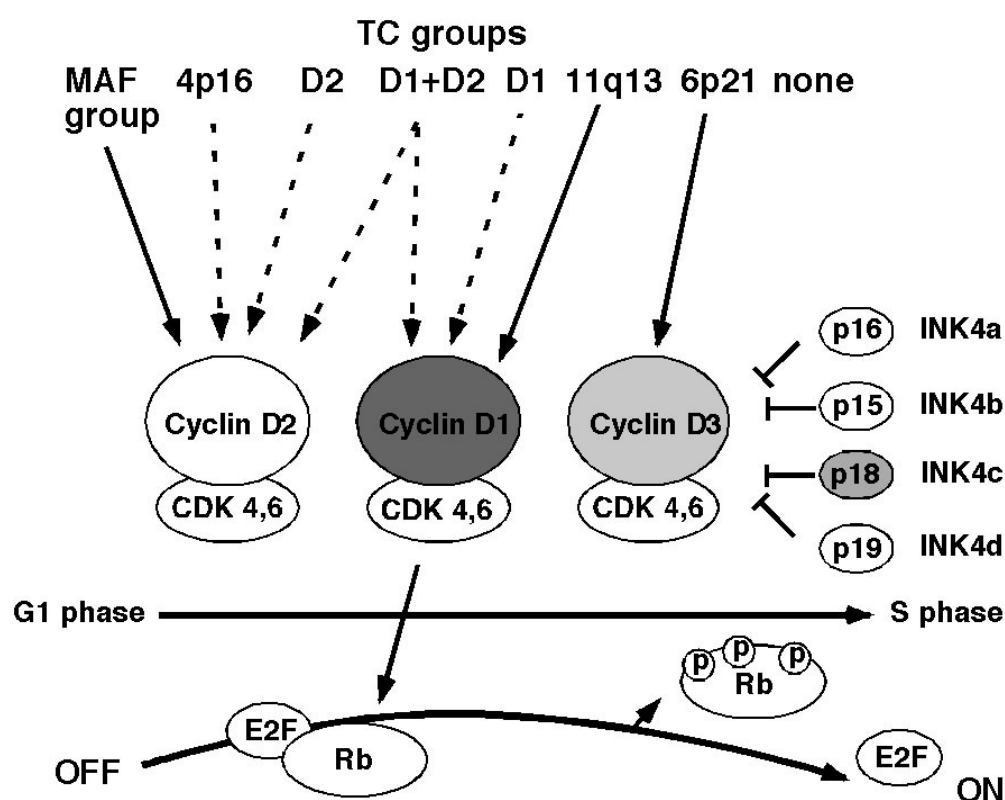

Figure 3. Alteration of RB pathway by both early and late pathogenic events. An early pathogenic event in tumors from seven of the translocation and Cyclin D (TC) groups is dysregulation of one of the three Cyclin D genes, either as a consequence of an Ig translocation (solid arrow), or by an unknown mechanism (dashed arrow). Increased expression of one of the Cyclin D proteins facilitates activation of CDK4 (or CDK6), which then phosphorylates and inactivates Rb so that E2F can facilitate G1>S cell cycle progression. This reaction is regulated by CDK inhibitors (INK4a-d), so that increased proliferation of some multiple myeloma (MM) tumors occurs only after a late oncogenic event that inactivates p18INK4c. (adapated from Kuehl and Bergsagel, ASH 2005)

According to the Cyclin D dysregulation and the presence of recurrent primary IgH translocations and a translocation/Cyclin D (TC) expression classification can be generated in MM. These TC groups (Table 1) can be distinguished on the basis of

the present Ig translocation and Cyclin D expression: 11q13 (16%) and 6p21 tumors (3%) express high levels of either *CCND1* or *CCND3* as a result of an Ig translocation; D1 tumors (34%) ectopically express moderate levels of *CCND1* despite the absence of a t(11;14) translocation; D1+D2 (6%) in addition express *CCND2.* D2 tumors (17%), which are a mixture of hyperdiploid and non-hyperdiploid tumors that do not fall into one of the other groups, express increased *CCND2* compared to normal PC. Tumors in group None (2%) do not have increased expression of a Cyclin D-type compared to normal bone marrow PC. 4p16 tumors (15%) express high levels of *CCND2*, and also *MMSET/FGFR3* as a result of a t(4;14) translocation; MAF tumors (7%) express the highest levels of *CCND2*, and also high levels of either *C-MAF* or *MAFB*, consistent with the possibility that both Maf transcription factors up-regulate the expression of *CCND2* (data MAFA unknown).

Table 1: Translocations and Cyclin D (TC) groups

Group (TC)	Primary translocation	Gene(s) at breakpoint	Cyclin D	Ploidy	Proliferation index	Bone disease (%MRI Pos)	Frequency (%)	Prognosis
6p21	6p21	CCND3	D3	NH	Average	100	3	? Good
11q13	11q13	CCND1	D1	D=NH	Average	94	16	Good
D1	None	None	D1	H	Low	86	34	Good
D1+D2	None	None	D1/D2	H	High	100	6	? Poor
D2	None	None	D2	H=NH	Average	67	17	?
None	None	None	None	NH	Average	100	2	? Good
4p16	4p16	FGFR3/MMSET	D2	NH>H	Average	57	15	Poor
MAF	16q23/20q11	CMaf/MAFB	D2	NH	High	55	5/2	Poor

Abbreviations: D, diploid; H, hyperdiploid; NH, non-hyperdiploid

(adapted from Kuehl and Bergsagel, ASH 2005)

The advantage of this TC classification system is that important biologic and clinical correlations can be associated within the TC groups (Table 1). For example, the TC D1 group of tumors is absent or under-represented in PCL and HMCL. This suggests that these tumors are particularly strongly dependent on an interaction with BM stromal cells. In addition, it is found that lytic bone disease correlates with the TC classification, with high prevalence (~ 90%) in TC 6p21, TC 11q13, TC D1 and TC D1+D2, and lower prevalence (~55%) in TC 4p16 and TC MAF. Earlier, it has been reported indeed that specific IgH translocations have a profound prognostic significance [58,91]. Patients with tumors that have a t(4;14) translocation (TC 4p16)

have a substantially shortened survival, and patients with a t(14;16)/t(14;20) (TC MAF) have a similarly poor if not worse prognosis [40]. By contrast, patients with tumors that have a t(11;14) translocation (TC 11q13) appear to have a better survival [92]. Similarly, it is known that the TC D1 group, representing most of the hyperdiploid patients, shares the good prognosis associated with hyperdiploidy. Further, the D1+D2 group, which have a higher proliferative index, and are overrepresented in relapsed patients, may have a poor prognosis. Because TC 6p21 has an overlapping gene expression profile as TC 11q13, these two can be grouped together [85]. Also, *C-MAF* and *MAFB* have an overlapping gene expression profile (this thesis) and can be grouped together and form one TC group.

All these results suggest that the TC classification, which appears to reflect the earliest events in pathogenesis, may be a clinically useful way to classify patients into groups that have distinct subtypes of MM tumors. Ultimately, this recently acquired method to subdivide the so far heterogeneous group of MM may prove crucial for a better therapeutic stratification.

5   A model for the molecular pathogenesis of MGUS and MM.

Based on the results described above a model for the molecular pathogenesis of MM is proposed (Figure 4) [50]. As summarized above, there are two pathways of pathogenesis: an NHRD pathway and an HRD pathway. Altogether, there are four early and partially overlapping events: IgH translocation mediated mainly by errors in the CSR in the GC B-cells, hyperdiploidy associated with multiple trisomies, loss of chromosome 13 sequences, and dysregulation of a Cyclin D gene. Although the increased expression of a Cyclin D gene in itself may not cause increased proliferation, it may make the cells more susceptible to proliferative stimuli. This may result in selective expansion of cells as a result of interaction with BM stromal cells that express IL-6, IGF-1, or other cytokines.

Additional mutations of K- or N-Ras, secondary MYC translocations, and inactivation of p53 by a variety of mechanisms are progression events. Mutations that constitutively activate the NF-κB pathway occur in approximately half of MM tumors. Perhaps these mutations facilitate independence from environmental factors that activate this pathway at earlier stages of pathogenesis. Secondary chromosomal translocations and other genomic alterations may occur at all stages of tumorgenesis.
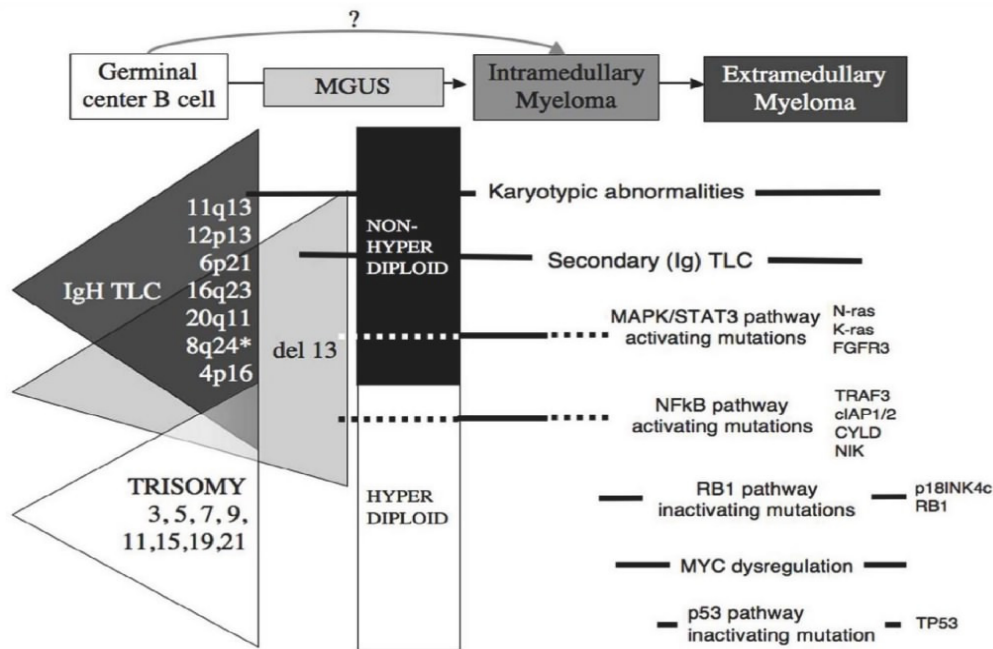
Figure 4: Disease stages and timing of oncogenic events in MM (see text for further details). The degree of intersection between the overlapping triangles estimates the percentage of each genetic subgroup with coexisting genetic abnormalities. The translocation partners in the IgH translocation (TLC) group are ordered according to increasing frequency of concurrent 13. The different mutations activating the different signaling or cell-cycle regulatory pathways are mutually exclusive, i.e., RAS and FGFR3 mutations always occur in different patients.

*The 8q24 partner referred to here is MAFA; C-MYC is also located on 8q24, but these are usually secondary IgH translocations (adapted from [50]).

6   Large Maf transcription factors.

6.1   *Large Maf transcription factors form a superfamily of bZIP proteins.*

The large Maf proteins form a distinct group of bZIP transcription factors [93]. The bZIP structural motif, comprised of a basic region and a leucine zipper, harbors an 18 amino acid long basic peptide that is considered the simplest structural motif for specific DNA recognition [94]. A leucine zipper domain [95] allows formation of homo- or hetero-dimers of mutually compatible bZIP proteins [96,97]. Other families of bZIP proteins are AP-1 [98], CREB/ATF [99,100], CNC [101,102], C/EBP [103,104] and PAR [105].

An extensive number of studies on bZIP have shown their critical function in the regulation of cellular differentiation by their association with a variety of signal transduction pathways. The hallmark of the AP-1 protein complex c-Fos/c-Jun is its role in regulation of proliferative responses [106]. CREB proteins regulate signal

transduction using cAMP as a second messenger and play specific functions in the long-term neurological memory [107]. The heterodimeric protein NF-E2 (p45/p18), from the CNC/small Maf group, regulates expression of βA-globin and other genes activated during erythropoiesis [108].

6.2   History, structure and DNA-binding of large Mafs.

The founding member of the Maf family, v-maf, was identified in 1989 from natural musculo-aponeurotic fibrosarcoma of chicken in a gag-maf locus from the replication-defective retrovirus AS42 [109]. Cellular counterparts - *C-MAFs*- were cloned from a number of vertebrate genomes using a probe containing a v-maf sequence. Endogenous *C-MAF* expression is not induced by mitogens in contrast to the AP-1 proteins c-Jun and c-Fos [109]. The protein encoded by *C-MAF* possesses a bZIP domain close to its C-terminus. Molecular dissection studies identified additional functional domains including the ancillary DNA binding region, hinge domain, and acidic transcriptional activation domain [93,110].

A retinal-specific cDNA related to Maf sequences was isolated by screening a subtractive cDNA library and named the neural retina leucine zipper (*NRL*) [111]. The *NRL* domain structure is similar to *C-MAF*, though its internal regions are shorter.

MAFB was cloned as an abundant gene expressed in the developing caudal hindbrain and was shown to match with the gene (*Krml1*) affected in the mouse kreisler (kr) mutation [112]. Homozygous kr mice exhibit hyperactive behavior, characterized by head tossing and running in circles. This is caused by abnormal segmentation of the hindbrain and defective otic development [113,114].

The last large Maf protein is MAFA (chicken homologue L-MAF) and was independently discovered in the quail retina [115] and chicken lens library [116]. An overview of the molecular structures of the large Mafs is shown in Figure 5.

In contrast to large Mafs, the genes *MAFK, MAFF*, and *MAFG* encode shorter proteins lacking the transcriptional activation domains. These proteins, which act as transcriptional repressors, are called small Mafs. Nevertheless, small Mafs can form heterodimers with cap'n'collar (CNC) proteins that harbor transcriptional activation domains.

The bZIP domain of the large Mafs is located near the C-terminus and is highly homologous to a similar domain in the AP-1 and CREB/ATF proteins. However, all large Mafs possess an adjacent homologous region, initially termed the

extended homology region. This region was subsequently shown to mediate DNA binding and was named the ancillary DNA-binding domain [94]. Large Mafs share an N-terminal transcriptional domain, consisting of approximately 100 amino acid residues rich in serine, proline, and tyrosine. The linker region between these domains is of variable length (Figure 5). *MAFA*, *MAFB*, and *C-MAF* contain histidine repeats between the transactivation domain and the ancillary DNA-binding domain. Another feature of this linker is the presence of a domain rich in glycine residues between the histidine repeats and the ancillary DNA-binding domain. *MAFA*, *MAFB*, and *C-MAF* lack introns, while *NRL* is encoded by four exons.
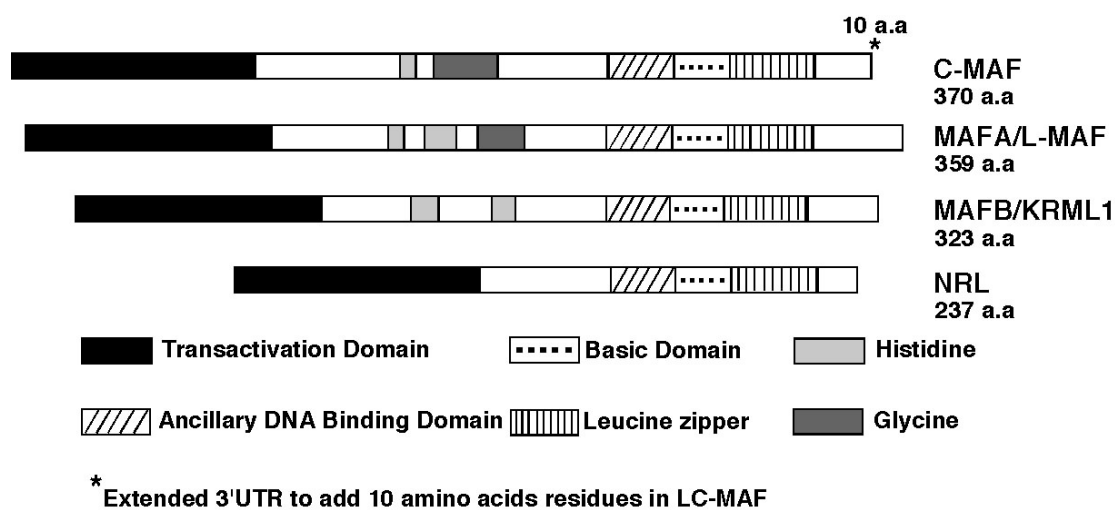

Figure 5: Molecular structures of human large Mafs.

The structural domains including the transactivation domains, ancillary DNA binding domains, basic regions, leucine zippers, histidine- and glycine-rich domains are shown in boxes

The leucine zipper allows the formation of Maf homo- and heterodimers with other compatible bZIP proteins to control gene expression [96,97,117,118]. The formation of heterodimers greatly expands the repertoire of DNA-binding specificities of these proteins. Heterodimers of C-MAF and NRL with c-Fos and c-Jun were observed in vitro [97,118]. MAFA, but not MAFB, also can heterodimerize with c-Jun [97,115]. It has been proposed that large Mafs can heterodimerize with each other [119]. Since a number of bZIP proteins are alternatively spliced, including CREB, CREM, and C-MAF, the number of potential heterodimers of this family is enormous. The mixing and matching of gene regulatory proteins to form Large Mafs binding sites are called Maf recognition elements (MAREs) and are derivatives of sites recognized by AP-1 and ATF/CREB proteins. *In vitro* selection of the optimal binding sites for *C-MAF*

generated two 13 and 14 base pair palindromic sequences, TGCTGACTCAGCA (T-MARE) and TGCTGACGTCAGCA (C-MARE) [96]. In support of this, T-MARE and C-MARE contain AP-1 (TPA-responsive element) and CRE consensus motifs in the middle region, respectively. Similar studies of C-MAF and NRL homodimers also identified binding to palindromic recognition sites with the consensus sequences $TGC(N)_{6-7}GCA$ [97], lacking the internal AP-1 site. In addition, Fos/NRL, Jun/NRL, and Fos/C-MAF heterodimers recognized the nonpalindromic consensus sequence $TGAC(N)_{3-4}GCA$. MAF binding sites found in rhodopsin, crystalline and other genes showed high conservation of 5'-TGCTGA-3' only in the 5'-half region. In the 3'-half site, the GCA motif is less frequent than the opposite TGC motif suggesting the important role of the TGC motif for MAF binding. All together, the TGCTGA sequence, comprised by a Maf motif TGC and an AP-1 motif TGA is conserved in most natural Maf binding sites. Furthermore, it was found that the 5'-AT-rich half-site of MARE next to the palindromic MARE is also an important binding site for the large Maf group [120].

6.3   *Expression pattern of MAFB and C-MAF during development.*

The expression of *MAFB* has been studied in mice, rats, chicken, and zebra fish. In mice, the analysis focused on the eye and brain. *MAFB* expression at the mRNA level was detected from E10.5 in mouse lens vesicle [121]. *In situ* hybridizations pointed to high levels of *MAFB* expression in lens epithelial cells [112,121-125]. It has been demonstrated that *MAFB* could activate several crystallin genes including the mouse αA-, αB- and γF-, chicken βB1-, and rat βB2-crystallin promoters [126-128]. However, none of these genes demonstrated the specificity of *MAFB* action. *MAFB* is also essential for renal development. *MAFB*(-/-) mutant mice display severe renal dysgenesis characterized by abnormal glomerular differentiation and nephritic tubular apoptosis associated with diminished expression of several kidney disease related genes [129].

In the mouse embryo, *C-MAF* is dynamically expressed in multiple tissues with different onsets of expression but *C-MAF* mRNA was mainly detected in the lens development [121]. Its expression is evenly maintained in lens vesicle, but is dramatically up regulated in primary lens fiber cells [130]. Mouse *C-MAF* is also widely expressed in regions such as the spinal cord, cartilage, spleen, kidney, heart, lung, intestine, muscle, uterus, and liver [124,130]. *C-MAF* has been shown to regulate

transcription of the L7 gene expressed in cerebellar Purkinje cells via two binding sites in the 5′-promoter [131]. These sites were both specific to *C-MAF* and NRL and were not activated by Fos or Jun. The mouse αA-, αB-, βB2, and γF-crystallin promoters are activated by *C-MAF* in lens and non-lens cultured cells [127,128,132,133]

6.4 The role of MAFB and C-MAF in the haematopoietic system.

MAFB has a well-established role in haematopoiesis as it is expressed only in monocytes/macrophages cells and can suppress erythroid differentiation by inhibiting erythroid gene expression via direct interaction with Ets-1 [134,135]. Exogenous *MAFB* leads to monocytic development of myeloid cell lines [136]. Another study showed that *MAFB* is highly expressed in CD34^{+} derived monocyte precursors as compared by granulocyte precursors [137]. By transducing leukemic monoblasts and cord blood CD34^{+} hematopoetic stem/progenitor cells with a "full-length" *MAFB* cDNA, induction of monomacrophage differentiation accompanied by inhibition of erythroid commitment was observed, indicating that *MAFB* is an important regulator of monocyte commitment in human hematopoesis. For myeloid dendritic cell maturation, down regulation of *MAFB* is required, regulated by balanced expression of *MAFB* and Ets family factor PU.1 [138]. Furthermore, *MAFB* is essential for F4/80 expression and actin organization in macrophages, suggesting a role of *MAFB* and perhaps MAFB/Fos complexes later in monocyte lineage development as shown in *MAFB*(-/-) fetal liver and spleen cells [129,139]. *MAFB* has been shown to act as an interaction partner and repressor of Ets-1, which represses transcription of the transferrin receptor [135]. During megakaryocyte differentiation, extracellular signal-regulated kinase (ERK) induces expression of *MAFB* and controls transcription of platelet GPIIb integrin (CD41) using DNA-binding GATA1 and Ets factors [140]. *MAFB* is not expressed anywhere during lymphopoiesis.

In hematopoesis, tissue-specific expression of the interleukin-4 gene in CD4^{+} T helper (Th2) cells is regulated by synergism between *C-MAF* and the nuclear factor for activated T cells (*NF-ATp*), interacting with two sites in the proximal promoter region [141]. High levels of C-MAF expression were found in angioimmunoblastic T-cell lymphoma (AILT) [142]. In combination with a study showing that over expression of *C-MAF* under the CD2 promoter contributes to T-cell lymphoma in mice [143], this suggests an important role for *C-MAF* in T-Cell lymphogenesis. Similar to *MAFB*, *C-MAF* plays an important role in monocytic differentiation [144] by interaction of *C-MAF*

with *C-Myb* that represses the *CD13/APN* promoter in myeolid cells [144]. Consistent with this role of *C-MAF*, it is observed that *C-MAF* expression is increased in MAFB-deficient macrophages [139]. This observation highlights the importance of the relative expression levels and functional interaction of different Maf members in haematopoietic differentiation processes [145,146]. *C-MAF* is not expressed anywhere during B cell development

7   Aim of this thesis.

In Multiple Myeloma patients, a translocation involving the IgH locus on chromosome 14 is an almost ubiquitous major event [49]. As a result of these translocations several genes may be up regulated and can act as oncogenes. In earlier work, our group identified a novel translocation in MM, i.e. the t(14;20)(q32;q11).

This thesis focuses on the molecular consequences of this novel t(14;20) in MM. Chapter 2 describes the cloning and the molecular characterization of the chromosomal breakpoint of the t(14;20), leading to the notion of *MAFB* over expression HMCLs and in patient samples.

A few studies have provided some insights into the molecular pathway and expression of *C-MAF* in MM. However, even though *MAFB* was placed in the same TC group as *C-MAF*, little to nothing was known about the role of *MAFB* in MM. In Chapter 3 we identified primary target genes of *MAFB* by using inducible *MAFB* cell lines followed by gene expression arrays at different time points. Comparing the *in vitro* data with gene expression profiles obtained from MM or PCL tumors carrying an activated *MAFB* gene validated the identified target genes *in vitro* and *in vivo*. Functional implications of these up regulated genes were studied by several assays.

In Chapter 4, we describe our novel anti-MAFB monoclonal antibody. With this antibody and using two different patient sets, MAFB oncoprotein expression in BM biopsies of MM patients was found only in patients harbouring the t(14;20).

To study the role of *MAFB* in MM *in vivo* we generated a *MAFB* transgenic mouse under the control of the human CD19 promoter and mouse Eμ enhancer. The results of this mouse study are described in Chapter 5.

Summarized, this thesis enhances our insights in the role of *MAFB* in MM.

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Chapter 2

The Recurrent Translocation t(14;20)(q32;q12) in Multiple Myeloma results in aberrant expression of MAFB; a molecular and genetic analysis of the chromosomal breakpoint.

The Recurrent Translocation t(14;20)(q32;q12) in Multiple Myeloma results in aberrant expression of *MAFB*; A molecular and genetic analysis of the chromosomal breakpoint.

Gienke R. Boersma-Vreugdenhil[1#], Jeroen Kuipers[1,4#], Esther van Stralen[1,4] ,Ton Peeters[1], Lucienne Michaux[2], Anne Hagemeijer[2], Peter L. Pearson[3], Hans C. Clevers[1,4], Bert J.E.G. Bast[1]

\# These authors contributed equally to this study

[1] University Medical Centre Utrecht, Department of Immunology, Utrecht, The Netherlands

[2] Centre for Human Genetics, KU Leuven, Leuven, Belgium

[3] University Medical Centre Utrecht, Department of Medical Genetics, Utrecht, The Netherlands

[4] Current address: Hubrecht Laboratory, Centre for Biomedical Genetics, Utrecht, The Netherlands

Summary

Chromosomal translocations of the Immunoglobulin Heavy chain gene region at 14q32 are regularly involved in B lymphoid malignancies; they may initiate transformation either by deregulation of existing (proto) oncogenes or creation of new hybrid genes with transforming properties. Previously, we reported a reciprocal novel translocation, t(14;20)(q32;q12), found in the myeloma cell line UM3. In this cell line, the t(14;20) is the only translocation involving the IgH locus. Using double color immuno FISH, we found the t(14;20) in the diagnostic bone marrow sample as well, excluding a possible *in vitro* artefact. We also have found this recurrent t(14;20) in four other cell lines and in additional patient material. We cloned the regions containing the breakpoints in the der(14) and der(20) chromosomes from UM3, and analysed ectopic mRNA expression of genes in the breakpoint regions of both derivative chromosomes. Ectopic gene expression was observed for the transcription factor *MAFB* in der(14). The breakpoint scatter in the five cell lines with a t(14;20) - all expressing *MAFB*- is comprised within a region of 0,8 Mb. Provisional data indicate that this t(14;20) is associated with an adverse prognosis. Aberrant expression of *MAFB* may be involved in the oncogenic transformation of myeloma cells that harbour the t(14;20).

Introduction

Translocations involving the Immunoglobulin Heavy chain (IgH) region at chromosome region 14q32 are regularly involved in human B cell malignancies and may up-regulate existing oncogenes or create new hybrid genes with transforming properties. For example, Burkitt lymphoma shows a specific t(8;14)(q24:q32). The proto-oncogene c-myc is translocated to the immunoglobulin heavy chain (IgH) locus at 14q32, resulting in increased expression of the oncogene due to the strong immunoglobulin enhancers[1]. In multiple myeloma (MM), a neoplasm of terminally differentiated plasma cells, translocations of the IgH locus are among the most consistent and common genetic changes. These translocations are found in the earliest stage of the disease suggesting that such translocations are an early and possibly initiating event in the disease development[2]. In addition, the frequency of IgH translocations increases with disease progression. Thus, t(14q32) has been detected in 74% of patients with MM, by Fluorescent In Situ Hybridization (FISH), and in 85% in plasma cell leukemia (PCL)[3], and we have found a t(14q32) in virtually all MM cell lines[4]. Recently, by using modified immuno-FISH techniques, which specifically identify plasma cells in a heterogeneous bone marrow cell population, we detected IgH translocations in as much as 96% of patients with MM at diagnosis[5].

In contrast to other lymphoid malignancies, translocations in MM occur mainly in or around the IgH switch regions, which are involved in the physiological process of isotype class switching[6]. Most of the 14q32 translocations cloned to date lead to dissociation of the intronic Ig μ enhancer and the α enhancer, so that potentially, oncogenes can be deregulated on each derivative chromosome. Recent molecular studies have recognized 4 major chromosome regions involving IgH translocations, i.e. 4p16 (*FGFR3* and *IgH-MMSET*), 6p21 (*CYCLIN D3*), 11q13 (*CYCLIN D1* and *MYEOV*) and 16q23 (*CMAF*), and a number of other regions, which are less frequently involved and as yet uncloned[2]. Previously, we reported a novel 14q32 translocation, involving chromosome 20 in a MM cell line (UM3)[4]. Now, we have found two additional MM cell lines (UM6 and EJM) as well as one myeloma patient having this t(14;20). To date, a similar, if not identical translocation has been detected in two MM cell lines (SACHI and SKMM1[7]) and has also been found in plasma cells from a patient suffering from PCL[7].

In the UM3 cell line, the t(14;20) is the only translocation in which the IgH locus is involved. We cloned the translocation breakpoints from this cell line and analysed aberrant regulation of gene expression in both derivative chromosomes and determined the variable positions of the breakpoints in the five t(14;20) containing-cell lines. Recently, it was shown that the t(14;20)(q32;q12) results in ectopic expression of *MAFB*, a transcription factor of the bZip family[7]. In this study we report this novel t(14;20) in three additional cell lines, all of which over express *MAFB* and we report the narrow breakpoint scatter (0.8 Mb) in the five cell lines with t(14;20) known to date.

