BCL6 – regulated by AhR/ARNT and wild-type *MEF2B* – drives expression of germinal center markers *MYBL1* and *LMO2*

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ABSTRACT

Genetic heterogeneity is widespread in tumors, but poorly documented in cell lines. According to immunoglobulin hypermutation analysis, the diffuse large B-cell lymphoma cell line U-2932 comprises two subpopulations faithfully representing original tumor subclones. We set out to identify molecular causes underlying subclone-specific expression affecting 221 genes including surface markers and the germinal center oncogenes *BCL6* and *MYC*. Genomic copy number variations explained 58/221 genes differentially expressed in the two U-2932 clones. Subclone-specific expression of the *aryl-hydrocarbon receptor* (*AhR*) and the resulting activity of the AhR/ARNT complex underlaid differential regulation of 11 genes including *MEF2B*. Knock-down and inhibitor experiments confirmed that *AhR/ARNT* regulates *MEF2B*, a key transcription factor for *BCL6*. *AhR*, *MEF2B* and *BCL6* levels correlated not only in the U-2932 subclones but in the majority of 23 cell lines tested, indicting overexpression of *AhR* as a novel mechanism behind BCL6 diffuse large B-cell lymphoma. Enforced modulation of *BCL6* affected 48/221 signature genes. Although *BCL6* is known as a transcriptional repressor, 28 genes were up-regulated, including *LMO2* and *MYBL1* which, like *BCL6*, signify germinal center diffuse large B-cell lymphoma. Supporting the notion that *BCL6* can induce gene expression, *BCL6* and the majority of potential targets were co-regulated in a series of B-cell lines. In conclusion, genomic copy number aberrations, activation of AhR/ARNT, and overexpression of *BCL6* are collectively responsible for differential expression of more than 100 genes in subclones of the U-2932 cell line. It is particularly interesting that *BCL6* – regulated by AhR/ARNT and wild-type *MEF2B* – may drive expression of germinal center markers in diffuse large B-cell lymphoma.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is an aggressive non-Hodgkin lymphoma. Expression array analyses have identified two molecularly distinct forms of the tumor, termed germinal center (GC) and activated B-cell (ABC) forms.[1] Primary mediastinal B-cell lymphoma is a third variant of this tumor marked by its characteristic gene expression signature.[2] Diverse molecular and cytogenetic alterations characterize the three subtypes of DLBCL.[3] Translocations affecting *BCL2* typically occur in the GC subtype while somatic mutations involving the NFκB pathway are more often found in ABC DLBCL.[3,4]

Cancers evolve under selective forces, including host immunity and drug inhibition, fuelled by mutational alterations explaining the clonal heterogeneity found in tumors including DLBCL.[5,6] Clonal evolution may also be the reason for the development of molecularly related tumors in one patient. Thus, up to 30% of nodular lymphocyte-predominant Hodgkin lymphomas transform into DLBCL.[7] Furthermore, clonal relationships have been reported for single patients with classical Hodgkin lymphoma and non-Hodgkin lymphoma.[8,9]

The U-2932 cell line was derived from a DLBCL patient with a history of Hodgkin lymphoma.[10] According to the results of gene expression analysis, U-2932 meets the criteria for an ABC DLBCL cell line.[11] We recently showed that U-2932 comprises two subclones (R1 and R2) derived from a presumptive mother clone with genomic *BCL2* amplification which acquired distinct sets of secondary rearrangements leading to the alternative overexpression of *BCL6* or *MYC* in the respective daughter clones.[12] Immunoglobulin gene hypermutation analysis showed that R1 and R2 represent subclones of the original tumor.[12] Thus, the two U-2932 subclones seemed to be ideal to study the cellular consequences of clonal evolution. More than 200 genes showed >10-fold expression differences between R1 and R2.[12] Hence, the two U-2932 subclones sensitively model the cellular consequences of clonal evolution *in vitro*. Here, we set out to identify molecular causes for subclone-specific gene expression, a necessary first step in addressing the serious therapeutic challenges posed by tumor heterogeneity.