Materials, Methods and Patients

Cell lines

The UM3 cell line is an IgG1-κ producing plasma cell line, established in our lab. The cell line was generated from bone marrow of a 51-year-old female patient with active myeloma, before treatment was started. Other MM cell lines that were used in this study are: UM1, UM6, and UM8 (established in our laboratory), XG1 (gift of dr. B. Klein), U266B1 (American Tissue Culture Collection; ATCC), L363 (German Collection of Micro organisms and cell cultures). RPMI8226 (ATCC), SKMM1 (gift of dr. A. Houghton[8]), SACHI (gift of dr. S. Iida[7]), and EJM (gift of dr. I.C.M. MacLennan[9]). A clinical and genetic overview of the cell lines carrying t(14;20) is presented in Table 1. The cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal calf serum. UM6, UM8 and XG1 were cultured in the presence of exogenous IL6 (1.25 ng/ml rhIL6, Roche, Almere, The Netherlands).

Cloning of the chromosomal breakpoint at der(14)

Genomic DNA isolated from the UM3 cell line was partially digested with *Sau3A*, and >10kb fragments were isolated on a sucrose gradient. Ligation into *BamH1* digested EMBL3 vector arms (Stratagene, La Jolla, CA, USA), and packaging using Gigapack III XL (Stratagene, La Jolla, CA., USA) was performed according the manufacturers' instructions. The packaged phages were used to infect XL1blue MRA(p2) bacteria and the library was plated and screened without amplification, hybridizing with a 1.5 kb *Sma1* fragment from a genomic IgG1 sequence. Since this probe recognizes all IgG subclasses, the positive plaques were replated and screened with an IgG1 specific oligo (GCTGAACCTCGCGGACAGTT). From the positive phages the insert was subcloned into bluescript (Stratagene, La Jolla, CA) and were used as FISH-probes on metaphase spreads from normal phytohaemagglutinin (PHA)-stimulated human lymphocytes. Only one clone contained the breakpoint from the der 14 chromosome as seen by a split signal on both chromosome 14 and 20. This clone was further characterized by sequencing.

DNA sequencing and sequence alignment for the reciprocal breakpoint at der(20)

Automated sequencing was performed using an ABI PRISM automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were checked against the Genbank and European Molecular Biology Laboratory (EMBL) databases using the Basic Local Alignment Search Tool (BLAST) and FASTA programs made available through the Human Genome Mapping Project Resource Centre (HGMP-RC; Hinxton Hall, Cambridge, UK)[10].

Based on the sequences around the t(14;20) breakpoint at der(14), the following primers were selected to amplify and analyse the reciprocal breakpoint at der (20): GCTCAGCTAAGAAATACTAGC and GAGACGGATCTCACTGTCAC. DNA Amplification using these primers results in a product of 723 base pairs.

Northern blot analysis

For the analysis of expression of putative target genes, Northern blot analysis was performed by routine methods. Based on genomic data provided by the HGMP genomic database, several expressed sequence tags (EST's) and genes from chromosome 20 up to a distance of more than 1 Mb at both sides of the breakpoint, were selected. cDNA's for these genes were obtained from the HGMP-RC to be used for northern blot analysis. The following I.M.A.G.E. cDNA clones were used: 0824985 (*TIMAP*); 490553 (*CHAPERONIN 10*); 0789040 (*C-SYN*); 2307154 (*MAFB*); 2163509 (*TOP1*); 362553 (*PLCG1*); 1753533 (*KIAA 0398*); and EST clones: 2689478; 1736745; 783638; and 610225.

Quantitative-PCR

To confirm the results of the northern blots we performed quantitative polymerase chain reaction (Q-PCR) to analyse the expression levels of *MAFB*. The PCR analysis was performed by using an ABI PRISM 7700 sequence detector. The primers were 5' TGTCCTCCACACCGCTCAG 3' and 5' TGCTCGCCATCCAGTACAGA 3'. For the PCR we used the SYBR Green PCR kit (P.E. biosystems, Warrington, UK) and cDNA's for this PCR were obtained by the use of de 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Applied Science, Indianapolis, IN, USA).

Clinical sample of multiple myeloma plasma cells

A 71-year-old woman was presented with stage III IgA k multiple myeloma. The bone marrow aspirate disclosed 45% plasma cells. A 72-hours culture without PHA stimulation was performed, and conventional karyotyping showed t(14;20) among complex karyotypical aberrations:

43, XX, del(1)(q32), t(1;3)(p31;q21), t(2;9)(q14;p21), der(6)t(6;12)(q27;p11), der(7)t(7;20)(q31;q12), -12, -13, der(14)t(7;14)(q31;q32), der(14)t(14;20)(q32;q?), der(16)t(1;16)(q21;p13), der(17)t(11;17)(?;p), der(19)t(1;19)(q32;p13),-22[2]/
43, XX, del(1)(q32), del(1)(p12p21), der(6)t(4;6)(q?;q25), der(7)t(7;20), t(7;11)(q11;q23), -10, der(11)t(10;11)(q21;p15), -12, -13, der(14)t(7;14), der(14)t(14;20), der(16)t(1;16), der(17), der(19)t(1;19), -22, +mar[3]/46,XX[1]

The chromosomal aberrations were further characterized by Multicolor-FISH, using the SpectraVysion probes (Vysis, Downers Grove, IL, USA) and the Quips software and with a dual color break-apart probe for the IgH locus (LSI IGH Dual Color, Break Apart Rearrangement Probe; Vysis). For the interphase FISH we used uncultured CD138+ sorted plasma cells.

Fluorescent in situ hybridization studies

Dual color- FISH (DC-FISH) was performed according to standard procedures, with some minor modifications. To detect 14q32 translocations a dual color probe set spanning the IgH locus was used as described earlier[5]. The IgH2 probe, located telomeric from the IgH locus was labeled with digoxigenin, and a probe hybridising to Cα2, located at the centromeric position in the IgH locus was labeled with biotin; both probes were detected with either a fluorescein isothiocyanate (FITC) or CY3 fluorochrome (Figure. 1A). The probes give a fusion signal at a normal IgH locus (yellow spot), but are separated in case of translocation of the IgH locus (segregated red and green spots).

The partner chromosome was recognized in metaphase preparations by the translocation of the IgH2 signal, and identified by 4',6-diamidino-2-phenylindole (DAPI) counterstaining of the chromosomes. In case of a putative t(14;20), the identification of the partner chromosome was confirmed by a second set of probes, using Cα2 and the *MAFB* probe (RP1-644L1) located at 20q12 (Figure. 1A).

To analyse the localization of the breakpoints at chromosome 20, plasmid artificial chromosome (PAC) and bacterial artificial chromosome (BAC) clones derived from chromosome 20 (obtained from the Sanger Centre, Cambridge, UK) were used (for their respective localizations see Figure 2). Hybridisation of these probes was combined with a probe for chromosome 14 (centromere 14 or Cα2).

For the detection of t(14;20) in bone marrow material from patients with MM, DC-immuno-FISH was performed as described previously[5]. Briefly, this technique combines immunological staining of Ig light chains with conventional DC-FISH; here we used probes for the IgH locus (Cα2) and for the *MAFB* locus (RP1-644L1) at chromosome 20.

Results

Detection and molecular cloning of the breakpoint of t(14;20).

The UM3 cell line was used for the detection and cloning of the breakpoint of t(14;20). G-banding of the UM3 cell line revealed its complex karyotype featuring hypo-tetraploidy with a 14q+ chromosome and monosomy 13 among other abnormalities (Table 1). FISH analysis of the UM3 cell line identified a t(14;20) and one normal, non-translocated chromosome 14[4].

Cell line	Age/sex	Status; survival	Ig	Cytogenetic karyotype	FISH	Gene alteration
UM3	51/F	Active MM; 9	IgGκ	44,X,-X,del(1)(?),-13, der(14) del(14)(q?q?) ins(14;1)(q?;?) add(14)(q?),-16,-20, +der(?)t(?;16)(?;?), +mar/hypotetraploidy	t(14;20)(q32;q12)	MAFB
SKMM1	55/M	PCL; 13	Ig-κ	33-45,-X,Y, -1, del(1)(p ?),t(4 ;19)(?;?), del(6)(q ?),t(8 ;10)(q ? ;q ?),-9, +del(11)(q11), t(11 ;15)(q ? ;q ?), der(13)t(1 ;13)(q ? ;p ?),add(14)(p13), add(14)(q32),-15,-18,-20.	t(14;20)(q32;q12) t(6;14)(p25;q32)	MAFB MUM1/IRF4-IGH
EJM	58/F	Refractory MM; 12	IgGλ	51,X,-X,+1,del(3)(q ?),-4, +5,del(6)(q21), +del(9)(q11),+ del(9)(q11), +del(11)(q11), +del(11)(q11), +del(12)(p11), -13,-13,-14,i(14)(q10),-15,-15,-16,del(18)(q11),+20,-21,-22,+7mar	t(14;20)(q32;q12) t(14q32)	MAFB P53 mutation
SACHI	47/F	PCL; 16	IgGλ	Hyperdiploid, add(14)(q3?2).	t(14;20)(q32;q12) t(8;14)(q24;q32)	MAFB c-MYC
UM6	61/M	PCL;13	IgAκ	Hypertriploid, add (14)(q ?)	t(14;20)(q32;q12)	MAFB
L363	36/F	PCL; n.a.	IgG	49(45-50)<2n>X, -X,-X, -6, +7, +8, +11, +11, +14, +19, -22, t(5;8)(q12/13;q24.32), der(8)t(5;8) (q12/13;q24.32), der(8)t(7 ;8)(q11.2 ;p22), del(11)(q13)x2, add(14)(q32), del(17)(p12), add(19)(q13) ; sideline with add(13)(q34)	t(6;20)(p?;q12)	MAFB P53 mutation

Table 1. Clinical and genetic characteristics of 5 MM cell lines with t(14;20)(q32;q12). PCL: plasma cell leukemia. Note that t(14;20) was not detected by classical karyotyping in any of the cell lines. Survival after diagnosis is given in months.

Metaphase FISH analysis was performed on the UM3 cells using a dual color IgH probe set and showed a translocation of the IgH locus at chromosome 14 and one or two normal IgH loci. The distal probe was detected at chromosome 20 (Figure 1B). The identification of the partner chromosome was confirmed by FISH-detection using a probe located at 20q12 (Figure 1C). Furthermore, this t(14;20) was detected in the clinical bone marrow sample obtained from the patient at the time of diagnosis (Figure 1D), thereby excluding the possibility that t(14;20) would be an artefact of cell culture.
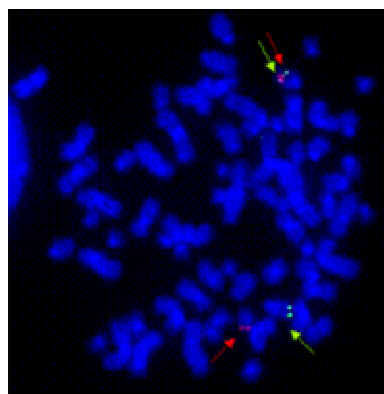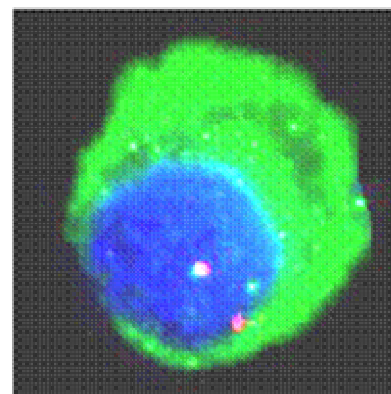
t(14;20) results in MAFB expression

A
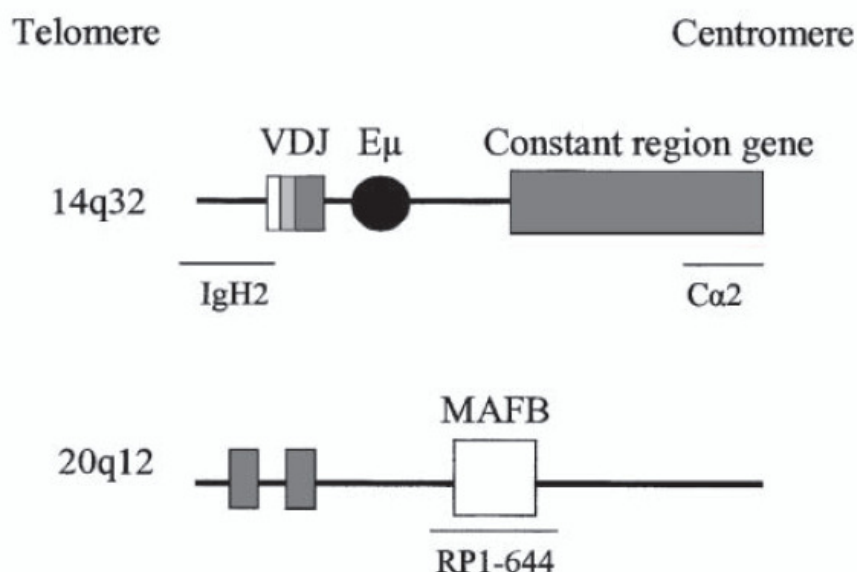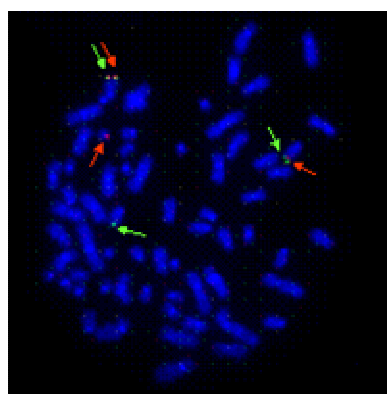
B   **C**   **D**

Figure 1. Detection of t(14;20)(q32;q12) in UM3 cell line and the initial clinical bone marrow sample at time of diagnosis.

A Diagram of 14q32 and 20q12 with the location of the probes, used for the DC-FISH

analysis (not in scale).

B DC-FISH analysis of a representative metaphase of UM3 cells hybridised with the IgH2(red)/Cα2(green) probe set. A single fusion (yellow) represents the normal IgH allele, and separate red and green signals indicate a translocation at the IgH locus. Note that this metaphase contains three chromosomes 14 and there is one chromosome 14 involved in the t(14;20).

C DC-FISH analysis using the Cα2(green)/20q12(red) probe set on metaphases of UM3 cells. Here, a fusion signal represents a translocation of IgH and 20q12. The probe for 20q12 (RP1-644) is shown in red and the probe for 14q32 (Cα2) is shown in green.

D DC-immuno-FISH analysis of a bone marrow sample from the patient, from which the UM3 cell line was derived, using the Cα2/20q12 probe set. Note that in the plasma cells (cytoplasmic Ig staining in green) the chromosome 20 probe (shown in red) co-localizes with the Cα2 probe (shown in green).

As described earlier by our group, the breakpoint of the UM3 cell line was detected around the switch γ1 region of the IgH locus using fibre-FISH analysis[4].

Therefore, a genomic phage library of the UM3 cell line was screened with a Cγ1 probe to identify clones that may contain the breakpoint. By screening the library, a genomic DNA clone of 11 kb was picked up and found to span the translocation breakpoint. This was verified by using this clone as a probe in FISH analysis on the UM3 cell line. The probe, indeed, hybridised to chromosome 14, the der(14) and der(20). Furthermore, sequence analysis of the clone confirmed the presence of sequences from the IgH locus as well as chromosome 20. By BLAST and FASTA analysis, the breakpoint was found at position 39436 of the BAC clone Emb: AL133419 (corresponding to bA101 in Figure 2), which was cytogenetically located at 20q12. The breakpoint in the IgH locus was located at position 91046 of the Emb: X97051.1. Interestingly, the breakpoint was found to be within the enhancer of the μ gene, adjacent to the sequence of the switch μ region.
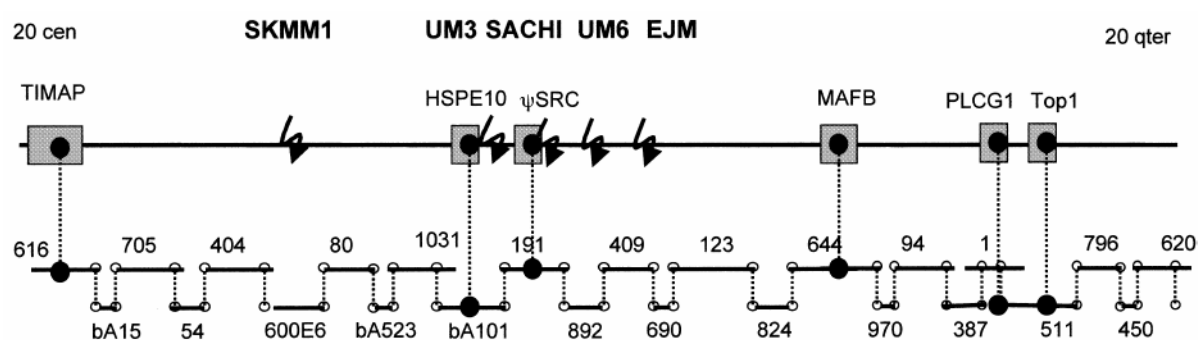

Figure 2. Chromosomal breakpoints identified in MM cell lines carrying t(14;20). Chromosomal breakpoints were analysed by FISH analysis using BAC and PAC clones derived from chromosome 20. The scheme represents the breakpoint scatter in 5 MM cell lines carrying t(14;20). The established barcode for chromosome 20 is depicted at the bottom. Jagged arrows indicate the breakpoints of indicated MM cell lines.

Next, we analysed the reciprocal breakpoint at der(20). Therefore, we selected PCR-primers from the distal sequence of the IgH locus and from chromosome 20, centromeric to the position of the breakpoint as detected at der(14). The breakpoint region was amplified by PCR analysis, and subsequently analysed by sequence analysis. Alignment to the Genbank demonstrated that the reciprocal translocation resulted in a loss of 35 nucleotides.

MAFB expression is associated with t(14;20)

Translocation of the IgH locus results in juxtaposing genes from the partner chromosome to the strong α-enhancer and regulatory units, which are found

centromeric from the IgH locus. According to the HGMP database, the breakpoint at chromosome 20 is located in a gene-poor region. The most proximal gene that is located telomeric from the breakpoint is *MAFB* at a distance of approximately 700 kb. Furthermore, the genes for topo-isomerase 1 (*TOP1*), and phospholipase C gamma 1 (*PLCG1*) were found at a distance of up to approximately 1 Mb. An EST and a pseudo-gene (*ψSRC*) have been reported in the same genomic region and were included in the analysis (Figure 3).
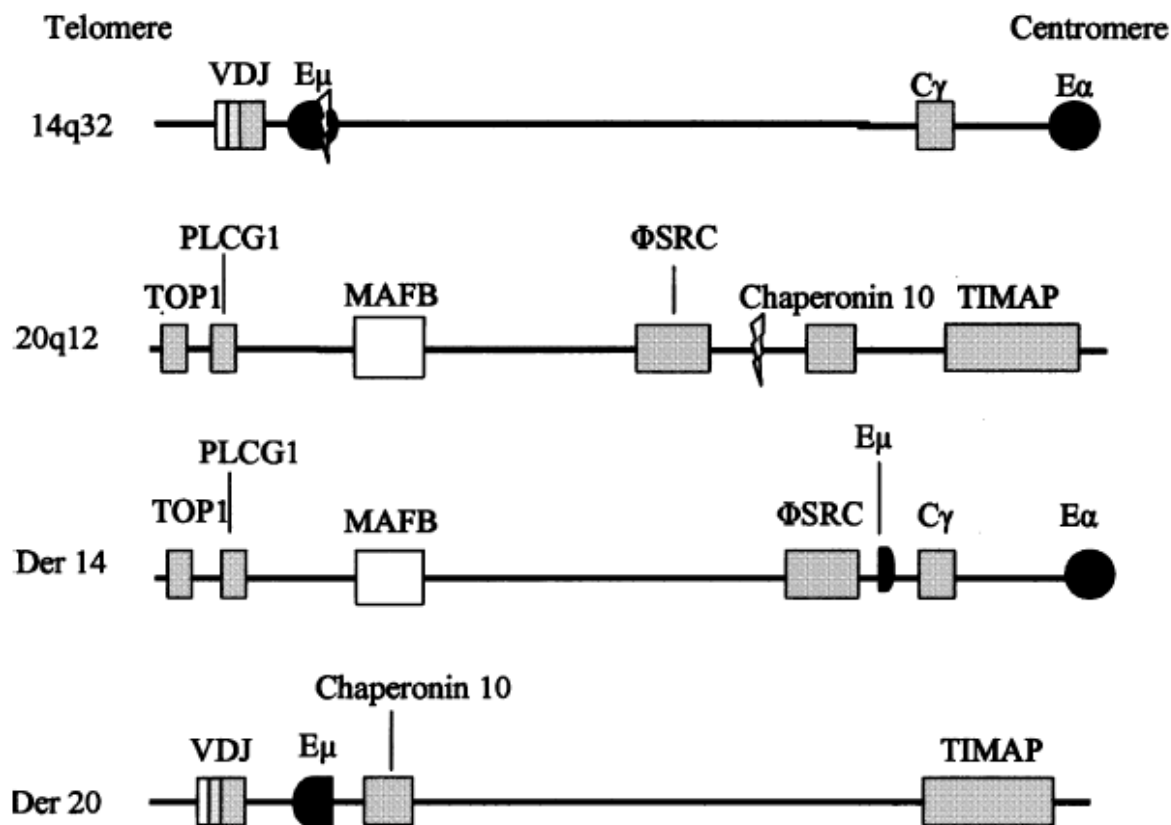

Figure 3. Potential target genes in the reciprocal t(14;20).

Diagram of the breakpoints in 14q32 and 20q12 (not in scale)

The potential target genes of 20q12 are juxtaposed to the strong α-enhancer on 14q32 and target genes of 14q32 are juxtaposed to the truncated μ-enhancer as a result of the reciprocal t(14;20).

Indicates breakpoints in the UM3 cell line

To investigate whether the translocation t(14;20) resulted in aberrant expression in any of these genes or EST's, we examined the expression of each of their mRNAs by means of Northern blot analysis of MM cell lines with t(14;20): UM3, UM6, SKMM1, EJM and SACHI. As control cell lines, several MM cell lines with different IgH translocations were included. Expression of the EST and pseudo-SRC gene was not detected in any of the cell lines tested (data not shown). *TOP1* and

PLCG1 were expressed in all cell lines tested, with comparable levels (data not shown). Interestingly, the *MAFB* gene was almost exclusively expressed in the t(14;20) containing cell lines (Figure 4A). However, *MAFB* expression was absent in our panel of control MM cell lines with exception of the L363 cell line. FISH analysis showed that the L363 cell line did contain a translocation also involving 20q12 but with chromosome 6 as partner chromosome (data not shown), possibly accounting for the deregulated *MAFB* expression.

To confirm the expression of *MAFB* in the MM cell lines we performed quantitative-PCR. Expression of *MAFB* was found in the five cell lines with a t(14;20) and in the cell line L363 with a t(6;20) (Figure 4B).

MAFB plays a role in erythroid differentiation and is in the haematopoietic system expressed in immature myeloid/monocytic cells but not in lymphocytes[11]. Therefore, expression in MM cell lines with t(14;20) is considered ectopic.
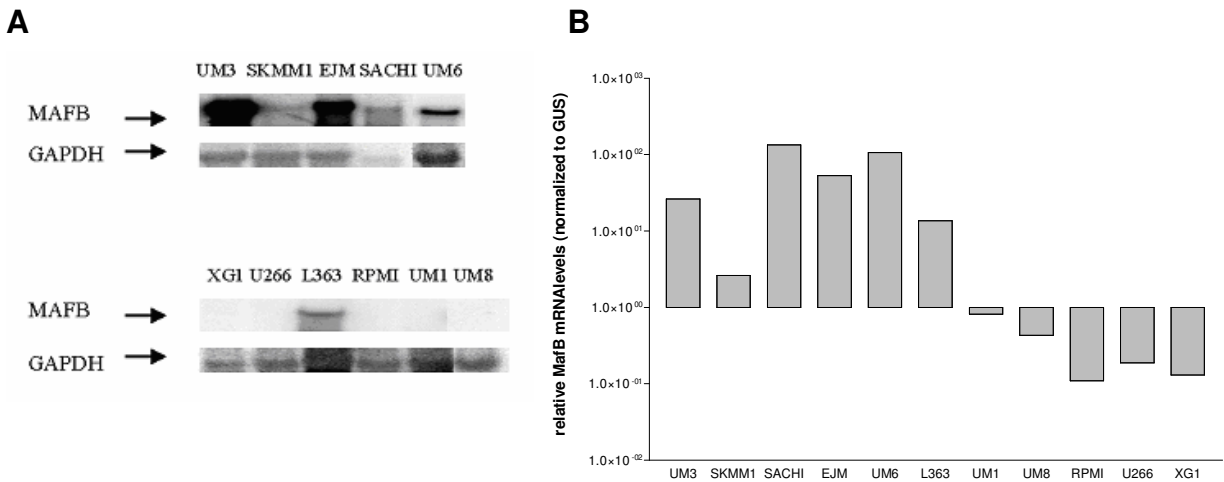
Figure 4. Ectopic *MAFB* gene expression in MM cell lines carrying t(14;20).

A *MAFB* mRNA expression was evaluated by means of Northern blot analysis in five cell lines carrying t(14;20): UM3, SKMM1, SACHI, UM6 and EJM (upper panel) and in control MM cell lines with different IgH translocations (lower panel). As a loading control the expression of GAPDH mRNA is shown below.

B Relative *MAFB* mRNA expression analysed by Q-PCR. The expression of *MAFB* was normalized to the GUS housekeeping gene

Chromosomal breakpoint scatter of t(14;20) in MM cell lines.

In order to analyse the breakpoint regions in the different t(14;20) cell lines, DC-FISH analysis was performed on metaphase chromosomes. BAC and PAC clones covering the sequence of chromosome 20 located between the markers D20S810 (20q11.23) and D20S850 (20q12), were detected with a FITC conjugate. The Cα2 probe that hybridised in the IgH locus on chromosome 14 was visualized with a CY3 conjugate. Hybridisation of the Cα2 probe with a probe situated centromeric to the breakpoint on chromosome 20 resulted in segregated signals while hybridisation with a probe telomeric to the breakpoint resulted in a fusion of the red and green signals on the der(14) resulting in a yellow signal. Using this strategy we confirmed the localization of the chromosomal breakpoint of UM3 and identified the breakpoints of SKMM1, SACHI, EJM and UM6 as shown in Figure 2. The breakpoints were situated within a region of maximum 800 kb as estimated from the PAC/BAC data with respect to size and sequence. This breakpoint scatter reflects a variation in distance of the IgH locus to the *MAFB* gene of between 500 kb and 1 Mb.

The t(14;20) is present in different sub clones of a patient with MM.

Besides the patient from which the UM3 was derived and apart from the confirmed expression of the t(14;20) in clinical material of the patient yielding the UM6 (data not shown), the t(14;20) was present in different sub clones of a additional MM patient. The t(14;20) was determined cytogenetically and characterised by R-banding (five metaphases) and Multicolor-FISH (six metaphases). In all abnormal metaphases analysed, both 14q32 were abnormal i.e. t(7;14) and t(14;20) in 8 cells and t(13;14) and t(14;20) in three cells.

FISH analysis with the IGH Dual Color, Break Apart Probe and subsequently whole chromosome paint 14 on metaphases after culture, confirmed the presence of two genetically different sub clones. One clone contained a balanced translocation of 14q32 and a deletion of the whole IgH locus (one green and one red pattern). In the second clone there was also a balanced translocation of 14q32 but it had a deletion of the IgH variable region (one green and two red pattern).

Interphase FISH with the IGH Dual Color, Break Apart Probe showed two main populations in purified plasma cells (magnetic-activated cell sorted CD138), one with two balanced translocations (two green, two red signal patterns in 39 % of

the cells) and the second with one balanced translocation and IgH variable deletion (one green, two red signal pattern, in 42 % of the cells). When tested with the Cα2/*MAFB* probe combination, the t(14;20)(q32;q12) was found in 62% of the cells. Altogether, these findings suggest that the t(14;20) is the primary change and that the other translocations (t(7;14)and t(13;14)) are secondary and less stable and associated with deletions.

The t(14;20) is associated with short survival time after diagnosis.

The patient described above died within 19 months after diagnosis. The cell lines containing t(14;20) were all derived from patients with a rather progressive form of MM and/or PCL (see Table 1). Mean survival time after diagnosis of the three patients reported in this study, and of the patients giving rise to the SACHI, EJM and SKMM1 cell lines, was less than 14 months[8,12] and Hamilton, personal communication 2004. In addition, the high occurrence of this t(14;20) in human myeloma cell lines (11 %[13]) suggests that *MAFB* has a high transforming capacity. Our (unpublished) results in raising MM cell lines (attempted with material from 50 patients, succeeded in 10 instances, two of which harbour this t(14;20)), fit with this notion.

Discussion

This study describes the cloning and molecular characterization of a novel recurrent translocation t(14;20) in MM. This translocation was now detected in five independently derived MM cell lines and resulted in ectopic expression of *MAFB*. The ectopic expression of *MAFB* is supposedly influenced by enhancer capacity of the strong α-enhancers in the IgH locus, which are known to be capable of acting over a long distance[14,15]. In addition, although this study was not intended to define the presence of this t(14;20) in primary patients material, we found it in three instances.

Translocations with breakpoints that occur inside regions of physiological recombination mediated by B-cell-specific mechanisms are considered as primary translocations[2]. Accordingly, the recurrent translocations involving chromosome regions 4p16, 6p21, 11q13 and 16q23 seem to have resulted from mistakes during the IgH switch process[16], whereas other "secondary" translocations resulted from subsequent chromosomal instability and tumour progression.

The t(14;20) described here involves the enhancer rather than the switch region and might be considered therefore to be a secondary translocation. However, here we present data indicating that the t(14;20) may be regarded as a primary translocation instead. First, in the UM3 and in the UM6 cell lines, the t(14;20) was the only simple and reciprocal translocation found involving the IgH locus. Secondly, in a MM patient carrying t(14;20), the plasma cells presented different clonal populations of which one exhibited a t(14;20) only, a second carried both t(14;20) and t(7;14), and in a third population the t(14;20) seemed to be accompanied by a t(13;14) (data not shown). The consistent presence of t(14;20) in all subpopulations of plasma cells is suggestive that t(14;20) is a primary translocation event followed by secondary translocations at the other loci. The t(14;20) has recently been regarded as a primary translocation indeed[13].

The functional implication of high level expression of *MAFB* in plasma cells as a result of t(14;20) and its putative role in the pathogenesis of MM is still unknown. *MAFB* has been shown to transform chicken embryonic fibroblasts when it is artificially over-expressed[17], indicating that it can act as an oncogene under certain conditions. *MAFB* is the avian homologue of the murine Kreisler gene product (*KRML*) and belongs to the family of Maf proteins (including c-Maf involved in the

t(14;16)(q32;q23) in MM), a subgroup of AP-1-type bZip transcription factors[18]. It can form homodimers via its leucine zipper domain that recognizes specific DNA consensus sequences termed Maf response elements or MARE [18]. Maf proteins have been implicated in several differentiation processes including haematopoiesis. It has a dual role as repressor of the erythroid differentiation by direct interaction with ETS1, and selectively stimulates monocyte/macrophage differentiation[18,19]. *MAFB* seems to be ubiquitously expressed in various tissues, while its expression in haematopoietic cells is restricted to myelo-monocytic lineage and macrophages[11]. Thus, expression in myeloma cells, originating from plasma cells can be regarded as ectopic. *MAFB* negative mutant mice exhibit defects in hindbrain patterning[20]. A possible aberrant regulation of cellular proliferation and/or apoptosis (underlying the oncogenic potential in plasma cells) is currently being addressed in studies using short interference RNA to knock down *MAFB* in the cell lines with t(14;20) and transgenic mice where the *MAFB* gene is placed under control of the IgH promoter and enhancers.

In the UM3 cell line the translocation breakpoint in the IgH locus was detected to be within the Ig μ enhancer (Eμ). Thus, a truncated enhancer gene was translocated to chromosome 20. According to its sequence, this truncated gene encoded several active domains of the enhancer[15]. It has been reported that in t(4;14)(p16;q32) containing cell lines, over expression of the MMSET gene at der (4) is found, apparently because of the influence of the translocated Eμ enhancer[21]. We investigated whether such a bi-directional up regulation was found in the t(14;20) as well. After cloning of this truncated μ enhancer (Δ3') into a luciferase reporter vector and transfection in the UM3 cell line, approximately 60% of the capacity of the full length Eμ was found (data not shown). Genes at der(20) might become aberrantly up regulated due to translocation of the truncated Eμ to chromosome 20. Indeed, initial results of Northern blotting with the *TIMAP* probe (see Fig. 4) seemed to show such an up regulation, but these findings could not be reproduced using Q-PCR.

Recent studies provided support for a molecular classification based on the translocation partner chromosome, and this grouping may represent clinically different patient populations[22,23]. The several MM cell lines with t(14;20)(q32;q12), and several patients described with this translocation indicate that this is a recurrent translocation. Studies on the frequency of this translocation at the time of diagnosis

need to be determined. Based on the analysis of the breakpoint scatter in the five cell lines with t(14;20), the PAC clone RP1-644L1 would be a suitable probe to use in DC-FISH analysis of patient material, since this probe will move to chromosome 14.

Studies on the prognostic value of this translocation need to be done as well. Provisional data suggest that the occurrence of this t(14;20) is correlated with a poor prognosis. Actually, Barillé *et al* (2003), included provisionally the t(14;20) together with the other maf-related translocation (t(14;16)) within a single clinical entity, characterized by a poor prognosis and high cyclin D2 activity. Evaluation in a large cohort of MM patients is needed to document more precisely the clinical correlation of t(14;20) with prognosis and response to treatment.

Acknowledgements

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t(14;20) results in MAFB expression

Chapter 3

Identification of primary MAFB target genes in multiple myeloma.

Identification of primary MAFB target genes in multiple myeloma.

Esther van Stralen[1,2], Marc van de Wetering[2], Luca Agnelli[3], Antonino Neri[3,4], Hans C. Clevers[2], Bert J.E.G. Bast[1],

[1] University Medical Center Utrecht, Department of Immunology, Utrecht, The Netherlands.

[2] Hubrecht Institute, Center for Developmental Biology and Stem Cell Research, Utrecht, The Netherlands.

[3] Laboratory of Molecular Genetics and Gene Expression, Fondazione IRCCS Policlinico, and

[4] Department of Medical Sciences, University of Milan, Milan, Italy.

Summary

Objective: in multiple myeloma (MM) seven primary recurrent translocations involving the Immunoglobulin Heavy chain (IgH) locus have been identified. One of the partner loci maps to 20q12 and involves the MAFB gene resulting in its ectopic expression. We attempt here to identify MAFB target genes in MM.

Design and methods: we used an inducible system to up regulate MAFB in MM cell lines not carrying the t(14;20). Micro array expression analysis was used to detect gene expression changes upon MAFB expression. These genes were further evaluated comparatively with gene expression profiles obtained from MM or plasma cell leukemia (PCL) tumors carrying an activated MAFB gene. Functional implications of these up regulated genes were studied by testing their promoter activity in reporter assays. C-MAF was included comparatively as well.

Results: the inducible cell lines identified a total of 284 modulated transcripts. After further evaluation using ex vivo data 14 common up regulated genes were found, common to the C-MAF pathway as well. The promoter activity of some of these secondary genes proved a functional relationship with MAFB. In connection with one of these secondary genes (NOTCH2) even tertiary up regulated genes were found. Functional studies indicated that inducible MAFB expression conferred anti-apoptotic effects.

Conclusion: we identified 14 up regulated genes, and their downstream consequences in the combined MAFB/C-MAF pathway. 11 of these genes are novel in the C-MAF pathway as well. These direct target genes may be responsible for the oncogenic transformation of MAF expressing myeloma cells.

Introduction

Multiple Myeloma (MM) is a malignancy of terminally differentiated plasma cells characterized by karyotypic complexity including numerical and structural chromosomal aberrations. A major event in MM is translocations involving the Immunoglobulin Heavy chain (IgH) region at chromosome 14q32. These are simple reciprocal translocations where the partner chromosome is juxtaposed to one of the strong Ig enhancers and are defined as primary translocations. As a result of this translocation genes located on the partner chromosome can act as oncogenes. There are seven well-known primary translocations identified in MM, causing over expression of specific genes - t(4;14)(p16;q32): *MMSET/FGFR3* (15%); t(6;14)(p21;q32): *CYCLIN D3* (2%); t(11;14)(q13;q32): *CYCLIN D1* (15%); t(12;14)(p13;q32): *CYCLIN D2* (<1%); t(14;16)(q32;q23): *C-MAF* (5%); t(14;20)(q32q12): *MAFB* (2%) and t(8;14)(q24.3;q32): *MAFA* (<1%) [1]

MAFB, *C-MAF* and *MAFA* belong to the MAF family of basic leucine zipper (bZip) transcription factors [2] and have trans-activating functions [3]. Ectopic expression of *MAFB* was found in several MM cell lines and additional patient material all carrying t(14;20) [4,5]. Studies of the prognostic value of the t(14;20) suggest that its occurrence is correlated with a high *CYCLIN D2* activity and a poor prognosis [6].

All primary translocations have been classified in Translocation and Cyclin (TC) groups, based on the respective dysregulated oncogene and Cyclin D expression. This system can be used to classify patients into groups that have distinct subtypes of MM. *MAFB* and *C-MAF* are classified in TC group 5 which expresses the highest levels of *CYCLIN D2* and is detected in 7% of all newly diagnosed MM patients [7].

To define the molecular basis of MM and delineate better the changes in gene expression occurring in different forms of plasma cell dyscrasias, gene expression profile analyses have been performed [8-10]. These studies describe dysregulated genes in patient material caused by primary translocations. In vitro studies for *C-MAF* and *FGFR3* were done to understand the pathway regulated by induced over expression of both genes in MM cell lines [11-13]. So far, the molecular consequences of *MAFB* over expression in MM have not been characterized.

Here, we studied the role of *MAFB* in MM. *MAFB* was over expressed in MM cell lines not carrying t(14;20). An *in vitro* model system was generated allowing

induced expression of *MAFB*. Gene expression patterns of the original MM cell line were compared to the modified MM cell line. *MAFB* target genes were identified by micro array analysis at different time points after *MAFB* induction. To validate the *in vitro* data, supervised analyses of *MAFB* positive patients compared to *MAFB* negative patients were performed. Here, we describe a set of genes that we show to be regulated by *MAFB* both *in vitro* and *in vivo.* Functional implications are shown by their promoter activity in reporter assays, and by their anti-apoptotic effect. Data on *C-MAF* regulated genes are included comparatively as well.

Material and methods

Cell lines

The following human myeloma cell lines (HMCL) were used in this study: UM1 (described earlier [14]) and U266B1 (American Tissue Culture Collection; ATCC). The cell lines were cultured in RPMI 1640 supplemented with 5% fetal calf serum and 1% Penicillin/Streptomycin.

Generation of stable MM cell lines expressing pcDNA4/TO-HAtag-MAFB

The coding region of *MAFB* was cloned into pcDNA4/TO (Invitrogen) using the *EcoRI* and *XbaI* sites. A Human influenza hemagglutinin (HA)-tag was placed in frame between the EcoRI site and the *MAFB* start site of transcription. Expression of *MAFB* was tested by Western Blot analysis. To generate stable clones expressing the Tetracycline repressor (TetR), the T-Rex system was used (Invitrogen). The cell lines were transfected by electroporation (Biorad) and cells were grown under Blasticidin selection. Resistant clones were tested for TetR activity by luciferase assays. 2.5×10^6 cells per well were transiently transfected with 4µg pcDNA4/TO-Luciferase [15] by electroporation. CMV-Renilla was used as an internal transfection control. Cells were seeded and induced with doxycycline (1 µg ml^{-1}). Luciferase activity was determined after 24h of induction using the Dual-luciferase reporter assay system (Promega) (data not shown). Cell lines expressing the TetR were re-transfected with pcDNA4/TO-HAtag-MAFB.

Immunofluorescence

MM cell lines expressing TetR and *MAFB* were induced with doxycycline (1µg ml^{-1}) for 48hours. Cytospins from induced and non-induced cells were prepared and fixed with 10% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in PBS and stained with a mouse anti-HA antibody (MαHA) (Sigma-Aldrich). Antibody binding was visualized by using a FITC anti mouse secondary antibody (FITCαM) (SouthernBiotech).

Western blotting

2.5 x 10^5 cells of the MM cell lines expressing TetR and *MAFB* were seeded in six-well plates and induced with doxycycline (1µg ml^{-1}) for 48 hours. Total cell lysates (TCL) were made from induced and non-induced cells whereby non-induced cells served as a control. Cells were washed with PBS and TCL were prepared by resuspending the cells in 2 x sample buffer (4% SDS, 12% glycerol, 50mM Tris-HCL pH 6.8, 0.01% bromophenol blue, 5% 2-β-mercaptoethanol). TCL were sonicated on ice and boiled for 5 minutes. Proteins were separated on 12.5% SDS-polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). After blocking in 5% skimmed powder milk in 0.1% Tween-20 blots were incubated with anti-HA antibody (Roche) and RAMPO (Pierce) as the secondary antibody. Immune complexes were visualized using an enhanced chemiluminescence kit (ECL plus, Amersham Life Science).

Northern blotting

To analyze expression of target genes, Northern Blot analysis was performed as previously reported [16]. The I.M.A.G.E. cDNA clones 4453777 *RhoE/*RND3, 6055379 *NOTCH2*, 4749611 *HES1*, 6204648 *HES5*, 5140396 *HES7* (RZPD, Germany) and 5933493 *ITGB7* (Geneservice Ltd, UK) were used to generate probes. cDNA of C-MAF was kindly given by Dr W.M. Keuhl, Mayo Clinic Arizona, USA.

Micro array analysis

Total RNA was isolated from U266/MAFB cells after induction for 5h and 10h and from non-induced cells. Syntheses of cDNA and Cy-3/Cy-5 labelings were performed using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). The samples were hybridized on Whole Human Genome Oligo 44K G4112A Micro arrays. Dye swaps were performed for each experiment and data analysis was done according to Agilent training manual instructions.

In vivo validation

The probe sets of the genes found by the Agilent array's were correlated with those represented on both Affymetrix GeneChip® HG-U133A and HG-U133Plus, identified by blasting the sequences of the Agilent probe sets using UCSC Genome

database. The expression profiles of the 279 HG-U133A selected probe sets were evaluated for 102 MM and 9 PCL patient samples from a proprietary data base deposited in NCBI Gene Expression Omnibus at *www.ncbi.nlm.mih.gov/geo* and accessible through GEO Series accession numbers GSE2113 and GSE2912). The expression profiles of the HG-U133Plus selected probe sets were evaluated for 248 MM patient samples from the dataset published by Zhan *et al* [10], publicly available at *www.ncbi.nlm.mih.gov/geo* and accessible through GEO Series accession numbers GSE2658. The supervised analyses were performed by means of the Significant Analysis of Microarrays software (SAM; Excel front-end, publicly available at *www-stat.stanford.edu/~tibs/SAM/index.html* [17]. The cut-off for significance was determined by tuning the Delta parameter on the false discovery rate (median FDR=0% and 90th percentile FDR=0%) and controlling the *q*-value for the gene list. The selected probe list was visualized using dChip software [18].

Promoter reporter assays

PCR was used to obtain the human promoter regions of target genes. 2000 bp from the ATG translation start site of these promoter regions were cloned in the pGL4.10 [luc2] reporter vector (Promega) in which the human promoter drives expression of luciferase. HEK 293T cells were co-transfected with the pcDNA4/TO-HAtag-MAFB/C-Maf expression construct and the promoter reporter vector. CMV-Renilla was used as an internal transfection control. Luciferase activity was determined after 24h of induction using the Dual-luciferase reporter assay system (Promega). Levels were normalized to CMV-Renilla expression.

Cell proliferation assay (MTS assay)

Functional effects of inducible MAFB expression were tested by proliferation under serum low circumstances and by testing drug resistance after addition of simvastatin, an HMG-CoA-Reductase inhibitor. *In vitro*, statins are cytotoxic against myeloma cells by inducing apoptosis and inhibiting proliferation [19]. Cells were seeded at a density of $1 * 10^5$ cells per well into 96-well flat bottom cell culture plates. For testing proliferation in low serum conditions, MM cell lines expressing TetR and *MAFB* were cultured in 100 μl medium with 0,5% FCS [20] and induced with doxycycline for 24h. For testing drug resistance, the MM cell lines were cultured in 5% FCS with simvastatin at different concentrations for 48h. After both these

procedures, the AQueous One Solution Cell Proliferation Assay (MTS assay) was used to estimate cell viability, according to manufacturer's instructions (Promega, Madison, WI, USA). This assay assesses cell proliferation colorimetrically by measuring the metabolic activity of cells through conversion of MTS by NADPH/NADH into a coloured formazan product that is soluble in tissue culture medium. Briefly, 20 μl of the MTS reagent was added to the medium of the cells. The absorbance was measured at 490 nm in a 96-well plate reader (Molecular Devices, Thermomax microplate reader) after an incubation period of 3-4 hours.

Results

In the current study we identified by inducible expression a set of genes regulated by *MAFB* both *in vitro* and *in vivo.* Downstream effects are shown, as well as the correlation with the C-MAF pathway.

HMCLs not carrying the t(14;20) inducibly express MAFB upon transfection

We used an inducible model system to express *MAFB* in HMCLs not carrying the t(14;20). Two MM cell lines U266 and UM1 were used for inducible over expression of *MAFB*. Previously, we showed that these cell lines do not express endogenous *MAFB* [4]. Furthermore, endogenous *C-MAF* is not expressed in the U266 cell line (Figure 1A and [11]), but it was weakly detected in the UM1 cell line (Figure 1A). Stable TetR-MAFB clones were tested for MAFB expression by immunocytochemistry (Figure 1B) and Western Blotting (Figure 1C). After induction with doxycycline *MAFB* was expressed in stable clones of both cell lines (data UM1 not shown).

Secondary genes in cell lines after inducible expression

To identify target genes of *MAFB* in MM, we performed an Agilent whole human genome 44K micro array on the U266 and UM1 cell lines over expressing *MAFB* (U266/MAFB, UM1/MAFB) after induction with doxycycline. A time course of *MAFB* expression following induction was determined by Western blotting (Figure 2A, data UM1/MAFB not shown). *MAFB* expression could be detected throughout the chosen time points as early as 2h following doxycycline treatment. The 10h time point was chosen for the first micro array comparing gene expression in induced and non induced U266/MAFB as well as UM1/MAFB cells. The results using the U266/MAFB were the most significant; to obtain a more comprehensive list of *MAFB* target genes we performed a second micro array at 5h after induction using U266/MAFB. In total 284 genes were significantly up- or down regulated at both time points in both arrays (P<0.05) (Supplementary Table 1, *share.ovi.com/media/Mafb.public/Mafb.10001*). To confirm the micro array data Northern Blot analysis was performed on a couple of genes (2 examples in Figure 2B).
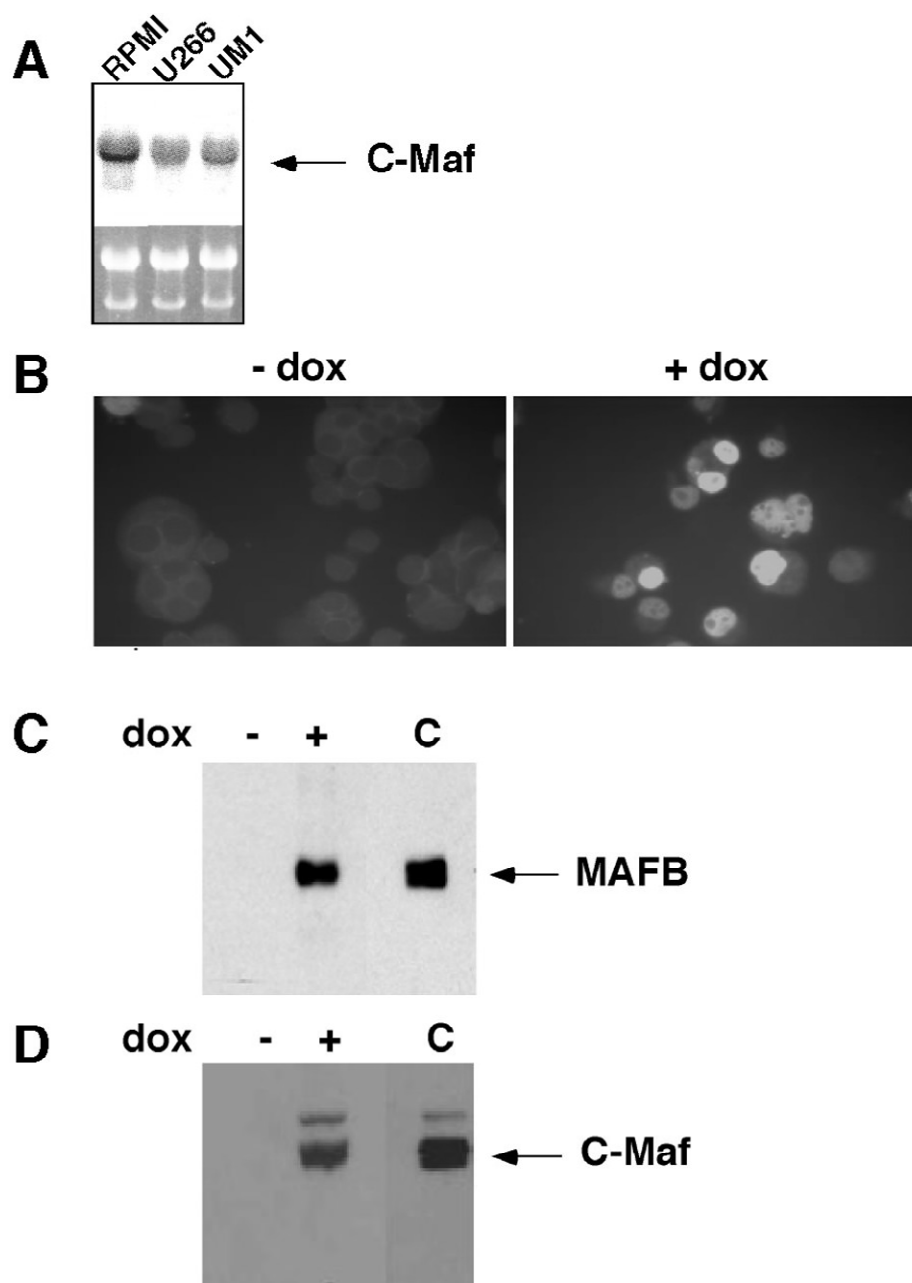
Identification of MAFB primary target genes

Figure 1: MAFB over expression in MM cell lines not carrying t(14;20)

A C-Maf mRNA expression in human MM cell lines. Northern Blot analysis showing no C-Maf expression in the U266 cell line and low C-Maf expression in the UM1 cell line. The RPMI cell line, which is known to have high C-Maf expression 11, was used as a positive control. Equal amounts of RNA were loaded, as shown by ethidium bromide staining

B MAFB is highly expressed in the nucleus of induced cells. Stable U266 cells expressing TetR and MAFB were generated (U266/MAFB). Expression of MAFB in stable clones was tested by immunofluorescence. U266 cells were induced with doxycycline for 48h and stained with an MαHA antibody (+dox). Staining was visualized using a FITCαM secondary antibody. Non- induced cells were stained as a control (-dox).

C,D Expression of MAFB/C-MAF protein analysed by Western Blot analysis. U266/MAFB and U266/C-MAF cells were induced with doxycycline for 48h (+) and total lysates were analysed. Non-induced cells (-) and a total lysate of 293T cells transiently transfected with MAFB/C-MAF were used as controls (c).
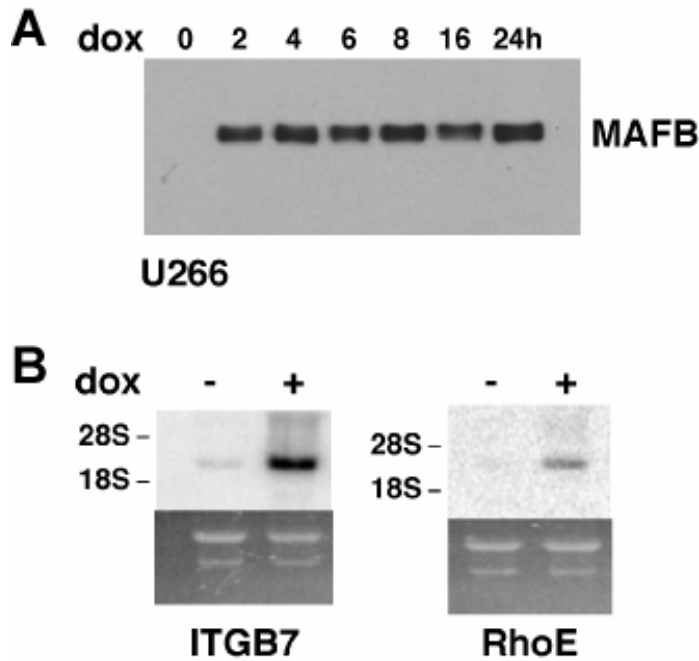
Figure 2: Micro array on MAFB negative cell lines ectopically expressing inducible MAFB.

A MAFB is induced as early as 2h after induction with doxycycline. Western blot analysis showing MAFB protein levels at indicated time points.

B Up regulation of MAFB target genes after induction with doxycycline. Northern Blot analysis showing ITGB7 and RhoE/RND3 expression after induction compared to non-induced cells. Equal amounts of RNA were loaded, as shown by ethidium bromide staining. To exclude doxycycline induced artefacts, U266 cells only expressing TetR were analysed after induction by Northern Blot compared to non-induced cells. No RNA expression of RhoE and ITGB7 was detected after induction in this cell line (data not shown). See supplementary Table 1 for the 284 genes found to be significantly up- or down regulated after performing gene arrays comparing these cells before and after induction.

Ex vivo validation of this induced up regulation

The comparison of our list with two publicly available data sets of patient samples led to the identification of 268 and 605 probe sets on the HG-U133A and HG-U133Plus array dataset, respectively. In both cases, we performed a supervised analysis between *MAFB* patients and the remaining samples of each dataset, and selected the probe sets at a *q*-value=0; this led respectively to the identification of 26 probe sets (21 genes) in the HG-U133A dataset (Figure 3A) and 39 probe sets (24 genes) in the HG-U133Plus dataset (Figure 3B). Of these, 14 genes resulted as common among the three selected lists (Figure 3C). Using the UM1/MAFB cells the up- or down regulation was not as high as that observed in the U266/MAFB cells. This may be due to the low endogenous *C-MAF* expression found in the UM1 cell line. Still, the common genes *ANG, BLVRA, CCR1, ITGB7* and *NOTCH2* emerged as well (data not shown).
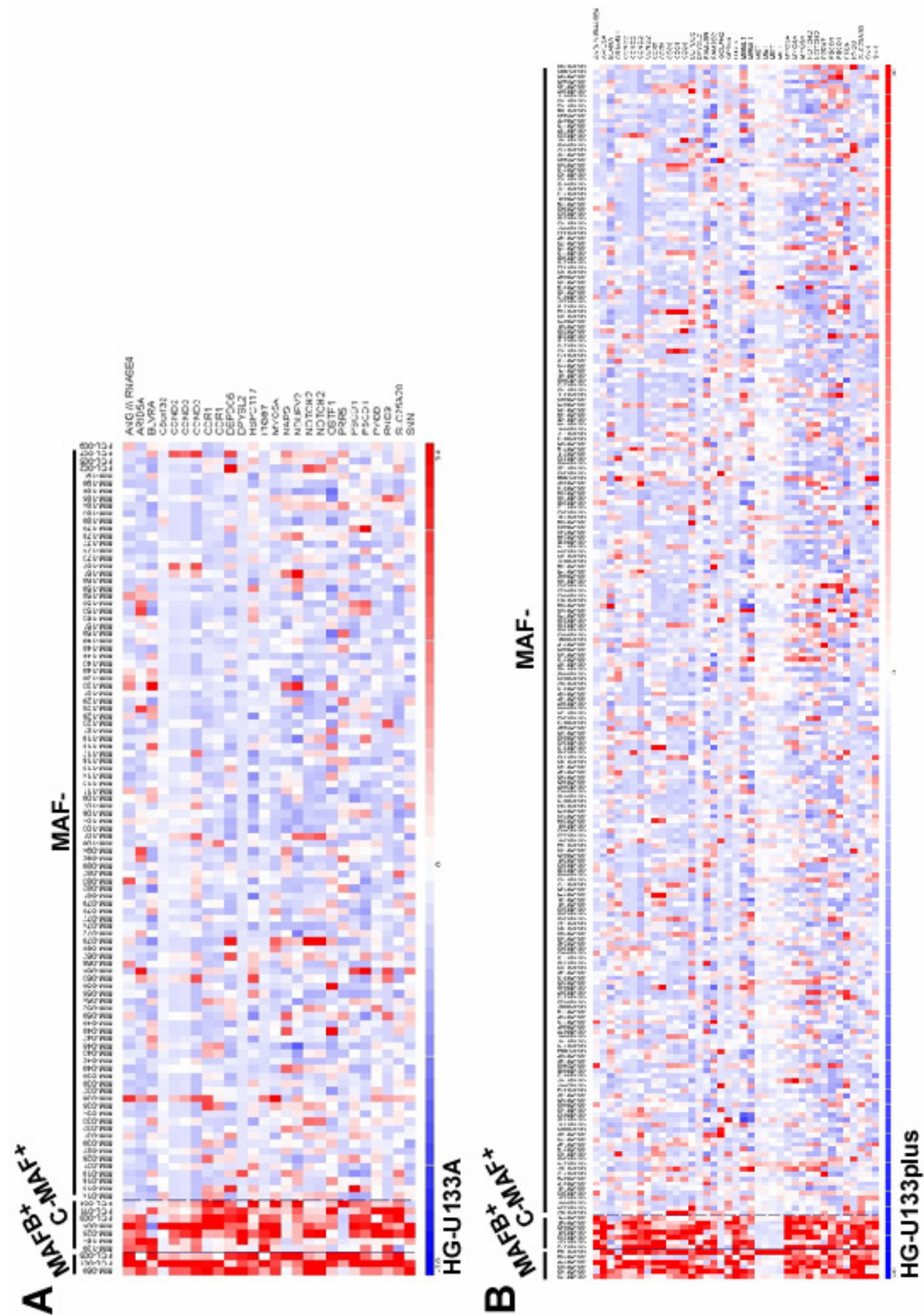
C

SAM_U133A			SAM_U133Plus		
Probe	**Name**	**Score**	**Probe**	**Name**	**Score**
205141_at	ANG /// RNASE4	8.625638	205141_at	ANG /// RNASE4	5.78
213138_at	ARID5A	5.60946	213138_at	ARID5A	20.1636
203773_x_at	BLVRA	4.132408	203773_x_at	BLVRA	4.00631
209829_at	C6orf32	5.102051	214772_at	C11orf41	7.81706
200951_s_at	CCND2	10.31853	200952_s_at	CCND2	10.2009
200952_s_at	CCND2	10.08682	200951_s_at	CCND2	9.33776
200953_s_at	CCND2	5.782666	200953_s_at	CCND2	6.73191
205098_at	CCR1	7.386164	1565735_at	CCND2	5.03862
205099_s_at	CCR1	6.96215	205098_at	CCR1	7.48556
218858_at	DEPDC6	4.294583	205099_s_at	CCR1	7.4549
200762_at	DPYSL2	14.68888	211861_x_at	CD28	7.61006
200042_at	HSPC117	7.52269	211856_x_at	CD28	7.41066
205718_at	ITGB7	8.315419	206545_at	CD28	7.34383
204527_at	MYO5A	6.279481	218858_at	DEPDC6	6.44045
210048_at	NAPG	4.590397	200762_at	DPYSL2	17.4539
202941_at	NDUFV2	4.962364	217916_s_at	FAM49B	4.66126
202443_x_at	NOTCH2	7.204415	65585_at	FAM86C	4.12275
212377_s_at	NOTCH2	6.177747	217771_at	GOLPH2	4.90403
204479_at	OSTF1	4.139671	206361_at	GPR44	7.03101
219168_s_at	PRR5	4.557545	205718_at	ITGB7	23.4349
202879_s_at	PSCD1	6.108931	218559_s_at	MAFB	48.2924
202880_s_at	PSCD1	4.727289	222670_s_at	MAFB	41.4007
201481_s_at	PYGB	7.903136	201153_s_at	MBNL1	4.55353
212724_at	RND3	7.32133	201151_s_at	MBNL1	4.17914
203658_at	SLC25A20	8.609013	213807_x_at	MET	6.52162
218033_s_at	SNN	4.436797	211599_x_at	MET	6.5065
			213816_s_at	MET	6.34354
			203510_at	MET	5.9729
			204527_at	MYO5A	11.686
			227761_at	MYO5A	8.44181
			241966_at	MYO5A	6.51002
			212377_s_at	NOTCH2	5.49862
			210756_s_at	NOTCH2	3.99505
			207091_at	P2RX7	6.19171
			202879_s_at	PSCD1	5.00165
			202880_s_at	PSCD1	4.13184
			211711_s_at	PTEN	4.45629
			212724_at	RND3	11.1992
			203658_at	SLC25A20	13.0808
			218033_s_at	SNN	6.37736
			218032_at	SNN	4.57683

Figure 3: Supervised analyses of genes up regulated by MAFB: in vitro and in vivo data.