Methods

Human cell lines and treatments

All cell lines are held by the DSMZ cell line bank (*http://www.dsmz.de*) and were cultured as described previously.[13]

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Knock-down experiments

Transfection with short interfering (si) RNA oligonucleotides was performed as described elsewhere.[14] Gene-specific siRNA oligonucleotides and AllStars negative control siRNA were obtained from Qiagen (Hilden, Germany). The siRNA (80 pmol) were transfected into $1x10^6$ cells by electroporation using an EPI-2500 impulse generator (Fischer, Heidelberg, Germany) at 350 V for 10 ms. Treated cells were harvested after 20 h.

Cell surface marker analysis and cell sorting

Cells were immunophenotyped and sorted on a FACSAriaIII (Becton Dickinson; Heidelberg, Germany). PerCP-Cy5.5-conjugated CD20 and APC-conjugated CD38 antibodies were purchased from Becton Dickinson, and APC-conjugated CD22, CD27 and CD59 antibodies were from Life Technologies (Darmstadt, Germany). The APC-conjugated CD24 antibody was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). U-2932 subclones R1 ($CD20^{++}/CD38^{++}$) and R2 ($CD20^{variable}/CD38^{variable}$) were phenotypically stable for more than 100 days in culture. During the period of this study, resorting was not necessary.

Cytogenetic analysis

CytoScan HD Array (Affymetrix; Santa Clara, CA, USA) hybridization analysis was performed to identify numerical aberrations. DNA was prepared using the Qiagen Gentra Puregene Kit (Qiagen; Hilden, Germany). Data were analyzed using the Chromosome Analysis Suite software version 2.0.1.2 (Affymetrix). Fluorescence *in situ* hybridization (FISH) was performed using bacterial artificial chromosome (BAC) clones (BACPAC Resources; Oakland, CA, USA) to analyze gene rearrangements at *BCL6* using protocols described previously.[15,16]

DNA microarray hybridization and quantitative genomic polymerase chain reaction analysis

Total RNA (500 ng) was used for biotin labeling according to the 3' IVT Express Kit (Affymetrix). The biotinylated cRNA (7.5 μg) was fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre). Samples were hybridized to an identical lot of Affymetrix GeneChip HG-U133 Plus 2.0 for 16 h at 45°C. Steps for washing and SA-PE staining were processed on the fluidics station 450 using the recommended FS450 protocol (Affymetrix). Images were analyzed on a GCS3000 Scanner with the GCOS1.2 Software Suite (Affymetrix). Data were analyzed using GeneSpring 11.5.1 (Agilent Technologies; Santa Clara, CA, USA). Signal intensities (raw data) were $\log_2$-transformed and normalized using RMA.

Quantitative genomic polymerase chain reaction was performed on a 7500 Applied Biosystems real-time polymerase chain reaction system using the SYBR green assay (Applied Biosystems; Darmstadt, Germany) with *ABL1* as the internal control and the diploid cell line NC-NC as a reference. Primers are shown in *Online Supplementary Table S1*.

Western blot analysis

Samples were prepared as described by Quentmeier *et al.*[17] We used the nuclear extract kit (Active Motif; La Hulpe, Belgium) to separate cytoplasmic and nuclear proteins. Bands on nitrocellulose membranes were visualized with the biotin/streptavidin-horseradish peroxidase system (GE Healthcare; Little Chalfont, UK) in combination with Renaissance Western Blot Chemoluminescence Reagent (Perkin Elmer; Waltham, MA, USA).

Statistical analysis

Data were organized using commonly employed spreadsheet programs. Statistical tests were performed using the R software, as described in *Online Supplementary Table S2*.[18] For analysis of transcription factor binding sites, we made use of a commercial bioinformatics service (Biobase; Wolfenbüttel, Germany).

Results

Numerical aberrations causing differential gene expression in subclones of the diffuse large B-cell lymphoma cell line U-2932

The DLBCL cell line U-2932 comprises two populations distinguishable by their expression of various B-cell markers including CD20 and CD38. According to the results of immunoglobulin hypermutation and cytogenetic analyses, the populations – named R1 and R2 - represent two subclones of the original tumor.[12] Here, we set out to determine why more than 200 genes showed gross (>10-fold) expression differences between R1 and R2 (*Online Supplementary Table S3*).