The core target gene program of MAFB overexpression in MM. The left (**A**: SAM_U133A; **B**: SAM_U133plus) panels shows the expression profiles of the core gene set in MAFB or C-MAF positive patients. The main part shows the expression profiles of the core gene set in MAF negative patients. The gene symbols are indicated as well as the sample numbers of the patients. For GenBank Accession numbers, see Supplementary Table 1.

C Identification of probe sets in the in vitro and both in vivo data sets. In yellow are the genes, which are common to the three lists.

Tertiary genes in cell lines after induced up regulation

By using the inducible *MAFB* cell lines as well as an inducible *C-MAF* cell line, which was generated in the same way as the *MAFB* cell lines (Figure 1D), we found higher expression of *NOTCH2* after induction with doxycycline (Figure 4A, data UM1 not shown). Indeed the resulting up regulation of *NOTCH2* has functional consequences as we have found high expression of the active NOTCH pathway target gene *HES1* in both U266 and UM1 induced cell lines (Figure 4B, data UM1 not shown). Also high expression of *HES5* and *HES7* was found (data not shown).
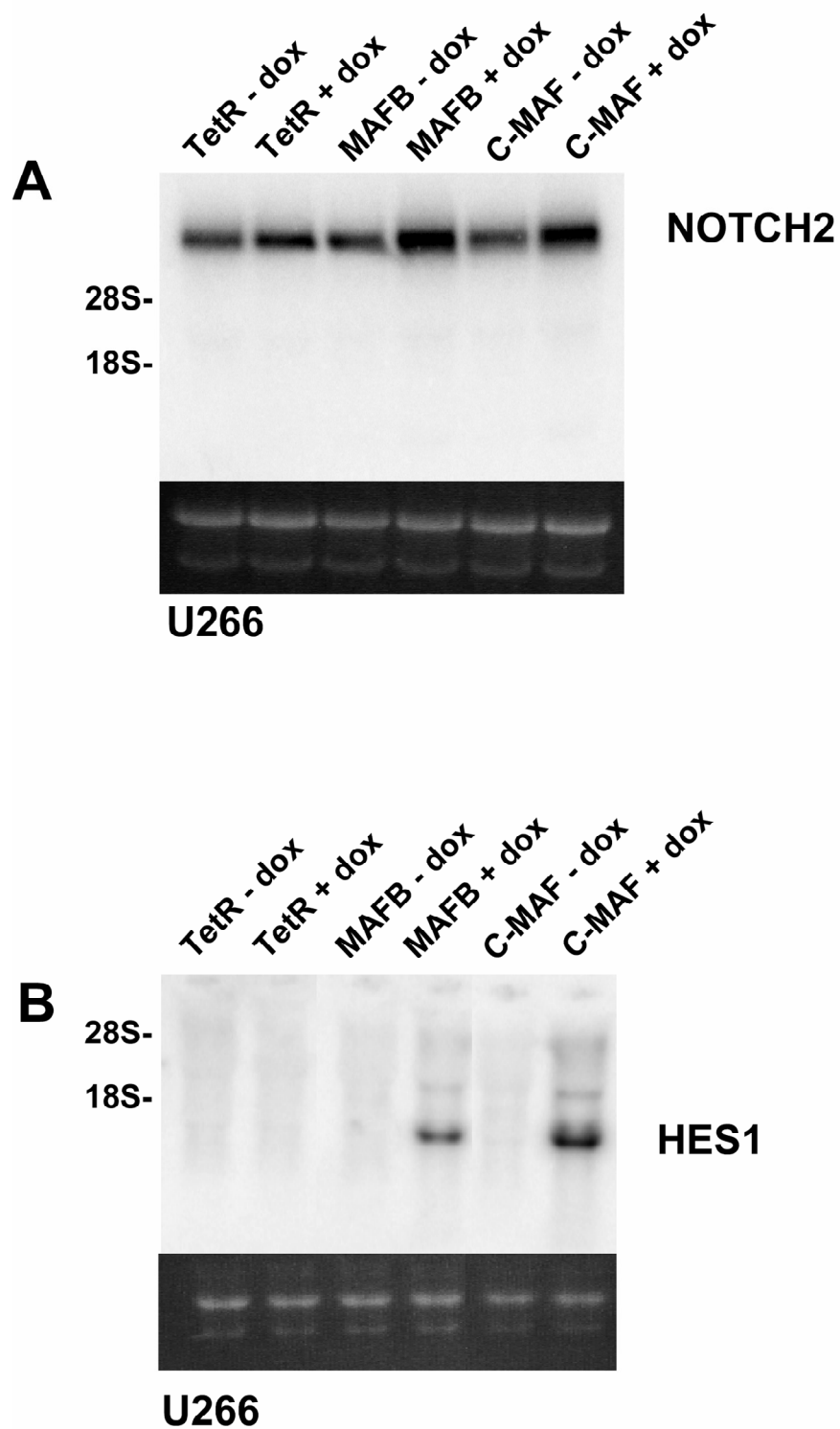
Figure 4: Functional Up regulation of NOTCH2 (A) and active NOTCH pathway target gene HES1 (B) by over expression of MAFB or C-MAF.

Northern Blot analysis showing NOTCH2 (**A**) and HES1 (**B**) up regulation after induction of MAFB or C-MAF compared to non-induced cells. Equal amounts of RNA were loaded, as shown by ethidium bromide staining. To exclude doxycycline induced artefacts, U266 cells only expressing TetR were analysed after induction by Northern Blot compared to non-induced cells.

Functional indication of this up regulation

To confirm that also other up regulated genes are direct targets of *MAFB* transactivation, we performed luciferase reporter assays by cloning the promoter regions of the target genes in a luciferase reporter vector. From 8 of the 11 new found target genes, the promoter regions express the MAFB/C-MAF conserved binding motif, so called Maf recognition element (MARE). This MARE is a long DNA sequence, TGC (N)6,7 GCA, where the flanking sites are important for MAF binding [21]. Five promoter regions were cloned in a luciferase reporter vector. By co-transfection of the promoter-luciferase construct and MAFB expression construct, we showed regulation of the promoter regions by *MAFB* (3 examples given in figure 5A). The same results were obtained by transfecting a *C-MAF* expression construct (Figure 5B). These data strongly suggest that the target genes found are direct targets of *MAFB*/*C-MAF* transactivation.

MAFB up regulation enhances proliferation under low serum conditions and protects MM cells from apoptosis induced by drugs.

To investigate the survival effects of MAFB up regulation in MM we tested cell viability of U266/MAFB and UM1/MAFB cells over expressing MAFB in low serum conditions. As shown in figure 6A, over expression of *MAFB* results in enhanced proliferation (20% increase in 24 hours) of MM cells (data UM1 not shown). We next examined the effect of up regulation of MAFB on drug sensitivity. We tested cell viability of cells from both cell lines over expressing MAFB and treated with simvastatin in different concentrations. As shown in figure 6B, simvastatin effectively killed ~50% of the control cells whereby MAFB over expressing cells were protected against apoptosis induced by simvastatin (data UM1 not shown). Taken together, these experiments demonstrate that up regulation of MAFB induces proliferation and protect cells from drug induced apoptosis.

A

Figure 5A: Regulation of promoter regions MAF target genes by MAFB.

Luciferase activity after binding of MAFB with the conserved MAF binding motifs in the promoter regions of the MAF target genes NOTCH2, SLC25A20 and ARID5A. HEK 293T cells were transiently co-transfected with the promoter/pGL4.10[luc2] reporter vector and a MAFB expression contruct. By transfecting increasing amounts of MAFB the higher luciferase activity was measured. Transfection of the empty pGL4.10[luc2] vector (EV) and only the promoter/pGL4.10[luc2)] reporter vector (PR) served as negative controls. Luciferase activity is indicated as an average of a duplicate assay from two independent experiments. Standard deviation is presented with error bars.
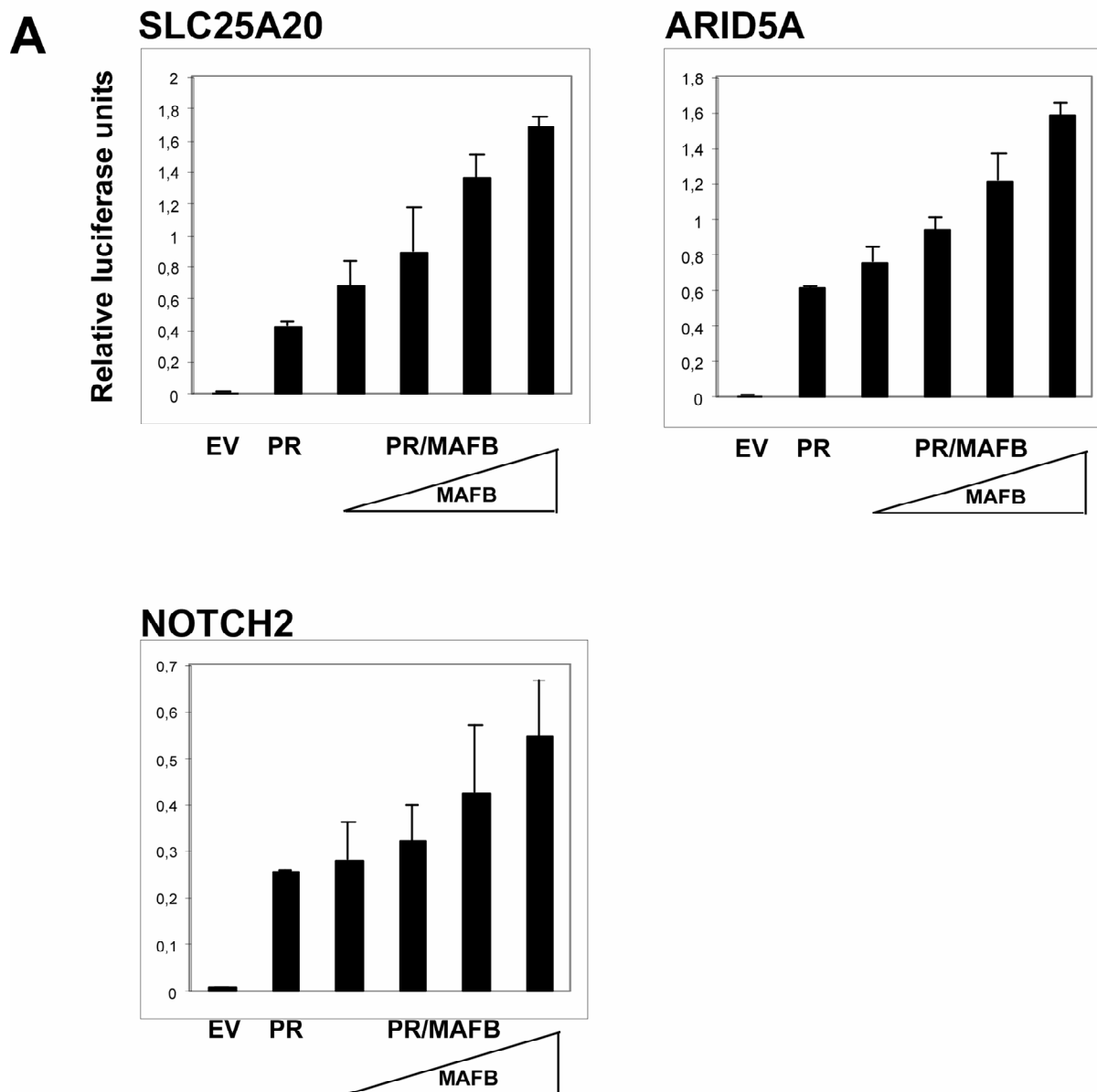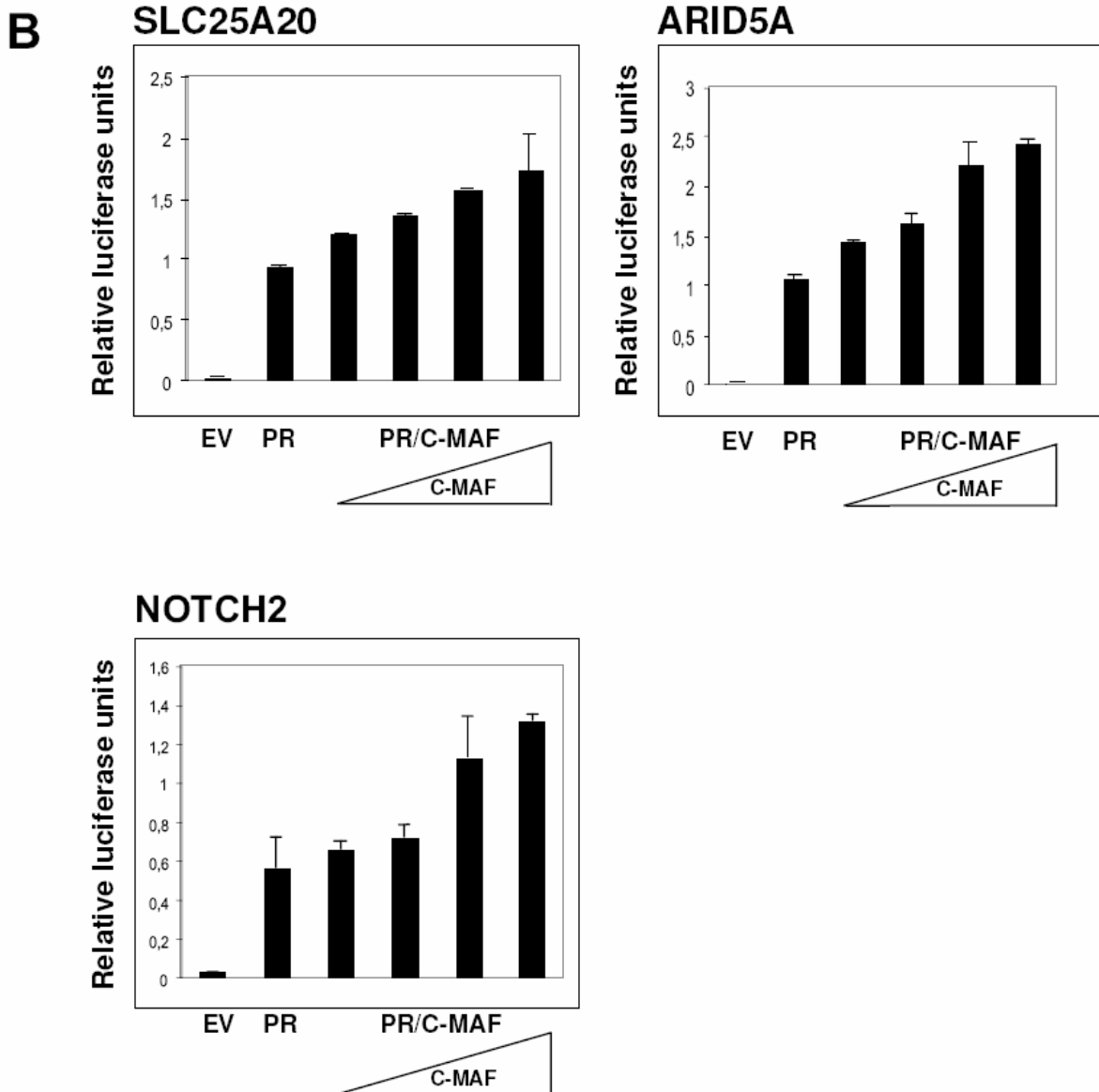
Figure 5B: Regulation of promoter regions MAF target genes by C-MAF.

Luciferase activity after binding of C-MAF with the conserved MAF binding motifs in the promoter regions of the MAF target genes NOTCH2, SLC25A20 and ARID5A. HEK 293T cells were transiently co-transfected with the promoter/pGL4.10[luc2] reporter vector and a C-MAF expression contruct. By transfecting increasing amounts of C-MAF the higher luciferase activity was measured. Transfection of the empty pGL4.10[luc2] vector (EV) and only the promoter/pGL4.10[luc2)] reporter vector (PR) served as negative controls. Luciferase activity is indicated as an average of a duplicate assay from two independent experiments. Standard deviation is presented with error bars.
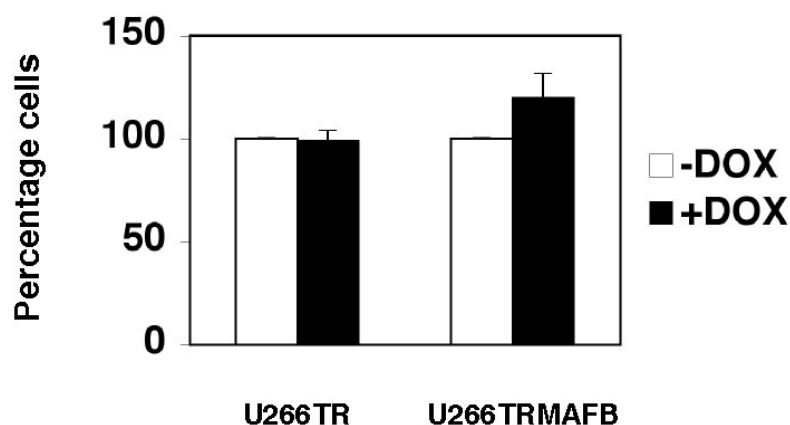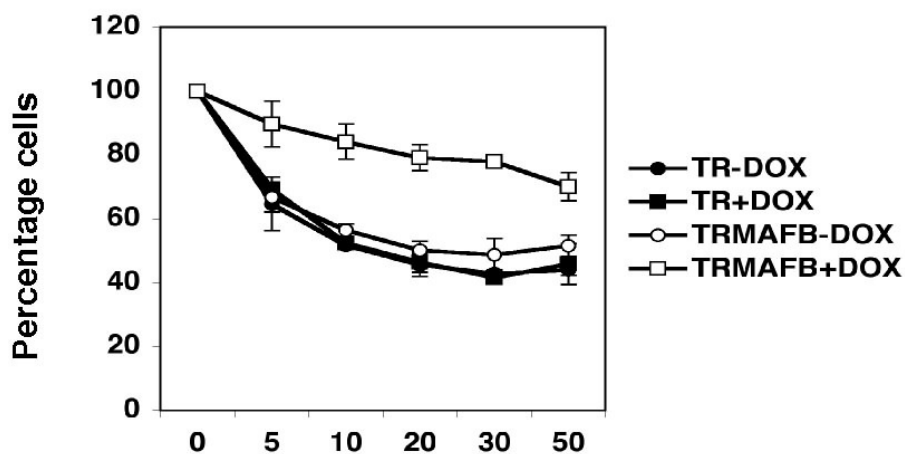
A

B

Figure 6: MAFB over expression induces proliferation and prevents MM cells from apoptosis.

A Induced proliferation after induction of MAFB. Doxycycline induced U266/MAFB cells show increased cell proliferation. Cell proliferation was measured as described in "Materials and Methods". Data is presented as a bar-graph and percentages of living cells are given. Induced cells were compared to non-induced cells which were set at 100%. Shown is 1 representative experiment of 2. Standard deviation is presented with error bars.

B Increased drug resistance after induction of MAFB. Simvastatin induced apoptosis in doxycycline induced U266/MAFB is lower than in control cells. Data is presented as a line-graph and percentages of living cells are given. Induced cells were compared to non-induced cells. Shown is 1 representative experiment of 2. Standard deviation is presented with error bars.

In both experiments (A and B), tests on U266 cells only expressing TetR excluded doxycycline induced artifacts after induction.

Discussion

To understand molecular events in MM, gene expression profile studies were performed. Distinct molecular patterns were dissected in different plasma cell dyscrasias with distinct IgH translocations [9] and a differential role of cyclin D species was seen leading to the discrimination of various translocation and cyclin groups [8,22]. A recent study demonstrated another molecular classification by analyzing 414 newly diagnosed MM patients [10]. In both approaches *MAFB* and *C-MAF* positive patients clustered together in the MAF group, characterized as well by high *CCND2* expression [6]. For identifying MAF target genes in vitro, Hurt et al tested induced over expression of *C-MAF* in HMCLs [11].

In current study we identified genes up regulated by *MAFB* in inducible cell lines. This experimental strategy has the advantage that it allows for highly controlled and reproducible expression profiling. Also, our inducible system provides the identification of genes that are directly up regulated by *MAFB* expression. Yet, the use of cell lines has to be validated, comparing with the *in vivo* transformation of myeloma cells. Therefore, the expression profiles of the selected transcriptional fingerprint were evaluated in two independent datasets including 111 and 248 primary tumors. This led us to identify 14 common up regulated genes.

Three of the *MAFB* common downstream genes (*CCND2, CCR1* and *ITGB7)* appeared to be common to the *C-MAF* pathway as well, as their up regulation has also been found after induced *C-MAF* over expression in HMCLs *in vitro* [11]. In addition to that study, we found in the *MAFB* pathway 11 more common up regulated genes, which were apparently up regulated in *C-MAF* positive patients as well (Figure 3). Some of these extra genes (i.e. *DEPDC6, DPYSL2, MYO5A, NOTCH2*) [8] or *ARID5A* [10] have been previously reported as up regulated in the whole MAF group *in vivo*. Notably, we found over expression of the three Hes genes (*HES1, HES5* and *HES7*) both after *MAFB* and after *C-MAF* induction. As particularly these Hes genes are directly regulated by *NOTCH* [23,24], our results suggest even stronger that over expression of *MAFB* as well as *C-MAF* activates the Notch pathway. Further, this study adds *RND3-RhoE, ANG, BLVRA, PSCD1, SLC25A20* and *SNN* to that list. As a result we may conclude that the new genes found after up regulation of *MAFB* are target genes of the whole MAF group. In addition as well, we found constitutive up

regulation of *RND3-RhoE*, *CCND1* and *NOTCH2* in the HMCLs UM3 and UM6, which due to the t(14;20) express endogenous *MAFB*[4] (data not shown).

In addition to the three secondary genes reported by Hurt et al in *C-MAF* pathway[11], this study highlights the role of 11 novel genes in the Maf pathway *in vitro* and *in vivo* altogether. One or several of the 11 novel primary MAFB target genes reported here may play a role in the survival of MM cells as we have shown that *MAFB* over expression induces proliferation and prevents cells from drug-induced apoptosis. Evidently, these *in vitro* indications are relevant for the infaust t(14;20) MM. From these genes, reportedly *NOTCH2* is involved in proliferation[25], or in growth arrest of MM[26]. Also the over expression of the NOTCH ligand JAG2 in MM is of note[27]. Further, recently treatment with γ-secretase inhibitor (GSI) alone was found to induce apoptosis of myeloma cells and enhances sensitivity to chemotherapy *in vitro* and *in vivo* via specific inhibition of Notch signalling. This might indicate again an important role for *NOTCH* in MM[28]. Recently as well, surpression of *CCND2* by kinetin riboside was reported to induce cell-cycle arrest, tumor cell-selective apoptosis (*in vitro* and *in vivo*) and inhibition of myeloma growth in xenografted mice[29]. The novel common gene *RND3-RhoE* may be of interest, due to its involvement in the organization of the cytoskeleton in response to growth factors[30]. The pathophysiological consequences of other novel common genes are not totally clear as yet due to lack of relevant information on these specific genes so far. They may merit attention however, especially in view of the adverse prognosis, associated with this *MAFB* up regulation[4].

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Chapter 4

MAFB oncoprotein detected by immunohistochemistry as a highly sensitive and specific marker for the prognostic unfavorable t(14;20)(q32;q12) in multiple myeloma patients

MAFB oncoprotein detected by immunohistochemistry as a highly sensitive and specific marker for the prognostic unfavorable t(14;20)(q32;q12) in multiple myeloma patients.

Esther van Stralen[1,2], Roos J. Leguit[3], Harry Begthel[2], Lucienne Michaux[4], Arjan Buijs[5], Heidi Lemmens[4], Jean-Marie Scheiff[6], Chantal Doyen[7], Pascal Pierre[8], Frédéric Forget[9], Hans C. Clevers[2] and Bert J.E.G. Bast[1]

[1] University Medical Center Utrecht, Department of Immunology, Utrecht, The Netherlands.

[2] Hubrecht Institute, Center for Developmental Biology and Stem Cell Research, Utrecht, The Netherlands.

[3] University Medical Center Utrecht, Department of Pathology, Utrecht, The Netherlands.

[4] University of Leuven, Center for Human Genetics, Leuven, Belgium

[5] University Medical Center Utrecht, Department of Medical Genetics, Utrecht, The Netherlands.

[6] UCL Saint-Luc, Department of Clinical Biology, Brussels, Belgium

[7] UCL Mont-Godinne, Department of Hematology, Brussels, Belgium

[8] Clinic of Saint-Joseph, Department of Hematology, Arlon, Belgium

[9] C.H. De L'Ardenne, Department of Hematology, Libramont, Belgium

Letter to the editor

Chang et al reported [1] the frequent expression of C-MAF nuclear oncoprotein in Multiple Myeloma (MM), not only in patients positive for the t(14;16)(q32;q23), which results in an up regulation of *C-MAF*, but also in a considerable proportion (25%) of t(14;16) negative patients. Here we have studied MAFB oncoprotein expression in MM patient samples, starting with samples tested for the t(14;20)(q32;q12) by Fluorescence In Situ Hybridization (FISH) analysis [2]. As a result, we only found protein expression of MAFB confined to t(14;20) positive patients. This is remarkable, as primary translocations involving *MAFB* and *C-MAF* are grouped together according their expression and correlation of high *CYCLIN D2* activity and poor prognosis in MM (reviewed in [3]).

Due to the t(14;20) translocation, *MAFB* is translocated to the immunoglobulin heavy chain locus on chromosome 14, juxtaposed to the strong IgH Eα enhancer, resulting in oncogenic over expression of *MAFB*. Primary translocations are major events in MM with a prevalence of about 40%.

Maf genes form a distinct group of bZIP transcription factors and belong to the AP-1 super family. Members from the large Maf group can form homo- and hetero dimers with other members of bZIP transcription factors and have transactivating and transforming abilities (reviewed in [4]). They are involved in about 7% of MM, whereas the prevalence of MAFB is about 2% (reviewed in [3])

In this study we developed a specific anti- MAFB monoclonal antibody to detect MAFB protein expression in primary MM tumor samples, as tested by immunohistochemistry (IHC) in two independent MM patient datasets. The first dataset represented a total of 73 patients diagnosed with MM treated in the UMC Utrecht. The median age was 57 years and represents 46 male and 27 female patients. 40 patients had an IgG M protein, 16 IgA, 1 IgD, 1 IgM and 15 light chain disease. 20 patients of this dataset were previously screened for t(14;20) by FISH analysis and 2 patients carried this t(14;20) [2]. In these particular patients, the tumors were very progressive and one patient died after 13 months [5]. From 53 patients the cytogenetic background with

respect to Maf was not known at the time of staining. The second dataset represented 2 MM (2 female) patients tested positive for t(14;20) by FISH analysis and 4 (3 male, 1 female) MM patients not harboring a t(14;20). The median age was 60 years and 4 patients had an IgG M protein, 1 IgA and 1 light chain disease. These patients were diagnosed and treated in UCL Mont-Godinne, Brussels, Belgium.

The anti-MAFB monoclonal antibody was raised as described previously [6]. A 971 bp PCR product encoding all amino acids of the full human *MAFB* coding region was used to produce recombinant protein in *Escherichia coli* and used for mouse immunizations. Four hybridomas were isolated that recognized full-length human *MAFB* when over expressed in COS cells, and the best one (clone 1F4, IgG1κ) performed well in Western Blot analysis and Immunofluorescence (Figure 1).
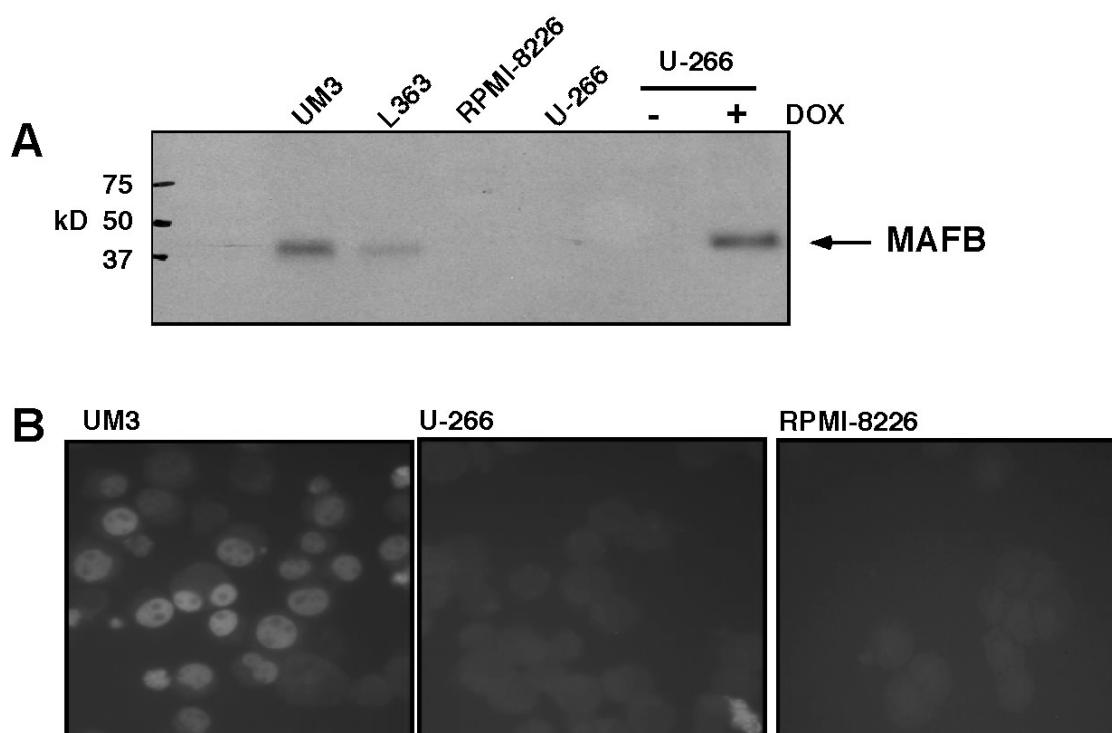

Figure 1: Specificity of monoclonal MAFB antibody.
A Expression of MAFB protein analyzed by Western Blot analysis using the specific anti-MAFB monoclonal antibody. Western blot analysis was performed on total cell lysates obtained from the UM3 and L363 MM cell lines expressing endogenous MAFB 5 compared to a cell line not expressing MAFB (U-266), a cell line expressing endogenous C-MAF (RPMI-8226) and an inducible U266-MAFB cell line which express MAFB after induction with doxycycline (submitted for publication). After protein separation and gel blotting, blots were incubated with monoclonal MAFB antibody and RAMPO (Pierce, IL, USA) as the secondary antibody. Immune complexes were visualized using an enhanced chemiluminescence kit (ECL plus, Amersham Life Science, UK). Expression of MAFB was detected in only in the UM3, L363 and inducible MAFB cell line after induction with doxycycline.

B MAFB detection in the nucleus of cells of the UM3 cell line carrying t(14;20), as tested by immunofluorescence. Cytospin centrifuged cells were made from the UM3, U-266 and RPMI-8226 cell lines and incubated with anti- MAFB monoclonal antibody. Binding was visualized using a FITCαM secondary antibody. As a result, MAFB was detected only in the UM3 cell line confirming the specificity of the antibody.

For detection of MAFB protein by IHC, this antibody was tested and proved positive on both formalin and Bouin fixed cells obtained from the *MAFB* positive UM3, L363 and UM6 MM cell lines [5] (data not shown); this was tested as bone marrow biopsies from both datasets were fixed differently, e.g. formalin fixation in the UMC Utrecht and Bouin fixation in UCL Mont-Godinne, Brussels. Paraffin sections were de-paraffinizated and heat induced for antigen retrieval. Non-immune mouse serum was tested as negative control. Paraffin sections of the U-266 and RPMI-8226 MM cell lines stained negative (data not shown), confirming the Western blotting.

From diagnostic bone marrow biopsy blocks, 5 µM thick paraffin sections were cut and stained for plasma cell detection and MAFB expression. *MAFB* is responsible for monocyte/macrophage differentiation in the haematopoetic system [7], and expression of MAFB in monocytes and macrophages (identified according to CD68 expression, data not shown) served as a positive control within BM tissue samples. Myeloma cells of all samples tested from both datasets were detected on the basis of positivity for cytoplasmatic light chain staining. Two independent observers estimated the percent MAFB protein stained nuclei. Cases with more than 20% MAFB nuclear expression were considered positive.

In all 20 MM patient samples from the first dataset which had been tested for t(14;20), MAFB nuclear expression was only observed in the plasma cells (>70%) from the two t(14;20) positive patients (Figure 2). In patients not carrying the t(14;20), no nuclear MAFB expression was detected in the plasma cells, both indicating the specificity of the anti-MAFB monoclonal antibody, as well as the restricted presence of MAFB in t(14;20) positive patients. Next, we studied MAFB expression in 53 randomly chosen MM patients from this first dataset not knowing the cytogenetic background. As a result, one of the 53 patient samples was positive for MAFB. In review, cytogenetic studies on cultured cells derived from peripheral blood of this patient diagnosed for plasma cell leukemia, had resulted in a

44,XX,del(6)(q21),del(12)(p12),-13,add(14)(q32),-16,-20,+mar[18]/46,XX[10] karyotype.
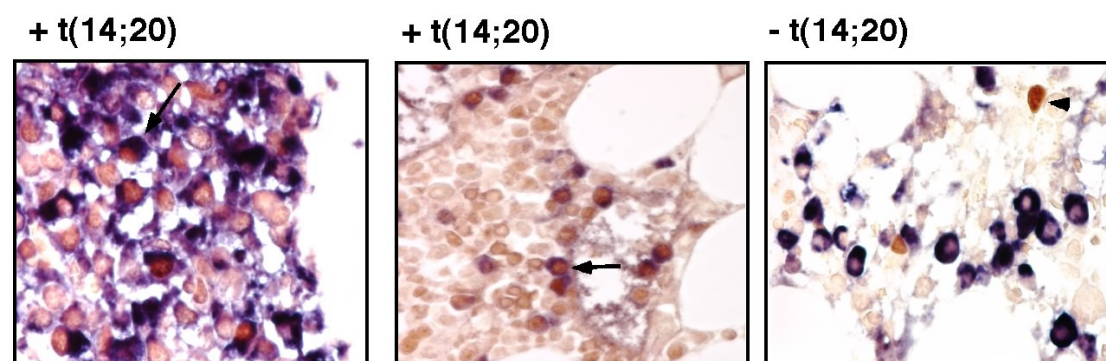
+ t(14;20)   **+ t(14;20)**   **- t(14;20)**

Figure 2: MAFB over expression by IHC in MM patient material with t(14;20)

In bone marrow biopsies from two t(14;20) positive patients (+ t(14;20)) the plasma cells (identified on the basis of cytoplasmic light chain positivity) showed positive nuclear staining with the anti-MAFB monoclonal antibody, whereas in a t(14;20) negative patient (- t(14;20)) only the macrophages (serving as positive control for the staining) were MAFB positive. An arrow indicates a plasma cell expressing MAFB; an arrowhead indicates a macrophage expressing MAFB. Scale bar is 10 μm.

For detection of plasma cells in cytoplasmatic staining, goat anti light chain antibodies conjugated with alkaline phophatase (AP) (Southern Biotech, USA) were used. Cytoplasmatic light chain expression was visualized using a mixture of Nitro-Blue Tetrazolium Chloride (NBT) and 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (BCIP) (Sigma-Aldrich Inc., USA). MAFB expression was visualized using peroxidase-conjugated mouse EnVision+ (DakoCytomation, Ca, USA) as a secondary antibody and revealed by incubation in 3,3'-Diaminobenzidine (DAB) solution.
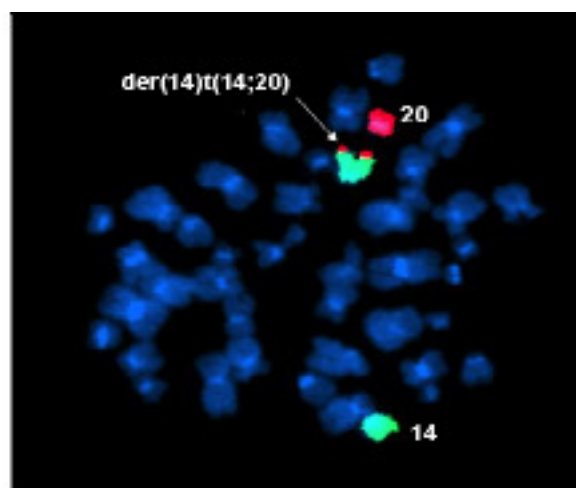

Figure 3: Identification of t(14;20) in IHC MAFB positive case

FISH analysis on cultured peripheral blood cells of IHC MAFB positive patient identified a derivative chromosome 14 as a result of a t(14;20) using whole chromosome paints 14 (green) and 20 (red) (Abbott Molecular Inc, Des Plaines, Il,, USA).

FISH analysis using whole chromosome 14 and 20 specific paints revealed a derivative chromosome 14 as a result of a t(14;20) with loss of derivative chromosome 20 (Figure 3). FISH analysis using a 14q32 *IGH* specific probe showed a 14q32 *IGH* rearrangement with loss of telomeric 5' *IGH* variable gene segments in accordance with the absence of derivative chromosome 20 (probe: LSI IGH, Abbott Molecular Inc., Des Plaines, Il, USA)(data not shown).

After analyzing samples from the second dataset the two *MAFB* positive MM patients according to the t(14;20), MAFB oncoprotein expression was also found in >70% of the plasma cells, whereas the t(14;20) negative MM patients in the same dataset scored negative for MAFB protein. In conclusion, MAFB oncoprotein expression in MM is strongly correlated with t(14;20).

Interestingly, in one MAFB positive patient we could detect 1% MAFB positive plasma cells after VAD therapy (data not shown), which might suggest that this staining may be used as a marker for minimal residual disease (MRD).

Taken together this is the first report to detect MAFB oncoprotein expression in MM patients. *MAFB* up regulation due to the t(14;20) coincides with an adverse outcome; here we show that the protein was detected exclusively in patients carrying this t(14;20). This resembles the strong correlation between t(4;14), a primary translocation resulting in over expression of *FGFR3* in MM, detected by interphase FISH and FGFR3 protein expression by IHC in MM [8]. Our results seem to be at variance with data on the C-MAF protein, which expression was detected in 30% of MM cases regardless harboring a t(14;16) [1]. Our results here and the restricted FGFR3 expression in MM [8] arouse the question why C-MAF expression has been found in such a higher proportion of MM patients, and what would be its significance – especially as in that study no prognostic impact could be attributed to the C-MAF oncoprotein expression. One factor in this aspect may be that (contrary to the polyclonal anti-C-MAF antibody used by [1], but in concordance with the monoclonal anti FGFR3 antibody, used by [8]) we have used a monoclonal anti-MAFB antibody here; indeed, comparatively we have

been using a polyclonal anti-MAFB antibody, but these results were not fully evaluable for us (data not shown). Furthermore, recently the high proportion of *C-MAF* mRNA over expression found by Q-PCR (50% of MM bone marrow samples [9]) has been questioned. Rasmussen *et al.* found also by Q-PCR *C-MAF* mRNA being over expressed in only 4.4% in MM patients [10], coinciding with the reported prevalence of t(14;16) in MM, i.e. ~5% (reviewed in [3]). Combined, these data suggests that the prevalence of C-MAF oncoprotein expression in MM remains a matter of debate.

Because we show that aberrant MAFB expression detection with paraffin ICH is an easy and reliable method, we suggest that our anti-MAFB monoclonal antibody may be used in routine diagnostic matters. Indeed, as MAFB protein detection was only found in patients carrying t(14;20) (which is associated with a bad prognosis), our results indicate that MAFB may be a prognostic marker in the risk stratification of MM patients.

Importantly, the exclusive presence of MAFB protein associated with this adverse prognosis may enable novel therapies targeting the *MAFB* gene expression pathway. Indeed, for expression of FGFR3 it has been demonstrated that inhibition of FGFR3 by an inhibitory anti-FGFR3 antibody inhibits proliferation and induces apoptosis in FGFR3-expressing human myeloma cells [11].

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Chapter 5

Transgenic mice that express MAFB under the control of the CD19 promoter region: a mouse model for Multiple Myeloma.

Transgenic mice that express *MAFB* under the control of the CD19 promoter region: a mouse model for Multiple Myeloma.

Esther van Stralen[1,2], Gemma M. Dingjan[3], Marjolein J.W de Bruijn[4], Sjef Verbeek[5], Frans M. A. Hofhuis[1], Harry Begthel[2], Marc van de Wetering[2], Hans C. Clevers[2], Rudolf W. Hendriks[4], Bert J.E.G. Bast[1],

[1] University Medical Center Utrecht, Department of Immunology, Utrecht, The Netherlands.
[2] Hubrecht Insitute, Center for Developmental Biology and Stem Cell Research, Utrecht, The Netherlands.
[3] Erasmus MC, Department of Immunology, Rotterdam, The Netherlands.
[4] Erasmus MC, Department of Pulmonary Medicine, Rotterdam, The Netherlands
[5] Leiden University Medical Center, Department of Human Genetics, Leiden, The Netherlands.

Summary

Over expression of oncogenes as a result of IgH translocations is a major event in multiple myeloma (MM). Ectopic expression of *MAFB* is a result of the primary translocation t(14;20)(q32;q12). Earlier, we have identified the primary target genes of *MAFB* by its over expression *in vitro* studies, but the role of *MAFB* for myelomagenesis *in vivo* is still unknown. To study the role of *MAFB in vivo* we created a transgenic mouse model expressing *MAFB* under the control of the human CD19 promoter and the strong Eμ Ig enhancer. Transgenic expression was found in two founder lines. Two groups of transgenic mice were formed to follow immune response and tumor growth. To stimulate B-cell development to post-germinal plasma cells, mice were immunized with T-cell dependent and T-cell independent antigens or LPS. B-cell development and serum M-protein of immunized mice were monitored for a year at different time points. After a year, changes in the development of B-cells, T-cells and Myeloid cells were analysed in a large group of transgenic mice compared to non transgenic mice. Despite of transgenic expression of *MAFB* in lymphoid organs, no MM phenotype was found as yet.

Introduction

The Maf proto-oncogene was originally identified within the genome of the avian musculoaponeurotic fibrosarcoma virus, AS42 [1]. Maf proteins form a distinct group of bZIP transcription factors [2] and can form homo- and heterodimers with other members of the bZIP transcription factors. Members of the large Maf proteins are MAFB, C-MAF MAFA/L-Maf and NRL; they contain a transcriptional activation domain in contrast to the small Maf proteins, which lack the transcriptional activation domain. Large Maf proteins have trans-activating functions [3] and play a key role in haematopoiesis [4,5]. Recently, it has been found that *C-MAF*, *MAFB* and *MAFA* are ectopically expressed in plasma cells (PC) from Multiple Myeloma (MM) [6-8], and putatively function as an oncogene (Chapter 3).

MM is a tumor of post-germinal center long-lived plasma cells (PC). It is an incurable disease and accounts for approximately 10% of the haematological disorders. A common event in MM is translocations involving the IgH locus on chromosome 14, more specifically at the switch regions [9]. These IgH translocations are simple reciprocal translocations and result from the Ig remodelling event class switch recombination (CSR) (reviewed in [10]). As a result of these primary translocations, the partner chromosomes are juxtaposed to one of the strong Ig enhancers. Thus, genes located on the partner chromosomes may now act as oncogenes. There are seven well-defined primary translocations in MM and three of these translocations involve a Maf gene, i.e. t(14;16)(q32;q23): *C-MAF* (5%); t(14;20)(q32q12): *MAFB* (2%) and t(8;14)(q24.3;q32): *MAFA* (<1%). C-MAF and MAFB are expressed at high levels in MM cell lines carrying the translocations t(14;16) and t(14;20) respectively [6,11]. Together, translocations involving Maf genes are detected in about 7% of all newly diagnosed MM patients and studies of the prognostic value of the t(14;20) and t(14;16) suggest that its occurrence is correlated with a high *CYCLIN D2* activity and a poor prognosis [12,13].

So far, only *in vitro* studies have given insight in the molecular pathogenesis of *MAFB* and *C-MAF* up regulation in MM (chapter 3 and [8]). There is no evidence to date for the involvement of Maf genes in the development of MM *in vivo*. However, one study shows that over expression

of *C-MAF* under the CD2 promoter contributes to the development of T-cell lymphoma in mice [14]. Next to the Maf genes quoted above, the primary translocations in MM involve t(4;14)(p16;q32): *MMSET/FGFR3* (15%); t(6;14)(p21;q32): *CYCLIN D3* (2%); t(11;14)(q13;q32): *CYCLIN D1* (15%) and t(12;14)(p13;q32): *CYCLIN D2* (<1%) [10]. Several mouse models were generated to study MM *in vivo*; these involve the genes MYC [15,16] and XPB-1 [17] but none of these models involve genes that are up regulated as a result of primary translocations [15-17].

In this study, we have generated *MAFB* transgenic mice that over express *MAFB* in the B-cell compartment using the human CD19 promoter, which was shown to provide expression throughout the B-cell lineage [18-20]. In addition, a duplicated mouse Ig Eμ enhancer was cloned upstream of the CD19 promoter. The Eμ enhancer has been used in several mice studies showing that this specific enhancer achieves high levels of transcription and is expressed in the whole B-cell lineage [21-23]. To stimulate the development of B-cells into antibody expressing plasma cells (and thereby enabling putatively secondary oncogenic events), transgenic *Eμ-CD19-MAFB* mice were immunized with specific T-cell dependent (TD) and T-cell independent (TI) antigens or with the mitogen LPS. The immune response and the possible tumor growth of the transgenic *Eμ-CD19-MAFB* mice were monitored for one year by analyzing B-cell development, serum Ig levels and clonality at different time points.

Here, we describe the results of the *Eμ-CD19-MAFB* transgenic mice analyses compared to non transgenic mice. No MM phenotype was found after a year but increased levels of (antigen specific) IgM were observed in *Eμ-CD19-MAFB* transgenic mice.

Material and methods

Generation of Eμ-CD19-MAFB transgenic mice

The cosmid clone containing the 6.3-kb human CD19 promoter region cloned into cosmid vector pTL5 was described before [19]. The polylinker described here was modified into a new polylinker: *Bgl*II-*Mlu*I-*Xba*I-*Sma*I-*Sca*I-*Eco*RV-*Xho*I-*Not*I. The CD19 promoter was isolated by using the *Mlu*I-*Eco*RV sites and ligated into pBluescript SK vector containing 971 bp of the human *MAFB* coding region, a 651 bp β-globin intron and a 131 bp polyadenylation signal sequence. A Human influenza hemagglutinin (HA)-tag and a Kozac sequence were placed in frame between the *Eco*RI site and the *MAFB* start site of transcription. The Kozac sequence and reading frame of *MAFB* proved positive as tested before (chapter 3).

Two mouse Ig Eμ enhancers were obtained by PCR by using primers with unique restriction sites. The primers for the first Eμ enhancer were 5'-*Nde*I-GAGGTCTGGTGGAGCCTG-3' and 5'-*Xho*I-TCTAGATAATTGCATTCAT-3'. The primers for the second Eμ enhancer were 5' *Sal*I- GAGGTCTGGTGGAGCCTG-3' and 5' *Mlu*I-TCTAGATAATTGCATTCAT-3'. The purified enhancer PCR products were ligated between the unique *Nde*I and *Mlu*I sites in the pBluescript SK upstream of the CD19 promoter. The linearized ~11 kb Eμ-CD19-MAFB contruct was excised from the vector, gel-purified and microinjected into B6CBAF1 fertilized oocytes. Transgenic founder mice were identified by PCR analysis using two sets of primers: one primer set spanning the CD19 promotor and the β-globin intron (5' AAAACAGCGTGGCAGGGAGG 3' and 5' GGGTCCATGGTGATACAAGG 3') and one primer set spanning β-globin intron and *MAFB* (5'-ATACTCTGAGTCCAAACCGG-3' and 5'-TGATGATGGTGATGGTGCGG -3'). Transgenic founder mice were mated with C57BL/6 wild-type mice and offspring were identified by same PCR analysis described above.

Northern Blot analysis

To analyze expression of human *MAFB* in murine spleen and bone marrow cells, Northern Blot analysis was performed as previously reported [24]. The I.M.A.G.E. cDNA clone 5261053 *MAFB* (RZPD, Germany) was used to generate the probe.

Flow cytometric analysis

Preparations of single cell suspensions from lymphoid organs, four-color flow cytometry procedures and specific mAbs have been described previously [25-27]. For intracellular staining of cytoplasmic proteins, cells were first stained for cell surface markers and subsequently fixed in 2% paraformaldehyde and permeabilized using 0.5% saponin. Blood samples were depleted of erythrocytes by standard NH_4CL lysis before staining with mAbs. Events ($0.3-1 \times 10^5$) were scored using a FACS Calibur flow cytometer and were analyzed using FlowJo software (Tree Star, Inc).

Serum Ig detection, in vivo immunizations and immunohistochemistry

TNP-KLH and TNP-ficoll immunizations were performed as described previously [28]. To hyper stimulate the whole immune system, mice were immunized with 30 µg LPS [29]. Booster doses of TNP-KLH were given after 5 weeks. Clonality of serum Ig was tested by gel electrophoresis using the Sebia (Vilvoorde, Belgium) Hydrasys system. Levels of Ig subclasses or anti TNP specific antibodies in serum were measured by sandwich ELISA, as described previously [25]. Spleen tissues were fixed in 10% formalin, paraffin embedded, and sectioned at 3 to 6 µm for hematoxylin and eosin (H&E) staining. For cryosections, spleen tissues were snap-frozen in liquid nitrogen using Tissue Freezing medium (Leica). Tissues were sectioned and stained with a mouse monoclonal α *MAFB* IgG1κ antibody (chapter 4). To detect *MAFB* expression, as secondary and tertiary antibody, FITC conjungated α mouse IgG1 (BD bioscience, USA) and Rabbit α FITC-peroxidase (Dako, Denmark) was used.

Results

Transgenic expression of hMAFB under the control of the hCD19 promoter and m Eμ enhancer.

To generate transgenic mice expressing high levels of MAFB specifically in B-cells, the human *MAFB* cDNA was cloned into a vector downstream of a 6.3 kb genomic fragment containing the CD19 promoter. Upstream of the CD19 promoter, a duplicate of the mouse Eμ enhancer was cloned (Figure 1A). After microinjection into fertilized oocytes, two independent Eμ-CD19-MAFB transgenic mouse lines (2815 and 2826) were obtained and together with their transgenic progeny these founder lines were propagated and used in this study. To determine the genotype of the founder mice and the subsequent generations generated by crosses with C57BL/6 mice, PCR analysis was used with tail DNA as template (Figure 1B).

In the spleens and in the BM of transgenic mice, abundant mRNA expression of MAFB was detected by Northern blot analysis. Total RNA of mice from both founder lines was isolated and analyzed for *MAFB* expression. As a result, the highest mRNA expression was found in the founder line 2826 in both lymphoid organs compared to founder line 2815 (Figure 1C). To determine the expression of MAFB at the protein level, cryosections of spleen tissue of wild type and transgenic mice were stained with a specific monoclonal MAFB antibody (chapter 4) and MAFB expression was detected in a proportion of spleen cells only in founder line 2826 (Figure 1D). Due to the scarcity of MAFB positive cells, we were not able to determine the nature of the cells. Taken together, abundant mRNA *MAFB* expression was detected in lymphoid organs of *Eμ-CD19-MAFB* transgenic mice.

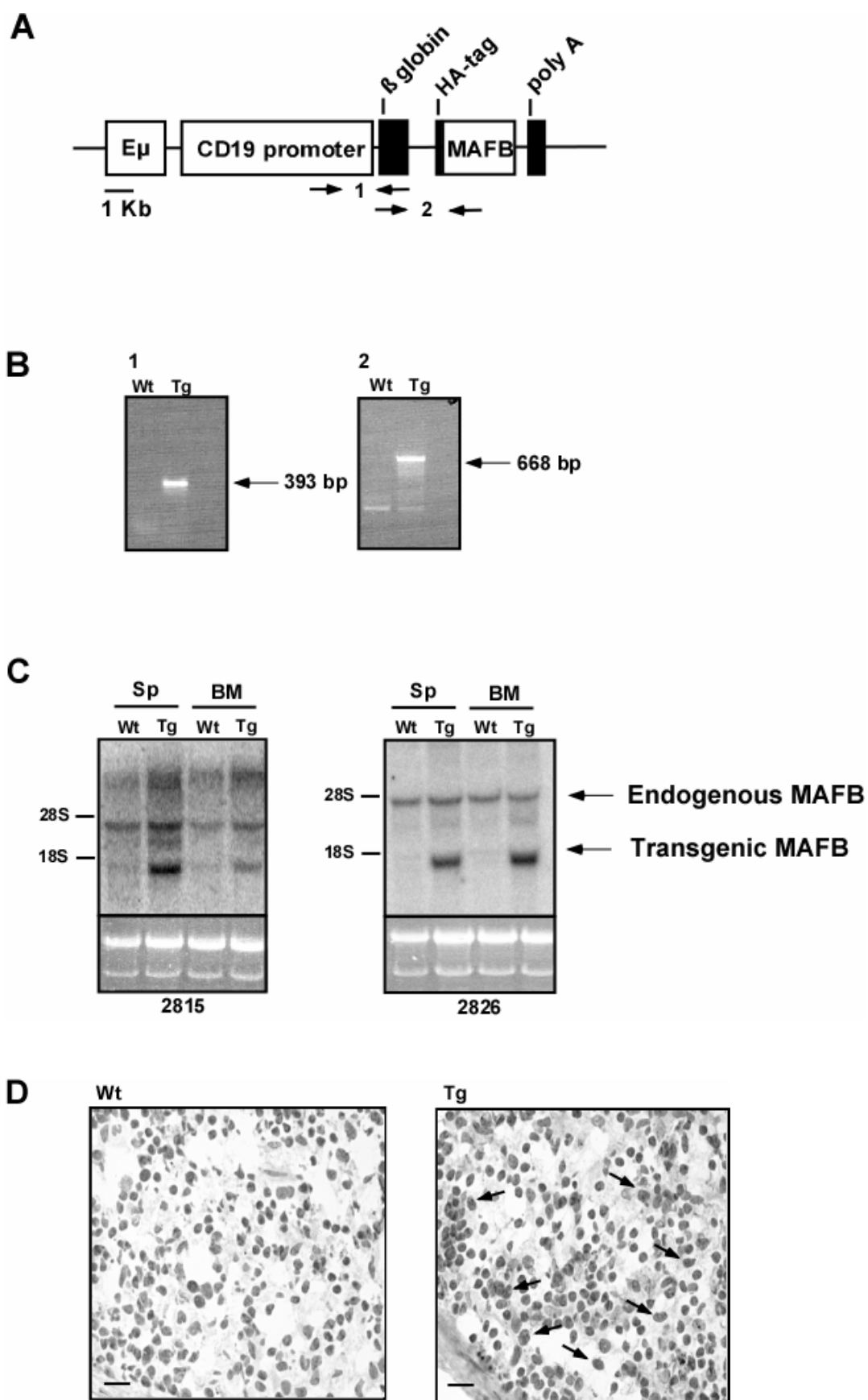**A**

B

C

D

Figure 1: Generation and characterization of Eµ-CD19-MAFB mice.

A Schematic representation of the transgenic construct. cDNA encoding MAFB was cloned downstream of the CD19 promoter and (duplicated) Eμ enhancer elements. A Human influenza hemagglutinin (HA)-tag was placed in frame between the EcoRI site and the MAFB start site of transcription.

B Characterization of Eμ-CD19-MAFB mice screened by PCR using tail DNA as template. Two primer sets were designed (see Fig 1A), one primer set spanning the CD19 promotor and the β-globin intron (1) and one primerset spanning β-globin intron and MAFB (2).

C Transgenic MAFB mRNA expression in Eμ-CD19-MAFB mice analyzed by Northern blot analysis. Splenocytes and bone marrow from wildtype and transgenic mice were obtained. Total RNA was isolated and probed for human MAFB. Equal amounts of RNA were loaded, as shown by ethidium bromide staining.

D Transgenic MAFB mRNA expression in Eμ-CD19-MAFB mice analyzed by IHC. MAFB expression (brown staining, some cells indicated by arrows) was detected by staining cryosections of spleen tissue obtained from wild-type and transgenic mice using a specific monoclonal antibody for MAFB. Scale bar is 25 μm.

No differences in B-cell development in Eμ-CD19-MAFB transgenic mice compared to wild-type mice.