We applied a whole genome array to investigate the extent to which subclone-specific gene expression might be attributable to numerical aberrations. Twenty-six percent (58/221) of the differentially expressed genes showed concordant numerical disparities between R1 and R2 (*Online Supplementary Table S3*). Statistical analysis of copy number aberrations and expression data revealed that numerical differences between the two subclones effectively predict differences in gene expression (sensitivity 0.64; specificity 0.94; accuracy 0.78). A McNemar chi-squared test with continuity correction rejected non-correlation of the two parameters with a *P*-value of 0.0036 (*P* values <0.05 are considered statistically significant). In fact, for several genes, the correlation between ploidy status and expression level was so stringent that a causal relation could be directly inferred: *CHMP2B* and *CGGBP1* on chr 3(p11.1-2) were highly amplified in R1 (13 n) and hemizygously lost in R2 (1 n) with corresponding differences in gene expression (Figure 1A,B, *Online Supplementary Figure S1*). Loss of expression in population R2 as a result of homozygous deletion was found for *ITM2B* and *RB1* on chr 13(q14.2) (Figure 1A,B, *Online Supplementary Figure S1*).

Thus, for a sizable minority of genes (≤26%) numerical aberrations provided a potential explanation, in some cases compelling, for the differences in gene expression between R1 and R2, leaving the question of what caused expression discrepancies for the remaining 163 genes unaffected by concordant copy number aberrations.

AhR/ARNT activity driving gene expression in U-2932 subclone R1

Constitutive activation of signaling chains can trigger aberrant expression of anti-apoptotic and proliferative genes, thereby promoting lymphomagenesis. Notable in this context was that the canonical AhR/ARNT target *CYP1A1* was high in R1 and low in R2 (Figure 2A). *AhR* levels paralleled expression of *CYP1A1*, suggesting that R1-high genes might be controlled by *AhR* expression (Figure 2A).

It is widely believed that ligand-activated AhR translocates to the nucleus where it binds to ARNT. *AhR/ARNT* regulation in B cells may operate differently. Normal, resting B cells express little *AhR*. Upon stimulation (e.g. with CD40 ligand) *AhR* is up-regulated and translocates to the nucleus in the absence of exogenous *AhR* ligand, explain-

ing how B-cell lymphoma cell lines show constitutive nuclear localization of the AhR/ARNT complex.[19-21] In accordance with this observation, AhR localized to the nucleus of U-2932 cells, independently of ligand stimulation in both subclones (*Online Supplementary Figure S2*). Thus, the major difference between subclones R1 and R2 concerned AhR expression level rather than subcellular localization (Figure 2A,B).

We, therefore, hypothesized that *AhR* regulation might underlie differential gene expression in the two U-2932 subclones. To find out how many of the R1-specific genes (126/221 genes with >10-fold expression difference were high in R1) were affected by AhR/ARNT activity, we treated U-2932 R1 cells with the AhR/ARNT inhibitor GNF351 (1 μM, 24 h). GNF351 treatment effected relocalization of AhR from the nucleus to the cytoplasm and led to down-regulation of the AhR/ARNT target *CYP1A1* (Figure 3A,B). Eleven of the 126 R1-high genes (9%) were inhibited by the AhR/ARNT inhibitor, confirming that the difference in AhR levels contributed to non-genomic divergences in gene expression between the two subclones (partially shown in Figure 3B).

AhR has been described as an epigenetically regulated gene.[22,23] To test whether *AhR* expression differences in DLBCL cell lines were subject to epigenetic regulation we treated *AhR*-positive (SU-DHL6, U-2932 R1) and *AhR*–negative (OCI-LY19, U-2932 R2) cell lines with inhibitors of histone deacetylation and DNA methylation. Both types of inhibitor significantly induced *AhR* expression in the negative cell lines, suggesting that differences at the epigenetic level were responsible for the expression of *AhR* in DLBCL cell lines (Table 1). This view was supported by the finding that the *AhR* promoter of the cell line most sensitive to inhibition of DNA methylation (OCI-LY19) was highly methylated (*Online Supplementary Figure S3*).