In the haematopoietic system, MAFB is normally expressed in monocytes and macrophages, but not in B-cells. As mentioned before, in MM, *MAFB* may act as an oncogene, dysregulating *CCND2* expression in post GC PC's. As a result of *CCND2* over expression, altered proliferation and/or reduced apoptosis of B-cells may be expected in *Eμ-CD19-MAFB* transgenic mice. To examine the effect of *MAFB* over expression in B-cell development total cell suspensions from various lymphoid tissues from 2-month-old transgenic and wild-type mice were analyzed by flow cytometry (Figure 2). Lymphoid tissues of two wild-type controls and two transgenic mice were analyzed from each founder line. In the early B-cell development no differences were found in the pro-/pre-B-cell fraction as shown by the expression profiles of B220/IgM on total lymphoid cell in the BM (Figure 2A).

To study the maturation of the B-cells in the periphery, single cells from spleen and lymph nodes were analyzed and normal IgM/IgD profiles were found in both lymphoid tissues (Figure 2B and 2C). Next, we studied different mature B-cell subsets in B-cell development: follicular B-cells and marginal zone B-cells in the spleen and B-1/B-2 B-cells in the peritoneal cells (Figure 2B and 2D). CD19$^+$/IgM$^+$ positive B-cells were gated and analyzed by their CD21/CD23 profile. Compared to wild-type mice no different pattern of marginal zone cells (CD21$^+$/CD23$^-$) and follicular cells (CD21^{low}/CD23$^+$) was found (Figure 2B). Also, no different pattern was found for B-1/B-2 B cells in peritoneal cells as shown by CD19/CD5 profile (Figure 2D). To exclude that

transgenic expression of *MAFB* has an effect on T-cell, erythroid and monocyte/myeloid cell development, we also analyzed single cells obtained from the thymus and BM. For T-cell development, the CD4/CD8 and CD69/CD3 profiles showed no differences between wild-type and transgenic mice and for the erythroid and monocyte/myeloid cell development the CD19/B220 and Ter119/MP20 profiles showed no differences (data not shown). In summary, no effects on the haematological compartment of *Eμ-CD19-MAFB* expression was found in both transgenic founder lines compared to wild-type mice.
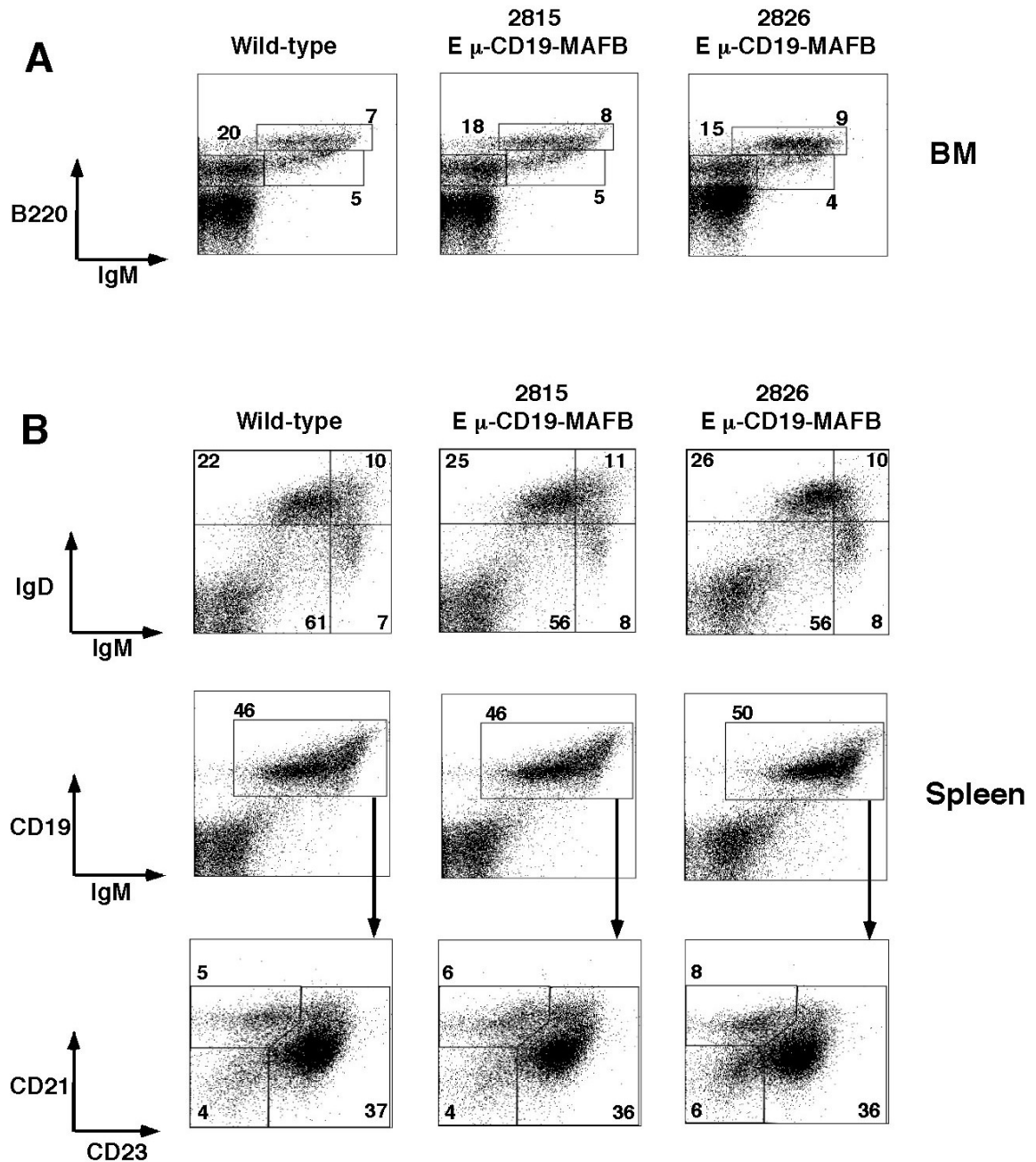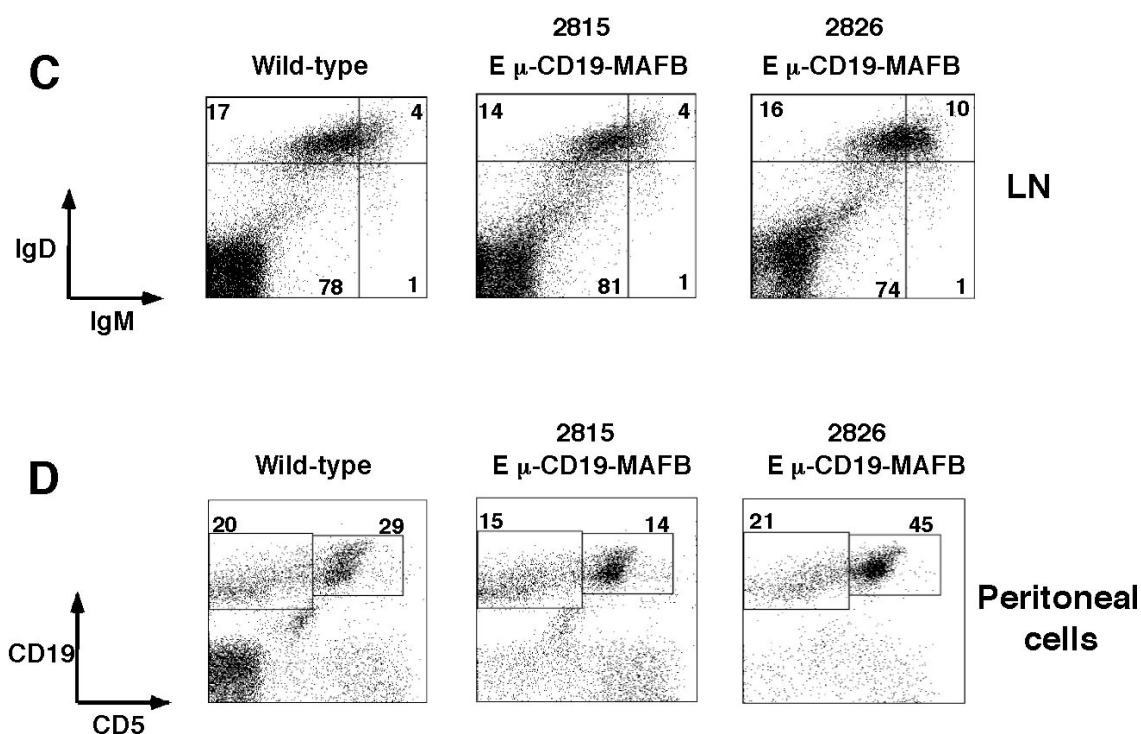
Figure 2: The effects of transgenic Eμ-CD19-MAFB expression on B-cell development.

Flow cytometric analysis of cells from BM (A), spleen (B), lymphe nodes (C) and peritoneal cavites (D) of the two transgenic Eμ-CD19-MAFB founder lines compared to non-transgenic mice.

A IgM/B220 profile of total bone marrow lymphoid cells.

B IgM/IgD and CD19/IgM profiles of total splenic lymphoid cells. The CD19+IgM+ B-cell fraction was gated and analyzed for the markers CD21/CD23.

C IgM/IgD profile of total lymphoid cells from the lymph nodes.

D CD5/CD19 profile of peritoneal cells.

Data are displayed as dot plots of total lymphocytes, which were gated on the basis of their forward and side scatter characteristics. Percentages of lymphocytes within the indicated gates are given. Data shown are representative for two wild-type and two transgenic mice of each transgenic founder line.

Increased total IgM levels in Eμ-CD19-MAFB transgenic mice.

To investigate whether expression of *MAFB* could have any effect on the immunoglobulin (Ig) levels, serum Ig levels were determined by Ig specific ELISA in *Eμ-CD19-MAFB* transgenic and wild-type mice. As shown in Figure 3A, the *Eμ-CD19-MAFB* transgenic mice had significantly increased (p=0.003) levels of IgM. A somewhat decreased level IgG1 was observed (not significantly) (Figure 3B) and all other Ig levels were normal. These data indicate that increased expression of *MAFB* might have an effect on the immunoglobulin expression in mature B-cells.

A
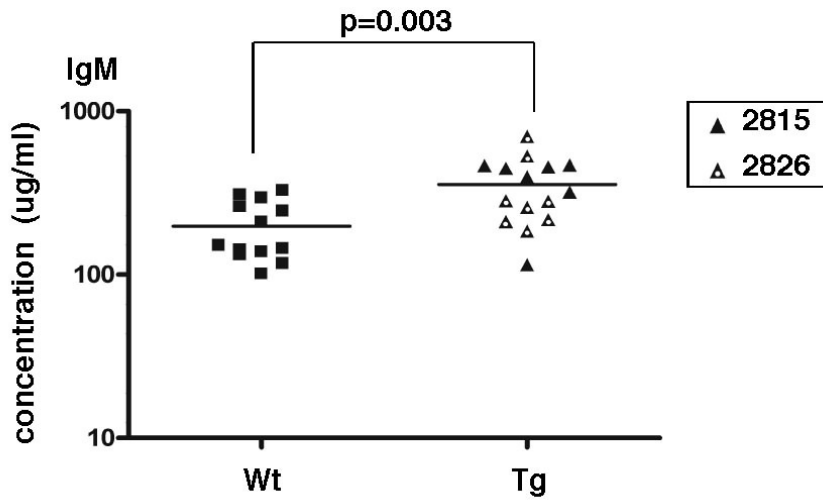
B
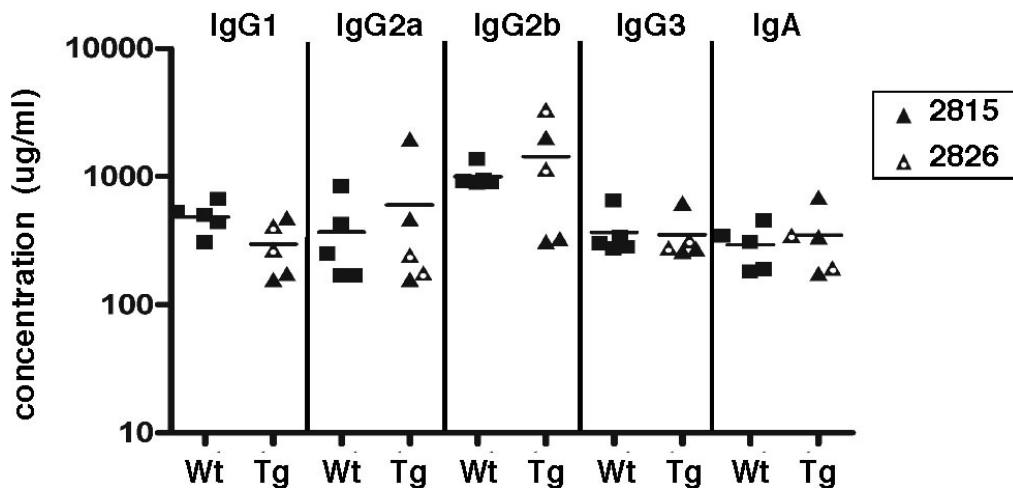
Figure 3: Increased IgM levels in transgenic Eμ-CD19-MAFB mice.

A Serum concentrations of IgM in the indicated mouse strains. Each symbol represents an individual animal.

B Serum concentrations of the IgG specific subclasses and IgA in the indicated mouse strains. Each symbol represents an individual animal.

Effect of MAFB on the immune response.

Panels of mice were formed to follow the immune response in transgenic mice. We immunized mice with a specific TD antigen, trinitrophenol (TNP) -keyhole limpet hemocyanin (KLH), a TI specific antigen, TNP-Ficoll, and with the mitogen lipopolysaccharide (LPS). Both transgenic founder lines and wild-type mice were immunized and blood serum samples were used for analysing anti TNP antibody or Ig levels. For analysing Ig levels in LPS immunized mice, Ig specific ELISA was used. Immunization with LPS gave

rise to a proper immune response as measured by total Ig in all groups (IgM and IgG3 shown in Figure 4A, other Ig data not shown). Apart however from the pre-existent higher IgM, shown above, the response to LPS immunization did not differ between transgenic vs wild-type mice. We tested here whether there was a quantitative difference in these responses among transgenic vs wild-type mice, and did not see that with respect to serum concentrations of total immunoglobulin due to LPS immunization.

Anti TNP antibodies were analyzed by TNP specific ELISA for mice immunized with TNP-KLH or TNP-Ficoll. As found with regard to total IgM, also pre-existent IgM type anti TNP antibodies were significantly (p=0.002) increased in the 2826 transgenic founder line already before immunization (Figure 4B), indicating that *Eμ-CD19-MAFB* transgenic mice have increased TNP-specific IgM antibodies despite of immunization. The immune responses to TD and TI antigen for all Igs were not dissimilar in *Eμ-CD19-MAFB* transgenic vs. wild type mice (Figure 4C).

A
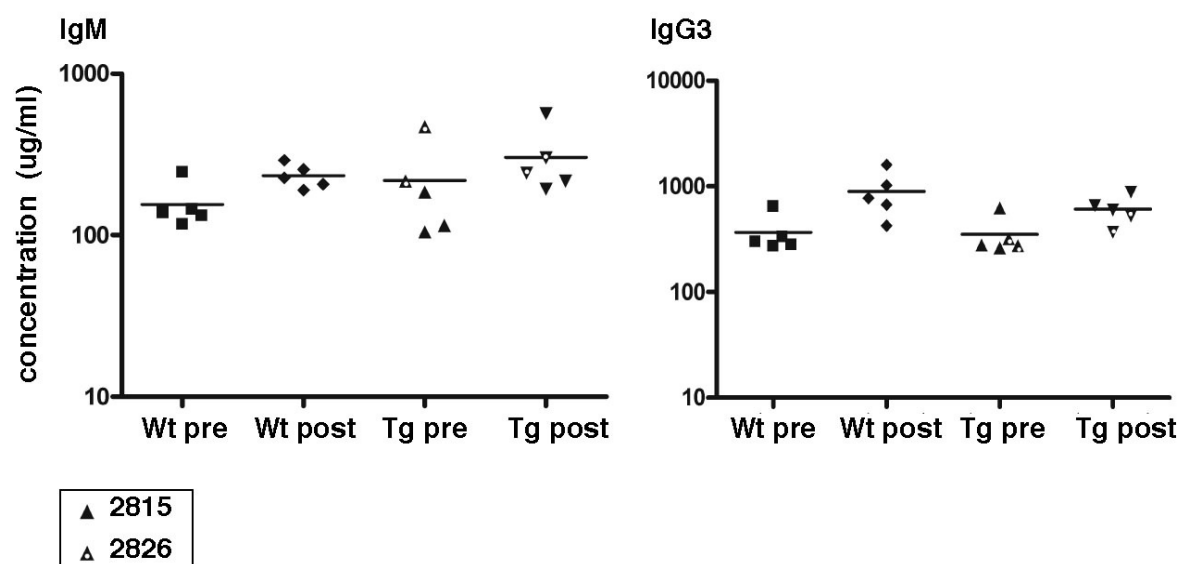
Figure 4: Immune responses in vivo to TD and TI antigens and mitogen LPS in transgenic Eμ-CD19-MAFB mice.

A Serum concentrations of IgM and IgG3 in indicated mouse strains after LPS immunisation. Each symbol represents an individual animal.
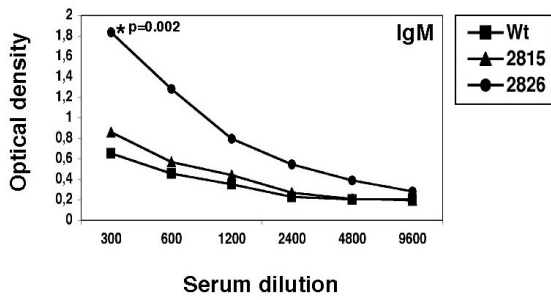
B

C
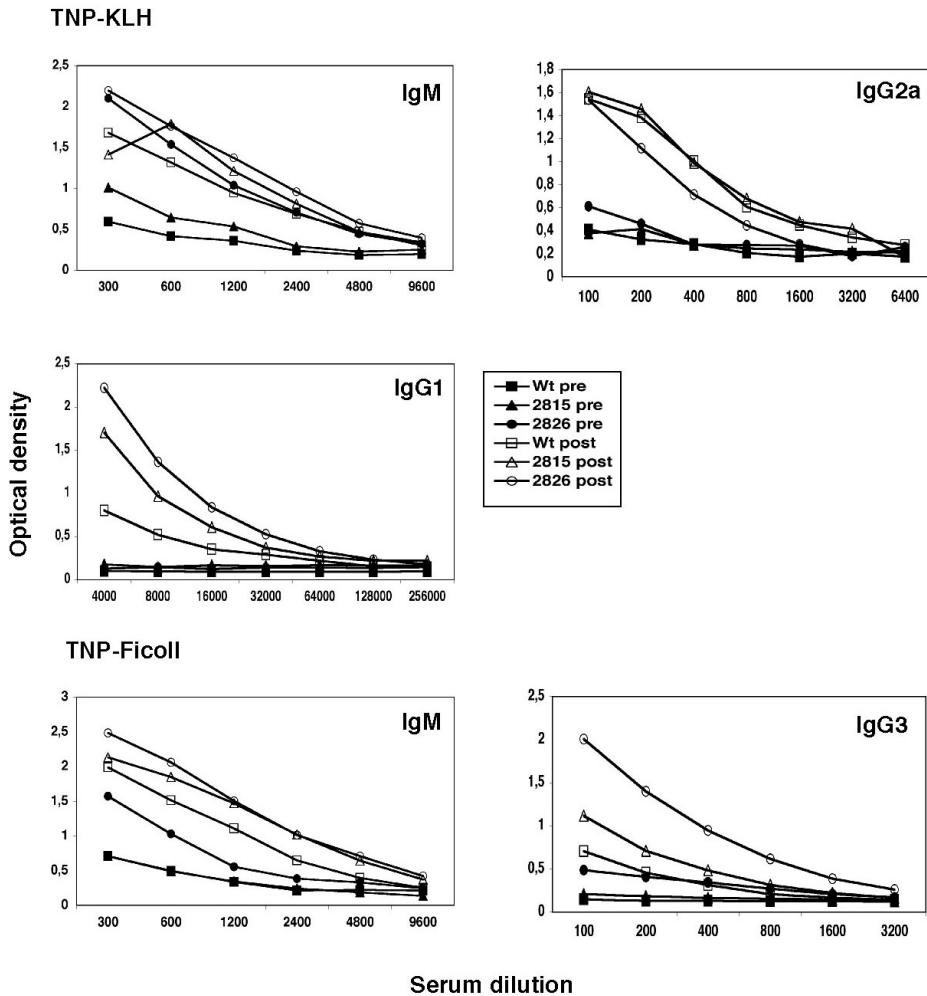
TNP-KLH

TNP-Ficoll

Serum dilution

Figure 4: Immune responses in vivo to TD and TI antigens and mitogen LPS in transgenic Eµ-CD19-MAFB mice.

B Increased pre-existent IgM type anti TNP antibodies in the 2826 transgenic founder line already before immunization. For each serum dilution, the ODs are shown as mean values from 9-10 mice in each group.

C Primary responses to TD TNP-specific IgM 7 days after in vivo TNP-KLH injection and secondary TD responses of the indicated subclasses (determined 7 days after an i.p. booster injection ~4 wk after the primary immunization).

Primary responses to TI TNP-specific of IgM and IgG3 7 days after in vivo TNP-Ficoll injection. For each serum dilution, the ODs are shown as mean values from 4-6 mice in each group.

Do Eµ-CD19-MAFB transgenic mice develop monoclonal PC expansion over time?

For following aberrations in B-cell development in transgenic mice, blood samples were analyzed at four different time points during a year of follow-up. The most characteristic feature of MM is the secretion of a large amount of monoclonal immunoglobulins that can be detected in the serum as a distinct band (M-protein). Serum Protein Electrophoresis (SPEP) was used to detect the M-protein spikes in serially bled mice. M-protein was followed at four time points; 20 out of the 83 evaluable mice showed a M-protein band after 200 days. These M components, however, were found scattered over the different experimental groups (Table 1), and had apparently not increased in size, when tested at retrospective days (data not shown); they may therefore not be taken as the result of secretion by possible tumor PCs.

To follow cellular B-cell aberrations in *Eµ-CD19-MAFB* transgenic mice, lymphocytes isolated from whole blood samples, were analysed by flow cytometry. This data demonstrated that most of the *Eµ-CD19-MAFB* mice do not accumulate differentiated plasma cells. No differences were observed in the CD19/IgM (Figure 5A) and Kappa/Lambda (data not shown) profiles of transgenic mice compared to wild-type mice. Also, immunization with TD and TI specific antigens and LPS showed no effect on B-cell development.

Table 1: Incidence of M-protein spikes in *Eµ-CD19-MAFB transgenic mice vs. wild-type*

	Control	TNP-Ficoll	TNP-KLH	LPS
Wt	n.d.	1/5	1/6	4/14
2815	2/10	1/8	0/8	2/8
2826	4/6	3/5	1/7	1/7

Incidence of M-protein spikes in a cohort of 25 immunized wild-type mice, 16 not immunized *Eµ-CD19-MAFB* transgenic mice and 43 immunized *Eµ-CD19-MAFB* transgenic mice after 200 days. n.d.: not determined

Effect of Eµ-CD19-MAFB transgenic expression on leukocyte development after a year of monitoring.

Panels of mice from the immune response group were sacrificed after a year to study the effect of *MAFB* over expression in B-, T-, and myeloid cell development. Splenic lymphoid cells were analyzed by flow cytometry. No

different results were observed in the IgM/CD19 and Kappa/lambda profiles for B cell development of Eμ-CD19-MAFB transgenic mice compared to wild-type mice (Figure 5B). As found for B-cell development, CD4/CD8 profiles for T-cell and Gr-1/MAC-1 profiles for myeloid cell development showed no abnormal cell populations in both founder lines. Therefore, we conclude that over expression of *MAFB* had no effect in B-, T-, and myeloid cell development in 1-year-old *Eμ-CD19-MAFB* transgenic mice.

Figure 5: Characterization of Eμ-CD19-MAFB transgenic mice after one year of monitoring.

A The effects of Eμ-CD19-MAFB transgenic expression and in vivo immunization on B-cell development. Lymphoid cells of whole blood samples of Eμ-CD19-MAFB transgenic compared to non-transgenic mice were stained with specific antibodies and analysed by flow cytometric analysis. Shown are the average results including the standard deviation of different types of immunisation: not immunized (control), TNP-KLH, TNP-Ficoll and LPS at four time points. Flow cytometry data is displayed as line graph of total lymphocytes, which were gated on the basis of their forward and side scatter. The relative percentages of CD19+IgM+ cells are indicated compared to wild type mice, which data was set at 100%. For B-cell development, total B-cells were gated on the expression of specific B-cell markers CD19 and IgM.
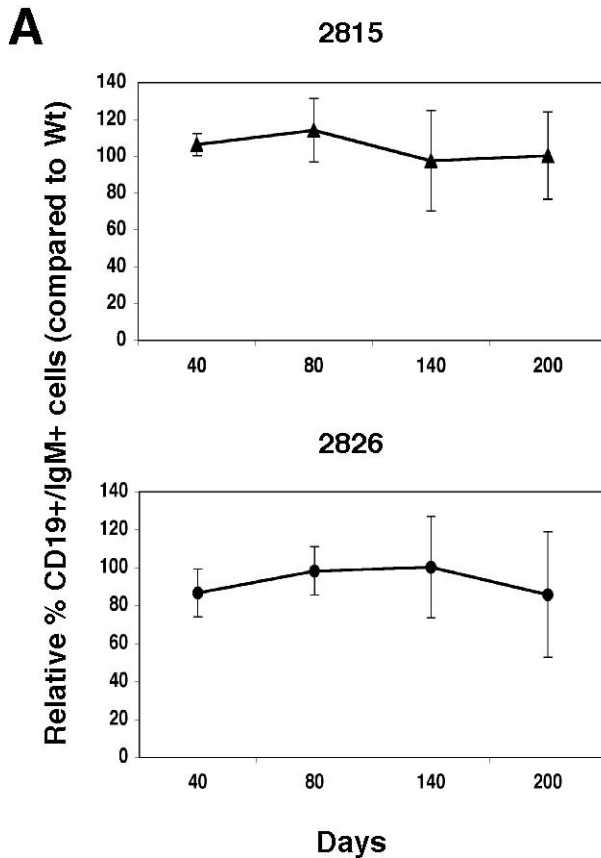
B
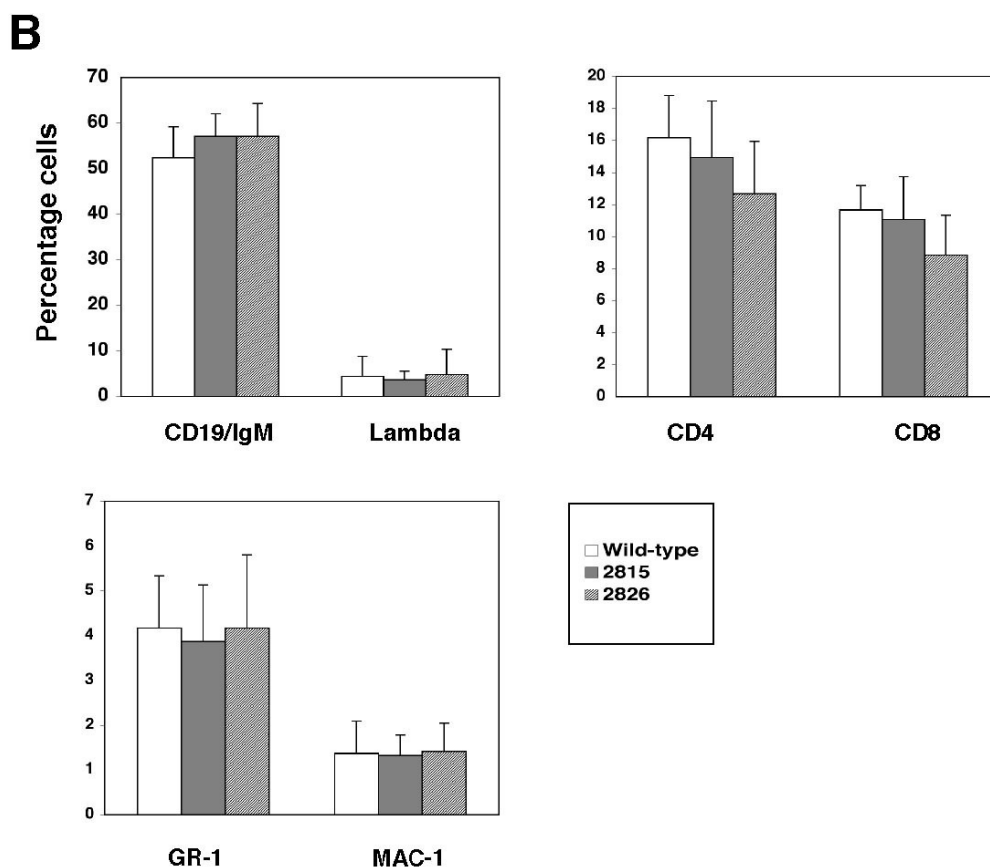
Figure 5: Characterization of Eμ-CD19-MAFB transgenic mice after one year of monitoring.

B The effects of Eμ-CD19-MAFB transgenic mice on B-cell, T-cell, and myeloid cell development. Total splenic lymphoid cells of Eμ-CD19-MAFB compared to non-transgenic mice were stained with specific antibodies and analysed by flow cytometric analysis. Flow cytometry data are displayed as bar graphs of total lymphocytes, which were gated on the basis of their forward and side scatter. The percentages of positive cells are indicated. For B-cell development, total B-cells were gated on the expression of specific B-cell markers CD19 and IgM. Kappa and lambda expression was investigated within the CD19+IgM+ cells. For T-cell development, total T-cells were stained with the specific T-cell markers CD4 and CD8. For myeloid cell development, granulocytes and monocytes/macrophages were stained with their specific markers GR-1 and Mac-1. Data shown are representative of 6-16 animals examined within each group. Standard deviation is presented with error bars.

Occurrence of aberrant B-cell development detected in three Eμ-CD19-MAFB transgenic mice.

In three transgenic mice regardless the type of immunization (2826: not immunized, 2826: TNP-Ficoll, 2815: not immunized), besides the normal B-cell population $CD19^+IgM^+$ kappa/lambda, an extra population of $CD19^-IgM^+$, 100% kappa$^+$ (Tg+) was observed in whole blood samples by flow cytometry analysis (Figure 6A). When tested, this $CD19^-IgM^+$, 100% kappa$^+$ extra population was found in one mouse (2826: not immunized) at different time points suggesting that it is an consistent event. Total single cell suspensions from different lymphoid tissues were generated from this specific Tg+ mouse

and B-cell, T-cell and myeloid cell development was analyzed by flow cytometry compared to a wild-type mouse and a transgenic mouse without the extra population (Tg-). The profiles tested for the specific B-, T-, and myeloid cell markers showed no differences between the Tg+, Tg- and wild-type mice (data not shown).

Figure 6: the occasional occurrence of aberrant B-cell development in Tg+ mice.
A CD19/IgM profile of Tg+ mouse compared to a wild-type mouse showing an extra population of CD19⁻IgM+, 100% kappa+ cells in blood analyzed by flow cytometry. The CD19⁻IgM+, 100% kappa+ cells were gated and analysed for the Kappa/Lambda markers. Data are displayed as dot plots of total lymphocytes, which were gated on the basis of their forward and side scatter characteristics. Percentages of lymphocytes within the indicated gates are given.
B IHC stainings from Wt, Tg- and Tg+ mice. Spleen sections were stained with H&E. Arrowheads indicate a population of abnormal spleen cells. Scale bar is 100 μm and 25 μm in magnification.

Furthermore, we examined the morphology of the spleen cells from the transgenic Tg+ by immunohistochemistry (IHC). As shown in figure 6B, IHC staining of spleen sections showed a population of abnormal cells in the

red pulpa of the spleen in Tg+ mice. The morphologically abnormal cells were not detected in wild-type and Tg- mice. To determine the nature of the cells, they were tested with different B-cell specific antibodies. Staining with anti-CD19, anti-B220, anti-IgM, anti-kappa light chain and anti-lambda light chain antibodies was negative on these cells. Because *MAFB* plays an important role in the monocyte-macrophage differentiation [5] and is essential for F4/80 expression in macrophages [30], we also performed IHC stainings with specific monocyte and macrophage markers. The abnormal cells stained negative for anti-CD11b, anti-CD11c and anti-Gr-1. So far, we were not able to determine the nature of these cells.

A second Tg+ mouse (2826: TNP-Ficoll) was analyzed in the same manner. Here, we detected in this mouse an increased number of B1-B-cells ($IgM^{high}IgD^{low}$ $CD5^+$) in spleen (Figure 6C). These two mice could not be evaluated for M proteineaemia. In the third Tg+ mouse (2815, not immunized) a slight M protein was found as well, but further none of the additional abnormalities with respect to splenic morphology or B1-B cells were observed. Summarized, all data suggest that besides expression of *MAFB* in lymphoid organs, no MM phenotype was observed in transgenic mice yet.
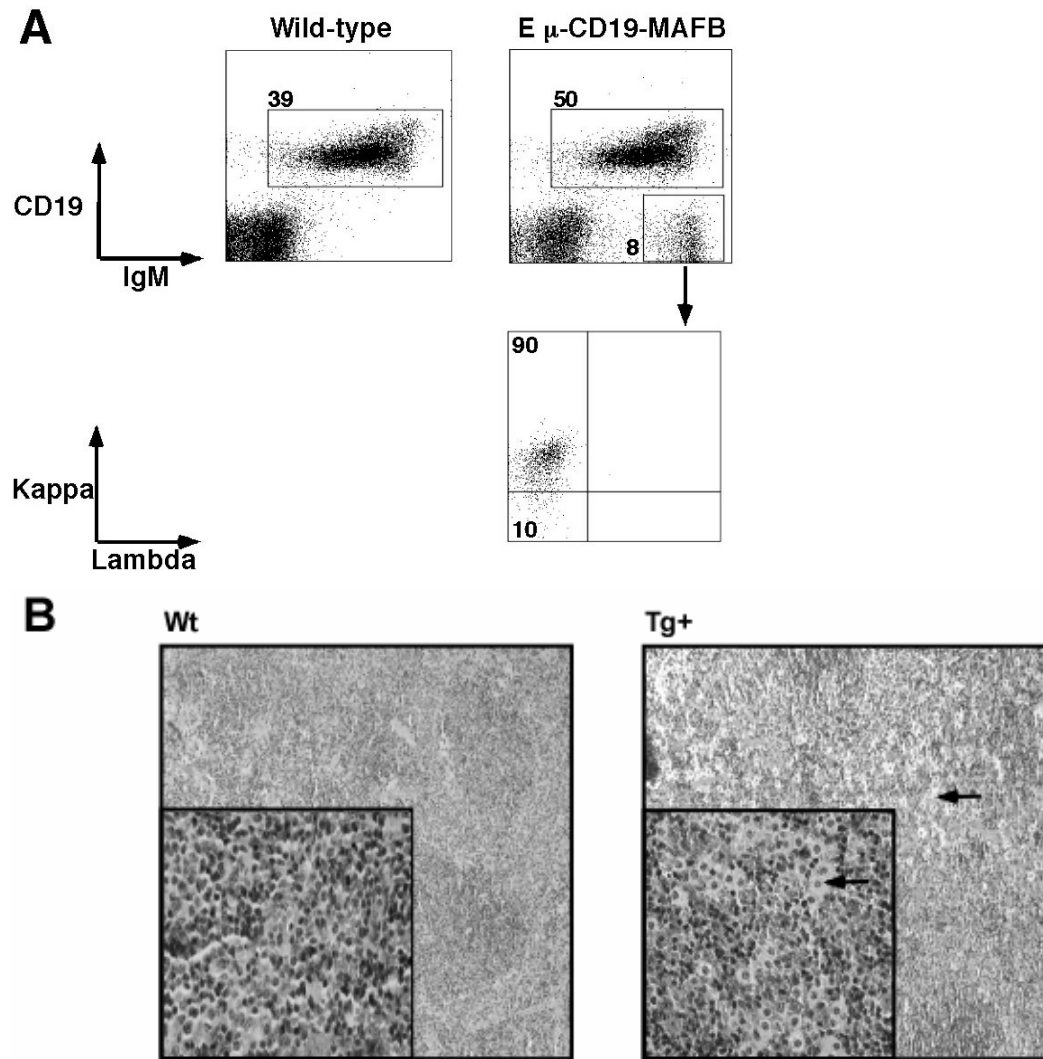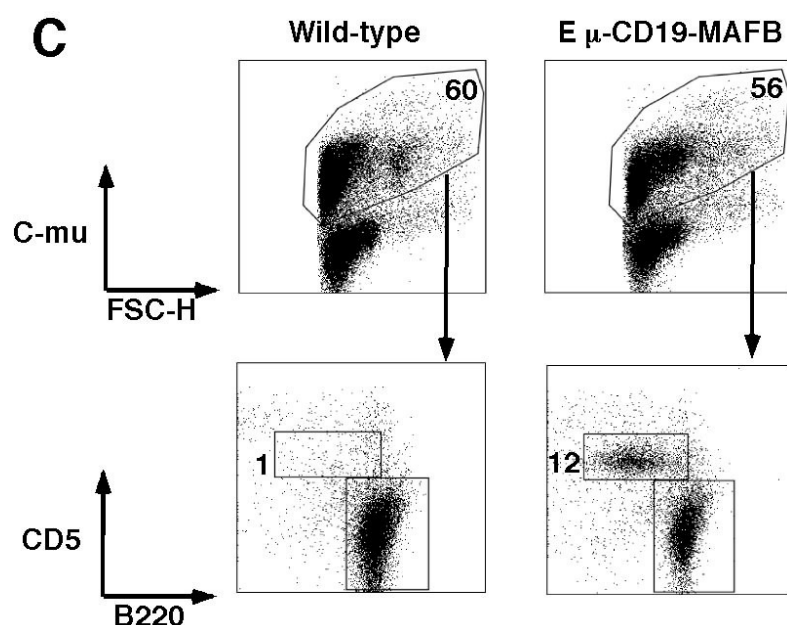

Figure 6: the occasional occurrence of aberrant B-cell development in Tg+ mice.

C CD5/B220 profile of Tg+ mouse compared to a wild-type mouse showing an increase of CD5+ B1 B-cells in spleen analyzed by flow cytometry. Total B-cells were gated on their expression of specific B-cell marker intracellular IgM (C-MU) and forward side scatter characteristics. Data are displayed as dot plots of total lymphocytes, which were gated on the basis of their forward and side scatter characteristics. Percentages of lymphocytes within the indicated gates are given.

Discussion

MAFB expression in B-cells under the control of hCD19 promoter and mEμ enhancer: set up of the study

In current study we tried to induce the formation of MM by generating a *MAFB* transgenic mouse. *MAFB* is a oncogene that is over expressed as a result of the primary t(14;20) in MM. To our knowledge this is the first transgenic mouse generated to date to study MM *in vivo* involving one of the seven up regulated oncogenes as a result of primary translocations in MM. During our work, one transgenic study described a T-cell lymphoma phenotype as a result of over expression of *C-MAF* where *C-MAF* was placed under the control of the human CD2 promoter [14], which finding lend support to our approach.

The *Eμ-CD19-MAFB* transgenic mouse was designed to obtain high expression of *MAFB* in mature B-cells. As a single primary oncogenic deregulation does not seem to suffice for tumor formation, and in view of the fact that monoclonal Ig components in MM have been found to have antibody reactivity [31,32], we tested whether antigenic stimulation might provide such a secondary signal. Therefore, we stimulated differentiation of B-cells into plasma cells after immunization with TI and TD specific antigens or with a mitogen. In the two transgenic founder lines obtained, *MAFB* expression was detected in total RNA of single cells obtained from two lymphoid tissues, spleen and BM. Using the specific monoclonal MAFB antibody to detect expression by IHC in cryosections of spleen tissue, expression was observed in a proportion of cells in founder line 2826. An explanation for the fact that we only found high *MAFB* mRNA expression and low MAFB expression at protein level in one founder line might be that expression of protein MAFB is below detection level in the majority of cells or protein MAFB is not stable enough for detection. Alternatively, the translation from mRNA might be disturbed by deficiencies in the construct. Both explanations seem to be improbable as the *Eμ-CD19-MAFB* construct was composed of enhancer and promoter elements, which were used in several other studies proving that this promoter/enhancer combination results in transgenic expression in targeted cells [17,19]. Furthermore, cDNA of *MAFB* containing the right Kozac sequence

was used in the gene expression array study to determine the direct target genes of *MAFB* (chapter 3).

B-cell development, Ig levels and immune response in Eµ-CD19-MAFB transgenic mouse.

In general, data of flow cytometry analysis showed that *Eµ-CD19-MAFB* transgene expression had no effect on B-, T- and myeloid cell development in two months old mice. Immunization with LPS and both T dependent or T independent forms of TNP gave rise to a proper and similar immune response for most Ig classes as measured by total Ig or specific antibodies respectively in all groups. However, both transgenic founder lines showed a significantly increased IgM level compared to wild-type mice. Furthermore, increased anti TNP antibody level of IgM was observed before immunization in the 2826 founder line, suggesting that *Eµ-CD19-MAFB* express IgM specific anti TNP antibodies despite immunisation Normally, natural antibodies with anti TNP activity or activity to other antigens can occur at low concentrations in mice [33,34]. Such natural antibodies were found to react with more than one antigen, i.e. so called multi-specific natural antibodies. Therefore, it is likely that the increased IgM antibodies levels found in *Eµ-CD19-MAFB* transgenic mice might be multi-specific IgM antibodies. Such multi-specific antibodies obviously overlap partly in antibody reactivity as seen in antigen specific ELISA and with high levels of pre-existent antibodies, the level of total IgM (although higher as well) is not necessarily higher to the same amount. A conceivable explanation of such an enhancing influence of *MAFB* transgenesis on natural antibodies (germ line preponderance?) may be not be easy at hand, however.

The role of natural antibodies in the pathogenesis of MM is not clear as yet. However, it is reported that some monoclonal Igs from MM patients exhibit similar specificities to that found with natural antibodies [35]. Furthermore, the role of natural antibodies in the pathogenesis of CLL has been reported whereby polyreactive antibodies are expressed by CLL B-cells [36]. Still, we cannot give a conceivable explanation wether *MAFB* over

expression has influenced the expression of natural antibodies and thereby it remains unclear what has influenced the high (specific) IgM levels.

Taken together, *Eμ-CD19-MAFB* transgenic mice show increased levels of IgM antibodies but it is not clear as yet what has influenced the high (specific) IgM levels and we were not able to elucidate this phenomenon.

Monoclonal PC development in Eμ-CD19-MAFB transgenic mouse.

Clonality of serum Ig is the mean feature in MM and usually is detected by analysing M-protein in serum. We have analysed the occurrence of an M-protein in serially bled *Eμ-CD19-MAFB* transgenic mice compared to wild-type, in different immunisation protocols. As a result, the M-protein was detected in ten *Eμ-CD19-MAFB* mice, which might indicate monoclonal expansion of tumor cells. Some arguments, however, seem to refute this conclusion; firstly, M components emerged in both wild type and transgenic mice; secondly, we did not see an effect of immunisation - which might have given secondary signals, required after *MAFB* transgenesis; and thirdly, we did not see an obvious increase of the M components in time.

Data of flow cytometry analysis showed that *Eμ-CD19-MAFB* transgene expression also had no effect on B-, T- and myeloid cell development after a year. Still, in three transgenic mice an extra population of $CD19^-IgM^+$, 100% kappa$^+$ cells was detected in blood analysis (Tg+ mice). In one mouse tested serially, this population of cells was found repeatedly, suggesting that one *Eμ-CD19-MAFB* transgenic mouse was developing a tumor-like monoclonal population of cells. After analyzing the whole B-cell compartment in different lymphoid tissues, the extra population was not detected anymore. The finding of extra $CD19^-IgM^+$, 100% kappa$^+$ cells in blood possibly might be due to aspecific binding of antibodies used in flow cytometry analysis are binding aspecifically to the Fc-receptors in monocytes an macrophages. However, this does not explain its repeated finding, and in only a low proportion of the mice tested.

In a second Tg+ mouse flow cytometry data showed only an increased numbers of B-1 B-cells ($IgM^{high}IgD^{low} CD5^+$) cells in spleen in Tg+ mouse compared to wild-type and Tg- mouse. B-1 B-cells represent a separate

subset of mature B-cells with distinct origins, cell surface phenotype, functional properties and anatomical distribution. These cells are called B-1 B-cells because it was shown that these cells arise from precursors in the fetal liver, whereas the conventional mature BM derived B-cells are also referred to as B-2 B-cells. It is known that B-1 cells contribute little in adaptive immune responses to most protein antigens, but contribute strongly to some TI antigens, like TNP-Ficoll. This might explane the increased numbers of CD5^{+} B-cells in this specific Tg+, which was immunized with TNP-Ficoll.

Transgenic over expression of MAFB caused by a primary translocation, a good model to mimic MM development in mice?

The development of animal models for MM has been an ongoing effort. Syngeneic (5TMM model) [37] or xenogenic (human MM into SCID-Hu or NOD-SCID) mouse transplant models were developed [38,39] and some time ago, Potter developed the transplantable Balb/c mouse peritoneal plasmacytoma model [40]. Despite some deficiencies of the latter model for MM, it was seminal for many fundamental discoveries, like the structure and rearrangements of Ig genes, Myc translocations and the role of IL6 and the microenvironment in the survival and growth of plasma cell tumors.

Recently, several transgenic mouse models that generated PC tumors were proposed: these include a mixed inbred of mice that co-expressed *C-MYC* and *BCL-X$_L$* driven by Ig enhancers [16] and insertion of *C-MYC* into the IgH locus, just 5' of the intronic Eμ enhancer [41]. In both models *MYC* was constitutively activated. Most of the tumors that were formed in these studies were of short latency and aggressive, e.g. extramedullary plasmacytoma. Also, in the latter study, besides plasmacytoma development, the mice developed lymphoblastic B-cell lymphomas with a human Burtkitt lymphoma-like morphology and diffuse large B-cell lymphoma. This mouse model mimicked some critical features of the human endemic Burkitt lymphoma t(8;14) and mouse plasmacytoma T(12;15). In some cases of both studies the BM was involved, sometimes associated with lytic bone disease, which is typical MM feature.

Very recently, two mouse models have been generated that appear to have a real MM phenotype [15,17]. In one model [17], the spliced isoform of the B-cell transcription factor *XBP-1* was over expressed under the control of immunoglobulin V_H promotor and the Eμ enhancer. These mice generated tumors that have the properties expected for MGUS and MM sequentially. However, most tumors formed in these XBP-1 transgenic mice produced IgM antibodies and only in a few examples IgG. Furthermore, only 50% of the mice had somatically mutated Ig, suggesting that most tumors were not a result of somatic hypermutation or class switching – both of which are a hallmark of human MM. The other MM mouse model [15], was an Activation-Induced Deaminase (AID) dependent *MYC* transgenic conditional mouse. Both somatic hypermutation and class switch recombination require the activity of AID, an enzyme that is expressed specifically in germinal-center B-cells [42]. In this model, a MYC transgene containing a stop codon and flanking Igκ regulatory sequences is activated sporadically in GC B-cells by AID-dependent somatic hypermutation that reverts the stop codon. As a result, MYC transgenic expression was activated only in B-cells that have undergone somatic hypermutation and detected in post-GC plasma cells. Most transgenic mice developed age dependent PC tumors that mainly produce IgG. There was a high frequency of Ig mutations and tumors were minimally proliferative and localized in the BM.

Compared to other MM mouse models, our initial results do not show that *Eμ-CD19-MAFB* transgenic mice give a MM phenotype *in vivo* as yet as well as aberrant progression of the B-cell development. Also the effect on the immune response, if present, seems to be rather subtle. Two transgenic MM mouse models discussed above show a clear MM phenotype but these features were obtained only after two years of transgenesis [15,17]. It is possible that *Eμ-CD19-MAFB* transgenic mice need more time to develop a MM phenotype. Indeed, we detected monoclonal serum Ig components in several mice indicating MGUS development. It is not sure whether this is an effect of transgenic expression of *MAFB* or the occurrence of spontaneous age-dependent serum M-protein in C57BL/6 mice. It is known that C57BL/6 mice develop age dependent MGUS [43,44]. The C57BL/6 mice had a 50%

occurrence of serum M-protein after 2 years of age (IgG>IgM) [43]. The level of M-protein was stable and associated with BM PC. This may explain the fact that M-protein was found in some wild-type mice, although it seems to be a rather early event here.

In addition to the analysis of the haematological compartment we also analysed the changes in gene expression in our *Eμ-CD19-MAFB* transgenic mice compared to wild-type mice by gene expression array's (data not shown). Two independent array's were performed by using total RNA isolated from single lymphoid cells obtained from spleens of *Eμ-CD19-MAFB* transgenic and wild-type mice. mRNA *MAFB* expression was confirmed by Northern Blot analysis before setting up the gene expression array's. Surprisingly, after evaluation of both gene expression patterns obtained, no common distinct up- and/or down regulated genes were found. As a result we were not able to describe a set of genes or transcription program that is dysregulated as a result of *MAFB* over expression. Also, none of the determined target genes of *MAFB* in the human situation, including *CCND2, ITGB7* and *CCR1* (chapter 3) were detected in both gene expression patterns. Taken together, these data may indicate that transgenic *MAFB* expression has very little and/or late effect on the haematological compartment besides abundant transgenic *MAFB* expression. Alternatively, it might be conceivable that constitutively up regulation of *MAFB* in transgenic mice has result in deregulation of other unknown pathways suppressing deregulation of known *MAFB* target genes. Ultimately, one might doubt whether human *MAFB* may be able to interplay with the mouse *MAFB* pathway.

To acquire more effect of *MAFB* might ensue after crossings of *Eμ-CD19-MAFB* transgenic mice with p53 deficient mice (defects in DNA repair [45]) where inactivation of p53 may induce a second hit in cells expressing *MAFB*. In human MM, it is presumed that IgH translocations usually represent primary, perhaps initiating, oncogenic events as normal B-cells pass through the GCs. Inactivation of p53 by variety of mechanisms is presumed to be a secondary progression event. Other possibilities are crossings with BCL-2/ BCL-X_L transgenic mice (apoptosis deficiency [46-48]).

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Chapter 6

General discussion

General discussion

The starting point of this study was the description by our group of a novel chromosomal translocation in cell lines, obtained from Multiple Myeloma (MM) patients, i.e. the t(14;20)(q32;q12) [1]. Subsequent work is reported in this thesis. In summary, we have shown in chapter 2 that as a result of this t(14;20) the transcription factor *MAFB* is up regulated. In addition we have shown that the t(14;20) is a primary translocation indeed.

In chapter 3 we describe the primary target genes that are up regulated by *MAFB* over expression *in vitro* and in primary patient BM samples. We found even secondary target genes and identified the Notch pathway as an important result caused by *MAFB* over expression. Furthermore, we show that the target genes found by over expression of *MAFB* also are up regulated by *C-MAF* over expression, suggesting that members of the large Maf group activate the same pathways in MM development.

In chapter 4 we describe a newly generated anti-MAFB monoclonal antibody. By using this antibody for staining BM biopsies by IHC, MAFB oncoprotein expression was detected in patients carrying t(14;20), but only there. Therefore this MoAb may be used as a prognostic marker in risk stratification of MM, and it may be used to detect minimal residual disease (MRD) as well.

To study MM development *in vivo* we generated a *MAFB* transgenic mouse, which results are described in Chapter 5. Human *MAFB* cDNA was placed under the control of the human CD19 promoter and a duplicate of the mouse Eμ enhancer. With this combination, high levels of *MAFB* expression were expected in the whole B-cell lineage but mainly during the late B-cell development. Higher IgM levels were detected in *Eμ-CD19-MAFB* transgenic mice compared to wild-type mice. However, after a year of monitoring the *Eμ-CD19-MAFB* transgenic mice, no aberrant B-cell development or a MM phenotype was observed yet.

t(14;20): a primary translocation in MM and associated with MAFB over expression.

Chromosomal translocations involving the IgH locus on chromosome 14q32 are a common event in MM. At the start of our studies four recurrent translocations involving the IgH locus had been found in HMCLs and MM patient material: t(4;14)(p16;q32): *MMSET/FGFR3*; t(6;14)(p21;q32): *CYCLIN D3*; t(11;14)(q13;q32): *CYCLIN D1* and t(14;16)(q23;q32): *C-MAF* [2-6]. As these translocations were simple reciprocal translocations mediated by errors in the class switch recombination mechanism, these translocations were presumed to be primary oncogenic events. As a result of these translocations genes that are translocated upstream of the strong enhancers on the IgH locus may now act as oncogenes. Now, we have found that a novel 14q32 translocation involving chromosome 20 can be considered as a primary translocation as well. This t(14;20)(q32;q12) was first detected in a MM cell line (UM3) generated in our institute (UMC Utrecht, the Netherlands) [1]. An other study described that as a result of the t(14;20) aberrant *MAFB* expression was detected in two HMCLs (SKMM-1 and SACHI) carrying this t(14;20) and in one patient suffering from PCL [7]. We show here in two additional MM cell lines (UM6 and EJM) as well as in patient material from which the UM3 and UM6 cell lines were derived and in an additional MM patient carrying this similar (if not identical?) t(14;20) (chapter 2). In the UM3, UM6 and EJM MM cell lines we also found ectopic expression of *MAFB*. Furthermore, after sequencing the breakpoint in the UM3 MM cell line and determining the breakpoints in the four other MM cell lines with t(14;20) by FISH, we report the narrow chromosomal breakpoint scatter (0.8 Mb) on chromosome der 20.

Before our results were presented, the t(14;20) was considered to be a secondary translocation, as the t(14;20) described in chapter 1 involves the enhancer rather than the switch region. However, our data indicate that the t(14;20) may be regarded as a primary translocation. Firstly, in the UM3 and UM6 MM cell lines, the t(14;20) is the only simple and reciprocal translocation. Secondly, we fortuitously found a patient with a t(14;20) in all malignant plasma cells, but additional different (presumably secondary) translocations in

subpopulations of these MM cells. Actually, after the presentation of our results, this t(14;20) is now regarded as a primary translocation indeed [8].

Maf genes up regulated as a result of a primary translocation deregulate common downstream targets in the pathogenesis of MM.

    For a better understanding of the molecular pathogenesis of MM a number of studies have been performed *in vitro* as well as *in vivo*. Gene expression arrays have improved our insight in important molecular events in MM *in vivo* [9,10]. In these reports *MAFB* and *C-MAF* positive patients clustered together in the MAF group, characterized as well by a high *CCND2* expression. For identifying MAF target genes in vitro, one group tested induced over expression of *C-MAF* in HMCLs [11]. As a result, *CCND2, ITGB7* and *CCR1* were identified as primary target genes of *C-MAF in vitro*. As described in chapter 3, we identified the up regulated genes by *MAFB.* In contrast with the *C-MAF* study we used inducible cell lines stably transfected with an expression vector containing human *MAFB* cDNA. This experimental strategy has the advantage that it allows for highly controlled and reproducible expression profiling. Also, our inducible system provides the identification of genes that are directly up regulated by *MAFB* expression. To validate the *in vitro* data, the expression profiles of the selected transcriptional fingerprint were evaluated in two independent datasets including 111 and 248 primary tumors. This led us to identify 14 up regulated genes.

    Three of this dysregulated target genes of *MAFB* (*CCND2, CCR1* and *ITGB7)* have been reported in the *C-MAF* pathway as well [11]. In addition to that study, we found in the *MAFB* pathway 11 more up regulated genes, which were apparently up regulated in *C-MAF* positive patients as well. Some of these extra genes (i.e. *DEPDC6, DPYSL2, MYO5A, NOTCH2*) [9] or *ARID5A* [10] have been previously reported as up regulated in the whole MAF group *in vivo*. Our study adds *RND3-RhoE, ANG, BLVRA, PSCD1, SLC25A20* and *SNN* to that list. More importantly, both after *MAFB* and *C-MAF* induction, we found over expression of the three Hes genes (*HES1, HES5* and *HES7*). Hes genes are directly regulated by active *NOTCH* [12,13] and our results indicates that over expression of *MAFB* as well as *C-MAF* functionally activates the Notch pathway. Notch is a heterodimeric cell surface receptor and initiation of

signalling requires interaction with one of the several Notch ligands (Delta-like or Jagged) present on an adjacent cell. Following interaction with a ligand, Notch is enzymatically cleaved, releasing a cytoplasmic fragment referred to as intracellular Notch (ICN). ICN then translocates to the nucleus where it binds to a transcription factor resulting in activation of a transcriptional process.

In MM, *NOTCH1, -2* and *-3* are expressed on tumor cells, as well as on bone marrow stromal cells (BMSCs) [14]. Interaction of MM cells with BMSCs that express Notch ligands, activates Notch signalling in MM cells. Also, interaction of MM cells with BMSCs activates Notch signalling in BMSCs leading to secretion of IL-6, VEGF and IGF-1 in BMSCs [15]. Furthermore, *NOTCH2* is involved in proliferation [16] and also the over expression of the Notch ligand JAG2 in MM is of note [15]. Seemingly at variance with these results, another group showed that Notch signalling is a potent inducer of growth arrest and apoptosis in a wide range of B-cell malignancies [17] indicating that more research is required to decipher the role of *NOTCH* in MM. According to our results, we can speculate that a primary event like IgH translocations involving a MAF gene can dysregulate the Notch pathway in the pathogenesis of MM. It is worth to examine whether specific gamma-secretase inhibitors (GSI), which inhibits the Notch pathway, have any effect on Maf MM positive cells. Indeed, a recent study shows that treatment with a GSI alone induced apoptosis of myeloma cells via specific inhibition of Notch signalling [18] indicating an important role for *NOTCH* in MM. Another point of interest may be to screen for activating mutations of *NOTCH* in MM since activating mutations of *NOTCH1* in human T-cell acute lymphoblastic leukaemia (T-ALL) were found [19]. *NOTCH1* was discovered as a partner gene in a t(7;9)(q34;q32) found in < 1% of T-ALLs. In this study, point mutations were found that involve the extracellular heterodimerization domain and/or the C-terminal PEST domain of *NOTCH1*. As a result of these mutations the Notch pathway is constitutively activated. These activating mutations were found in >50% of human T-ALLs and in 14 out of 30 T-ALL cell lines lacking the t(7;9).

All together, we may conclude that the new genes found after up regulation of *MAFB* are target genes of the whole MAF group. Studies on the

expression of these 14 primary target genes in MAF positive MM patients may enable their usage as a target for therapeutic purposes. Indeed, next to the *NOTCH* inhibition study [18] another study showed that suppression of *CCND2* by kinetin riboside induces cell-cycle arrest and tumor cell-selective apoptosis (*in vivo* and *in vitro*) and inhibition of myeloma growth in xenografted mice [20].

Detection of MAFB oncoprotein expression: a prognostic marker in risk stratification of MM.

In chapter 4 of this thesis, we describe a new anti-MAFB monoclonal antibody, which we have used to detect MAFB oncoprotein expression in MM patients by IHC. From the literature, it is known that *MAFB* gene activation is found in 2 % of all myeloma cases as a result of t(14;20). Together with C-MAF and MAFA, Maf expression was found in 10% of all myeloma cases positive for their primary translocations (reviewed in [8]). However, Chang et al reported the frequent expression of C-MAF nuclear oncoprotein (~30%) in Multiple Myeloma (MM), not only in patients positive for the t(14;16)(q32;q23), which results in an up regulation of *C-MAF*, but also in t(14;16) negative patients [21]. Additionally, *C-MAF* mRNA expression was detected in purified myeloma cells from bone marrow aspirates in 50% of the myeloma patients as determined by quantitative reverse transcription PCR [11]. These findings led the authors to suggest that additional mechanisms might play a role for expression of *C-MAF* in MM. Remarkably, however, this upregulation of C-MAF oncoprotein expression did not result in de same poor prognosis as reported for *C-MAF* gene up regulation. In our study we tested 20 MM patients for MAFB expression which had been tested for the t(14;20) and 53 MM patients without knowing their cytogenetic background. In addition we tested a second dataset including 2 MM patients carrying t(14;20) and 4 MM t(14;20) negative patients from another institute. In contrast to the C-MAF studies our results showed that MAFB oncoprotein expression is restricted to patients carrying the t(14;20), and provisional data confirm the poor clinical outcome. This coincidence of gene up regulation and protein expression is seen also in a study that shows a strong correlation between t(4;14), a primary translocation resulting in over expression of *FGFR3* in MM (as detected by interphase FISH) and FGFR3 protein expression by IHC in MM

[22]. Taken together, our results and the restricted FGFR3 expression in MM arouse the question why C-MAF protein expression has been found in such a higher proportion of MM patients. Contrary to the polyclonal anti-C-MAF antibody used by [21], we and [22] have used a monoclonal anti-MAFB or FGFR3 antibody respectively, suggesting that non-specific expression might have been detected in the C-MAF study. Indeed, we have been using a polyclonal anti-MAFB antibody also, but these results were not fully evaluable for us. Furthermore, the high proportion of *C-MAF* mRNA over expression has been questioned. Rasmussen *et al.* found also *C-MAF* mRNA being over expressed in only 4.4% in MM patients [23], coinciding with the reported prevalence of t(14;16) in MM, i.e. ~5% [8]. However, Hurt *et al.* reported *C-MAF* mRNA over expression in 50% of MM bone marrow samples of MM patients [11]. In both studies *C-MAF* over expression was detected by Q-PCR. Combined, these data suggests that the prevalence of C-MAF oncoprotein expression in MM remains a matter of debate.

Furthermore, this anti-MAFB monoclonal antibody may be used as a diagnostic marker in minimal residual disease (MRD). I.e, we were able to detect nuclear MAFB expression in plasma cells in BM sections of a patient defined as MAFB positive after tree times of VAD-treatment. The remaining few plasma cells evidently belonged to the malignant clone.

The detection of aberrant MAFB expression in paraffin ICH is an easy and reliable method, as immunoreactivity of our anti-MAFB monoclonal antibody was even found after two kinds of fixation, concluding that the antibody is specific and may be used routine diagnostic matters. Most importantly, as MAFB protein detection was only found in patients carrying t(14;20) (which have a bad prognosis), our results indicate that MAFB staining may be a prognostic marker in the risk stratification of MM patients. This may enable novel therapies targeting the *MAFB* gene expression pathway. Indeed, for expression of FGFR3 it has been demonstrated in two studies that inhibition of FGFR3 by an inhibitory anti-FGFR3 antibody or a tyrosine kinase inhibitor respectively inhibits proliferation and induces apoptosis in FGFR3-expressing human myeloma cells [24,25].

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Is an Eμ-CD19-MAFB transgenic mouse a good model to follow the effect of MAF genes in B-cell development?

In the last study of this thesis we describe the results of the effect of transgenic *MAFB* in B-cell development in the mouse. To date, this is the first transgenic mouse generated to study MM *in vivo* involving one of the seven up regulated oncogenes as a result of primary translocations in MM. Recently, several transgenic mouse models that generated PC tumors were proposed: these include a mixed inbred of mice that co-expressed *C-MYC* and *BCL-X$_L$* driven by Ig enhancers [26] and insertion of *C-MYC* into the IgH locus, just 5' of the intronic Eμ enhancer [27]. In both models *MYC* was constitutively activated. Most of the tumors that were formed in these studies were of short latency and aggressive, e.g. extramedullary plasmacytoma.

Very recently, two mouse models have been generated that appear to have a real MM phenotype [28,29]. In one model [28], the spliced isoform of the B-cell transcription factor *XBP-1* was over expressed under the control of immunoglobulin V$_H$ promotor and the Eμ enhancer. These mice generated tumors that have the properties expected for MGUS and MM. The other MM mouse model [29], was an Activation-Induced Deaminase (AID) dependent *MYC* transgenic conditional mouse. Most transgenic mice developed age dependent PC tumors that mainly produce IgG. There was a high frequency of Ig mutations and tumors were minimally proliferative and localized in the BM.

We generated an *Eμ-CD19-MAFB* transgenic mouse whereby *MAFB* cDNA was cloned downstream of de human CD19 promoter and a duplicate of the mouse Eμ enhancer. Both elements reportedly provide expression throughout the whole B-cell lineage [30-35] and especially the CD19 provides higher expression at the end of the B-cell lineage [33]. The cDNA of *MAFB* (containing a right Kozac sequence) was used in the gene expression array study as well as to determine the direct target genes of *MAFB* (chapter 3). This all suggest that the design of this transgenic MAFB construct will provide high MAFB expression in mature B-cells. Indeed, we detected high *MAFB* expression in RNA obtained from two lymphoid tissues, spleen and BM. We used our specific monoclonal *MAFB* antibody to detect expression by IHC in

cryosections of spleen tissue. MAFB protein expression was observed in a proportion of cells in one of the founder lines suggesting MAFB protein expression. This expression, however, was low, may be due to inherent limitations of the IHC. Also using other methods like Western Blot analysis and Immunoprecipitation, MAFB protein could not be demonstrated unequivocally.

After analysing the serum levels, a significant increased level of total IgM and was observed in Tg mice compared to wild type mice. Furthermore, by analysing the immune response, high levels of IgM specific anti TNP antibodies were observed in 2826 transgenic founder line before immunization. We suggest that the increased IgM antibodies found in *Eμ-CD19-MAFB* transgenic mice might be multi-specific natural antibodies. The role of natural antibodies in the pathogenesis of MM is not clear as yet. However, it is reported that some monoclonal Igs from MM patients exhibit similar specificities to that found with natural antibodies [36]. Furthermore, the role of natural antibodies in the pathogenesis of CLL has been reported [37] whereby polyreactive antibodies are expressed by CLL B-cells. Still, we cannot give a conceivable explanation whether and how *MAFB* over expression has influenced the expression of natural antibodies and thereby it remains unclear what has influenced the high (specific) IgM levels.

Besides the increased IgM levels, our results showed that *Eμ-CD19-MAFB* transgene expression had no effect on B-, T- and myeloid cell development in mice with the exception of three monotypic Tg+ mice. These three mice showed an extra population of CD19$^-$IgM$^+$, 100% kappa$^+$ cells detected in blood analysis. After further analysing, two of these mice showed different aberrancies in B-cell development. Due the minimal amount of Tg+ mice detected and the differences in B-cell development found, we cannot conclude that these results are consequences of transgenic *MAFB* expression. Also, no effect of transgenic *MAFB* expression was observed in monoclonal PC development. We detected monoclonal serum Ig components in several mice indicating MGUS development but it is not sure whether this is an effect of transgenic expression of *MAFB* or the occurrence of spontaneous age-dependent serum M-protein in C57BL/6 mice, which strain is included in

the background of our transgenic mice. It is known that C57BL/6 mice develop age dependent MGUS [38,39]: they have a 50% occurrence of serum M-protein after 2 years of age (IgG>IgM) [38]. In our study, the level of M-protein was stable and was found even in some wild-type mice.

As mentioned before, several mouse models were generated to study MM development *in vivo*. In most of these transgenic mouse models, *C-MYC* is used to induce B-cell tumors. In mice, *C-MYC* can be up regulated as a result of the translocation T(12;15)(F2;C), where *C-MYC* is translocated to the IgH locus on chromosome 12 under the control of one of the Ig enhancers. The human homologe of T(12;15) is t(8;14) causing endemic Burkitt lymphoma in humans, whereas in contrast mice harbouring the T(12;15) do develop plasmacytomas instead. Both malignancies were detected in one mouse model where *C-MYC* was inserted in the IgH locus upstream of the Eμ enhancer mimicking T(12;15) [27]. In another study, *C-MYC* was also inserted in the IgH locus, where *C-MYC* was inserted upstream the Eα enhancer [26]. Remarkably, this model showed no plasmacytomagenesis by *C-MYC* expression alone. Only after crossing with BCL-X$_L$ transgenic mice, the offspring developed plasmacytomas. Probably, this may be due to the *MYC*-dependent apoptosis of tumor precursor cells. Normally, MYC promotes cell growth and proliferation in the presence of growth factors [40,41]. Deregulated *MYC*, in the absence of growth factors, can force latent cells into active cell cycle [42,43] and that trigger apoptosis. *MYC*-induced apoptosis is a safeguard mechanism for eliminating aberrant cells with active *MYC* [44,45].

Why *C-MYC* transgene over expression alone had an effect on B-cell development in the first study [27] may be due to the insertion of *C-MYC* head-to-head juxtaposed to Eμ enhancer. *C-MYC* can now interact with Eα and Eδ in addition to Eμ. The crucial importance of MYC- Eα interaction for *MYC* deregulation in plasma cell tumors has been clearly shown [46]. In this study it is reported that four tissue-specific and cell stage-specific DNase I hypersensitive sites (HS1234) at the 3' of the IgH Cα gene, may function as a locus control region (LCR), deregulating *C-MYC* expression in T(12;15) plasmacytomas.

In human MM, *MAFB* is translocated up stream of the strong Eα enhancer resulting in *MAFB* over expression. Therefore, *MAFB* insertion juxtaposed to the Eμ enhancer in the IgH locus as described for *C-MYC* in [27] may be worth to consider. The usage of an Eα enhancer instead for *MAFB* transgenic expression may be considered as well.

C-MYC over expression was also used in the AID-dependent *MYC* transgenic conditional mouse [29]. This is the only model so far with a clear MM phenotype although it is suggested in this study that *C-MYC* is not responsible for direct MM development but that *C-MYC* dysregulation is a second event that drives one of the nascent MGUS tumors in C57BL/6 mice towards MM. Taken altogether, results of *C-MYC* studies in mice indicate that *C-MYC* is an important secondary event rather than a primary event since only aggressive tumors were found in both studies where *C-MYC* was inserted in the IgH locus. In humans, extramedullary MM is found after a secondary event like *C-MYC* translocations.

A transgenic mouse model using the oncogenes, which were up regulated as caused by primary translocations in MM is not studied to date. Several other studies in e.g. sarcoma have shown that over expression of up regulated genes as a result of specific translocations can result in associated phenotype in mice [47,48]. For example, synovial sarcoma is marked by a signature genetic event, t(X;18)(p11;q11). As a result of this translocation the SYT-SSX fusion protein is generated as the *SYT* gene on chromosome 18q11 is translocated and fused to either *SSX1*, *SSX2* or, very rarely, the *SSX4* gene on chromosome Xp11. A mouse model generated that conditionally express human SYT-SSX2 fusion protein resulted in synovial sarcoma-like tumors in mice expressing SYT-SSX2 [47].

Also, it is shown that transgenic expression of one of the MM related oncogenes (*C-MAF*) under the control of CD2 promoter results in a T-cell lymphoma phenotype [11]. Normally, *C-MAF* is expressed in Th2 cells and activates the expression of IL-4 and IL-10 in T-cells [49]. Furthermore, transgenic expression of XBP-1 result is an MM-like phenotype, whereas XBP-1 in the lymphoid system physiologically is responsible for plasma cell development. Enforced transgene expression of factors driving plasma cell

differentiation would enhance the development of a MM-like disease. These studies show that transgenic mouse models have been successfully generated involving genes, which have a role in (malignant) haematopoiesis.

In addition to the analysis of the haematological compartment we also analysed the changes in gene expression in our *Eμ-CD19-MAFB* transgenic mice compared to wild-type mice by two independent gene expression arrays. After evaluation of the two gene expression patterns obtained, no common distinct up- and/or down regulated genes were found besides *MAFB* expression in transgenic mice confirmed by Northern Blot Analysis. As a result we were not able to describe a set of genes or transcription program that is dysregulated as a result of *MAFB* over expression. Furthermore, *MAFB* or none of the determined target genes of *MAFB* in the human situation (chapter 3) were detected in both gene expression patterns. An explanation for the lack of *MAFB* detection in the expression pattern may be due to the fact that only one MAFB oligo was spotted on the array slide and that specifically that sequence was not present in our transgenic construct. Combined, these data may suggests that transgenic *MAFB* expression alone has very little and/or late effect on the haematological compartment. Alternatively, it might be conceivable that constitutively up regulation of *MAFB* in transgenic mice has resulted in deregulation of other unknown pathways suppressing deregulation of known *MAFB* target genes. Overall, the *Eμ-CD19-MAFB* transgenic mice did not result in a MM phenotype as yet, despite of abundant *MAFB* expression in lymphoid tissues. However, transgenic *MAFB* expression had some effect on B-cell development by showing increased levels of IgM levels. Nevertheless, we suggest reconsidering the design of a MAFB mouse model to directly delineate the involvement of *MAFB* in the development of B-cells and MM. Designing a knock-in MAFB mouse model whereby MAFB is inserted juxtaposed to the Eμ enhancer like the C-MYC knock-in model [27] is a good option.

Concluding remarks.

Taken together, this study has yielded novel insights in the role of *MAFB* in the development of MM. Despite of its low frequency of expression in MM patients, up regulation of *MAFB* is a primary and maybe initiating event in the pathogenesis of MM. Furthermore; we identified 11 new primary target genes that are deregulated by *MAFB* and by other members of the large Maf group in MM. As *MAFB* gene up regulation can be a prognostic marker in the risk stratification in MM patients, it would be interesting to test for *MAFB* expression or expression of deregulated *MAFB* target genes in routine diagnostics.

Together with the results of other groups, our results showed that cytogenetic changes is the major factor in MM, and this genetic evaluation has to be incorporated into clinical practice to give insight in prognosis and guide treatment. Indeed, a recent study describes 70 genes, which were linked to early disease-related death (70-gene model) [50]. Interestingly, 30% of these genes map to chromosome 1. From this 70-gene model, a 17-gene signature was derived which predict the clinical outcome as effective as the 70-gene model. This 17-gene signature was validated in two additional datasets of newly diagnosed and relapse patients. In both settings it was associated with poorer survival. Furthermore, it was found that patients having t(4;14) in addition to the high-risk 17-gene signature had an even shorter survival, indicating once more the important role of primary translocations in MM. This might suggests that both criteria act independently in the pathogenesis of MM, meaning that up regulated genes caused by a primary translocation differ from genes of the 17-gene signature – which is actually the case.

Summarizing, this all indicates an important role of genomics in MM, which can help to understand better the pathogenesis of MM. Next to new developments in genomics, standard procedures like conventional karyotyping and FISH (17p13 deletion) should be performed as well. Indeed with conventional karyotyping it is easily to detect deletion of chromosome 13 and aneuploidy (HRD and NHRD) and pre-identify high-risk patients.

We have shown in this study that up regulation of MAFB and as well as other genes by primary translocations in MM is an important event in the pathogenesis of MM. We suggest that MM patients should be screened for genes expressed by primary translocations for a better stratification of MM patients e.g. in a Dutch HOVON-trial. Real-time PCR gene expression assays rather than expensive gene arrays may be a useful tool. Such a proposed Q-PCR set (12 genes, see table 1) should be as concise and as informative as possible.

Table 1: real-time PCR gene set.

Translocated genes	Target genes from translocated genes	17-gene signature
MAFB	Cyclin D2	CKS1B
C-MAF	ITGB7	
MAFA	NOTCH2	
Cyclin D1	CCR1	
Cyclin D3		
FGFR3		
MMSET		

In this assay the seven oncogenes up regulated as a result of a primary translocation together with Cyclin D genes should be included. Some other well defined target genes like *ITGB7*, *NOTCH2* and *CCR1* should be included as well. The genes proposed here all pertain the Maf group; secondary genes of other translocated genes are scarcely known as yet. Screening for expression of *CKS1B* (located at chromosome 1q21) is interesting as well as this gene is one of the 17-gene signature-set, and mentioned earlier as an important gene in the pathogenesis of MM [51]. Indeed, it is shown that gain of sequences and corresponding increased gene expression at 1q21 occurs in 30-40% of MM tumors and is correlated with a poor prognosis [52,53].

Initially, these data may add to the risk stratification and should guide the choice of existent therapies – as different as they are currently. Ultimately guided by these data, MM patients may be treated with specific inhibitors of the specific deregulated pathways. Evidently, more (unique) pathways deregulated in high-risk patients have to be delineated in order to identify such novel therapeutic targets.

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Nederlandse samenvatting

(voor niet ingewijden)

Nederlandse samenvatting

In dit proefschrift wordt het onderzoek beschreven naar een specifieke chromosomale afwijkingen welke de oorzaak kan zijn van Multiple Myeloma (MM). MM of ook wel de 'ziekte van Kahler' is een kwaadaardige hematologische tumor, veroorzaakt door een ongeremde groei en deling van plasmacellen. Plasmacellen ontstaan uit B-lymfocyten, welke een onderdeel zijn van witte bloedcellen. Deze plasmacellen produceren de verschillende soorten antilichamen ten behoeve van ons afweer. Iedere plasmacel kan slechts één type antilichaam aanmaken. Als er ongeremde groei van één type plasmacel plaatsvindt wordt er een abnormale hoeveelheid van hetzelfde antilichaam geproduceerd, ook wel paraproteïne of M (monoclonaal) proteïne genoemd. De aanwezigheid van een M proteïne in een patiënt is een teken van abnormale toename van plasmacellen of de voorlopers hiervan. M proteïnes zijn niet specifiek voor MM, ze kunnen ook (dan meestal in lager hoeveelheden) aanwezig zijn bij andere (B cel) maligniteiten of zelfs zonder een (B cel) maligniteit.

B- en T lymfocyten worden in het beenmerg aangemaakt dat zich in het bot bevind. De woekering van plasmacellen verstoort de aanmaak van gezonde beenmerg cellen. Door bijvoorbeeld het zo ontstane tekort aan rode bloedlichaampjes of ander witte bloedcellen vergroot het de kans op bloedarmoede (moeheid) en infecties van MM patiënten. Ernstige vormen van MM kunnen leiden tot botpijn en botbreuken. Andere symptomen zijn ondermeer gewichtsverlies en neurologische afwijkingen. De ziekte komt vooral bij ouderen voor, zelden bij mensen onder de 40 jaar. MM is tot nu toe moeilijk te genezen en het verloop van de ziekte kan per patiënt zeer sterk verschillen.

Verwant aan MM is de ziekte MGUS (Monoclonal Gammopathy of Undetermined Significance). Dit betekent dat er M-proteïnen aanwezig zijn (monoclonal gammopathy), zonder dat er sprake is van woekering van kwaadaardige plasmacellen (undetermined significance). Deze ziekte leidt niet tot een kwaadaardige aandoening. Bij ongeveer 20% van de patiënten met MGUS kan zich op den duur MM ontwikkelen.

MM kan in verschillende gradaties voorkomen, van een milde tot een zeer ernstige vorm. In de milde vorm, ook wel smoldering MM genoemd, zijn er langzaam groeiende kwaadaardige plasmacellen aanwezig in het beenmerg maar de patiënten vertonen geen symptomen. Smoldering MM kan over gaan naar volledig ontwikkeld MM en uiteindelijk naar een zeer ernstige vorm van MM, ook wel extramedullary MM of plasmacytoma genoemd. In deze zeer ernstige vorm groeien de kwaadaardige plasmacellen sneller waardoor het beenmerg flink wordt aangetast. Tevens verplaatsen de tumorcellen zich ook naar andere bloedorganen zoals de milt en lymfeklieren. De gemiddelde overleving van MM patiënten is ongeveer 10 jaar met een grote spreiding, afhankelijk van de vorm van de ziekte. Een goed onderscheid tussen deze verschillende vormen MM op het ogenblik van diagnose (risico stratificatie) is dus van groot belang voor de keuze van de juiste therapie bij de juiste patiënt. Dit proefschrift draagt hieraan bij.

Chromosomale afwijkingen kunnen ten grondslag liggen aan een ongeremde groei van één type plasmacel. Een voorbeeld van een chromosomale afwijking in MM zijn translocaties, dwz de uitwisseling van erfelijk materiaal tussen verschillende chromosomen. Deze translocaties kunnen plaatsvinden tijdens de ontwikkeling van de plasmacel. Door een translocatie worden genen geactiveerd die in een normale situatie niet tot expressie komen in de ontwikkeling van een plasmacel. Tot de aanvang van onze studie waren er 4 translocaties bekend in MM: t(4;14) t(6;14), t(11;14) en t(14;16). Bij al deze translocaties is chromosoom 14 betrokken. Op chromosoom 14 ligt het 'Immunoglobulin Heavy Chain (IgH)' gen. Dit gen bepaalt welk antilichaam in een plasmacel wordt gevormd. In hoofdstuk 2 van dit proefschrift wordt een nieuwe translocatie bij MM beschreven namelijk t(14;20). We hebben deze specifieke translocatie uit kwaadaardige plasma cellen geïsoleerd en ontdekten dat een gen van chromosoom 20, te weten het gen *MAFB*, als gevolg van deze translocatie foutief onder invloed kwam van een regelend gen op chromosoom 14. Als gevolg hiervan wordt *MAFB* overactief (overexpressie). MM patiënten met deze specifieke translocatie hebben een zeer slechte prognose en overlijden gemiddeld binnen een jaar.

In Hoofdstuk 3 hebben wij ons de de vraag gesteld of overexpressie van *MAFB* een functie heeft in de ontwikkeling van MM. Om de rol van *MAFB* te bestuderen in MM is dit specifieke *MAFB* gen induceerbaar tot expressie gebracht in t(14;20) negatieve plasma cellijnen. Hierna hebben we gekeken welke genen al vroeg door dit abnormaal gereguleerde *MAFB* gen worden geactiveerd in deze cellijnen. Deze resultaten zijn vergeleken met gen expressie resultaten die zijn uitgevoerd op 400 MM patiënten. Door deze vergelijking hebben we uiteindelijk 14 specifieke targetgenen van *MAFB* gevonden in zowel plasmacellijnen (*in vitro*) als in MM patiënten (*in vivo*) . Deze 14 genen zijn de specifieke *MAFB* target genen. Tevens hebben wij aangetoond dat de *MAFB* target genen ook abnormaal worden gereguleerd bij patiënten met een andere translocatie: t(14;16). Als gevolg van deze translocatie komt het *C-MAF* gen tot overexpressie in plasmacellen. *C-MAF* en *MAFB* zijn afkomstig van dezelfde genfamilie en *C-MAF* patiënten hebben net zoals *MAFB* patiënten een zeer slechte prognose. De zo geïdentificeerde genen zijn dus de primaire target genen van de gehele MAF genen familie en deze genen lijken dus verantwoordelijk voor het slechte ziekte verloop van deze subgroep van MM.

Het is bekend dat de translocaties t(14;20)/*MAFB* en t(14;16)/*C-MAF* samen in ongeveer 10% van het totale aantal MM patiënten voorkomen. Dit is aangetoond met behulp van cytogenetisch onderzoek waarbij gebruik werd gemaakt van FISH (Fluorescence In Situ Hybridization), waarmee de getransloceerde *genen* (genen worden bij afspraak cursief geschreven) kunnen worden aangetoond in de kwaadaardige plasmacellen.

Onlangs had een andere wetenschappelijke studie aangetoond dat het eiwit (een eiwit (bij afspraak niet-cursief geschreven) wordt opgebouwd uit de genetische informatie die op een gen ligt) C-MAF ook tot expressie kan komen in MM patiënten, niet alleen in t(14;16) positieve patiënten, maar ook zonder de aanwezigheid van de translocatie t(14;16), en wel in ongeveer 30%. Dit vonden zij na kleuring van beenmergbiopten van MM patiënten met een specifiek antilichaam tegen het eiwit van C-MAF. Vreemd genoeg hadden deze "C-MAF positieve patiënten" niet allemaal de slechte prognose van de patiënten die *C-MAF* positief zijn vanwege de t(14;16)

Om MAFB eiwit expressie aan te tonen in beenmergbiopten hebben we een monoklonaal antilichaam tegen MAFB gemaakt. Dit wordt beschreven in hoofdstuk 4. Dit antilichaam kleurt plasmacellen van t(14;20) positieve patiënten aan . Plasmacellen van patiënten zonder deze translocatie kleuren niet aan. De gevoeligheid van deze kleuring is zo hoog dat slechts een gering aantal MAFB positieve tumorcellen goed aantoonbaar is. MAFB eiwit expressie is dus gecorreleerd aan de t(14;20) en een slechte prognose tot gevolg heeft. Met het gebruik dit antilichaam kunnen de 'slechte' patiënten veel sneller geïdentificeerd worden dan wanneer gebruik wordt gemaakt van de klassieke methode (aantoning van translocaties), en is dus goed te gebruiken in de diagnostiek.

In hoofdstuk 5 hebben we het effect van van *MAFB* in MM *in vivo* bestudeerd. Hiervoor hebben we een transgene (genetische veranderde) muis gemaakt waarbij *MAFB* afkomstig van de mens tot overexpressie werd gebracht in de B-cellen van de muis en dan voornamelijk in het late stadium van de B-cel onwikkeling (de voorlopers van de plasma cellen). Door de muizen te immuniseren met verschillende stoffen hebben we de B-cel ontwikkeling extra gestimuleerd wat zou kunnen leiden tot een versnelde vorming van MM. Vervolgens hebben we een verhoogde *MAFB* expressie aan kunnen tonen in cellen van de milt en in het beenmerg van *MAFB* transgene muizen. Dit is vergeleken *MAFB* expressie in normale muizen. In verloop van tijd vertoonde de *MAFB* transgene muizen verhoogde productie een bepaald immuunglobuline, namelijk IgM. De transgene muizen zijn echter niet ziek geworden en de verhoogde productie van IgM hebben ook niet geleid tot een MM fenotype of een andere hematologische aandoening in de tijd die wij hebben gebruikt voor deze studie. Tijd kan een beperkende factor zijn geweest, waardoor andere (meer langdurige) wetenschappelijke studies naar de ontwikkeling van MM *in vivo* wel resultaat hebben laten zien.

Hoofdstuk 6 van dit proefschrift is de discussie. Hierin wordt beschreven hoe door onze studie er meer duidelijkheid gekomen over de belangrijke rol van chromosomale translocaties in MM en de rol van MAFB hierbij. We hebben nieuwe target genen van MAFB aangetoond welke een rol kunnen hebben in de overleving van kwaadaardige plasmacellen. Tevens hebben we aangetoond dat MAFB overexpressie gecorreleerd is met een zeer slechte prognose. In de toekomst zou het voor belang kunnen zijn om een grote groep MM patiënten te screenen op MAFB overexpressie even als overexpressie van andere genen als gevolg van een chromosomale translocatie. MM patiënten kunnen op basis van deze resultaten geclassificeerd worden aan de hand van hun prognose en behandelmethoden kunnen hierop aangepast worden.

Dankwoord

Curriculum Vitae

List of publications

Abbreviations

Dankwoord

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Doeg Esther

Curriculum Vitae

Esther van Stralen is geboren op 29 mei 1974 te Hoorn, Nederland. Na de middelbare school heeft zij een studie biotechnologie (HLO) gevolgd aan de Hoge School van Amsterdam met als afstudeerproject: "Detectie van subtelomerische afwijkingen bij autistische kinderen met behulp van Fluorescentie In Situ Hybridization (FISH)". Deze studie is in 1997 met succes afgerond. Na deze opleiding is de schrijfster in diverse functies werkzaam geweest op verschillende cytogenetische laboratoria door het hele land. In 2003 is ze gestart met haar promotieonderzoek bij de afdeling immunologie, UMC Utrecht onder leiding van Prof. Dr. Hans C. Clevers en Dr. Bert J.E.G. Bast. Alle praktische werkzaamheden van het promotieonderzoek zijn verricht op het Hubrecht laboratorium in de groep van Hans Clevers. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

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Abbreviations

MM	Multiple Myeloma
BM	Bone marrow
MGUS	monoclonal gammopathy of undetermined significance
Ig	immunoglobulin
SMM	Smoldering Multiple Myeloma
HRD	hyperdiploid
NHRD	non-hyperdiploid
GC	Germinal center
BCR	B-cell receptor
SHM	Somatic hypermutation
CSR	Class switch recombination
FISH	Fluorescence in situ hybridization
PCL	Plasma cell leukemia
CML	chronic myelogenous leukemia
ALL	acute lymphoblastic lymphoma
MALT	mucosa-associated lymphatic tissue
DLCL	diffuse large cell lymphoma
HMCL	human MM cell lines
CGH	comparative genomic hybridization
GEP	gene expression profiling
TC	translocation
MARE	Maf recognition elements
AILT	angioimmunoblastic T-cell lymphoma
EST	expressed sequence tag
DC	Double colour
AID	Activation-Induced Deaminase
TetR	Tetracycline repressor
HA	Human influenza hemagglutinin
IHC	immunohistochemistry
TD	T-cell dependent
TI	T-cell independent
SPEP	Serum Protein Electrophoresis
ICN	intracellular Notch
GSI	gamma-secretase inhibitor
LCR	locus control region