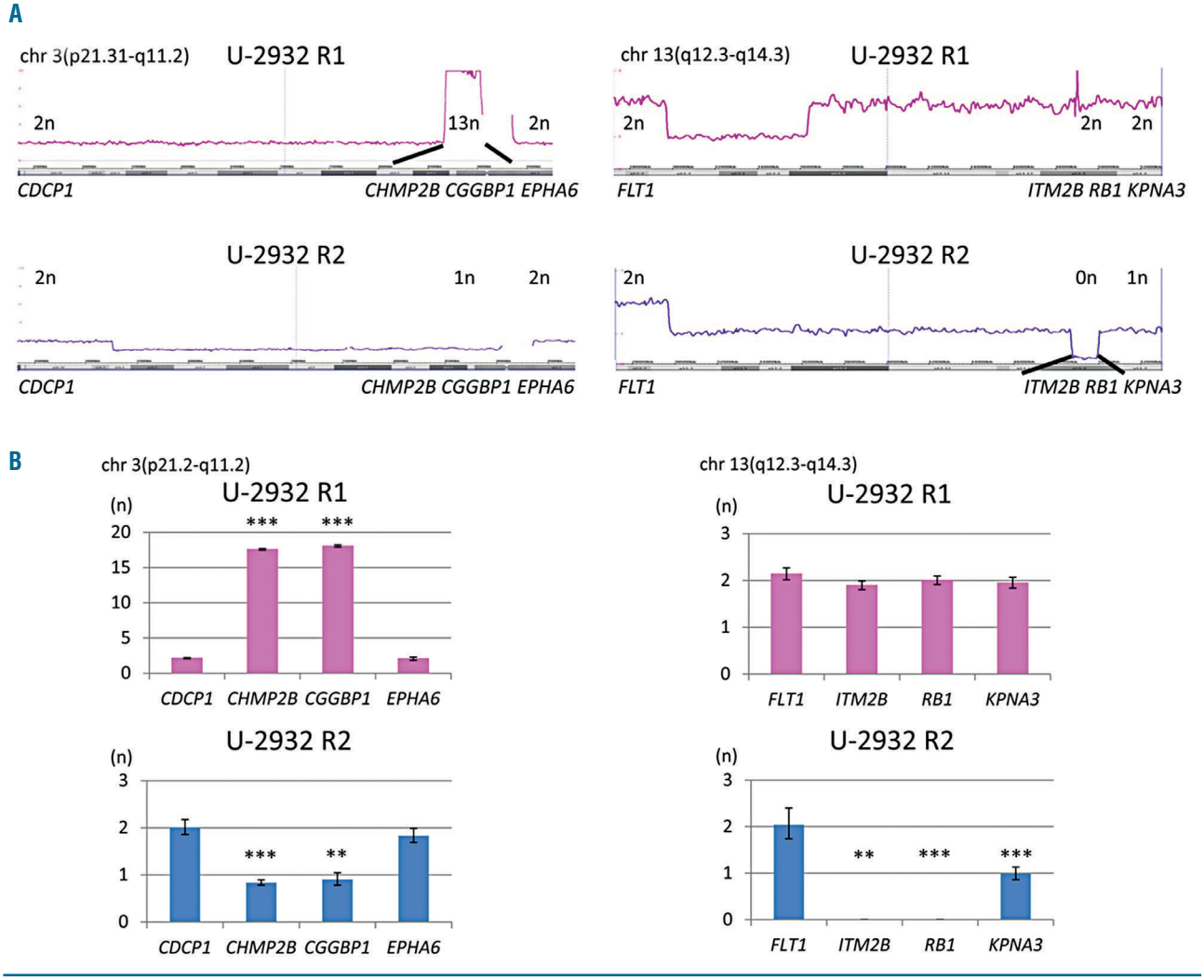

Figure 1. Copy number aberrations of U-2932 subclones R1 and R2. (A) Genomic copy number aberrations according to CytoScan HD Array analysis. (B) Results of quantitative genomic polymerase chain reaction showing the ploidy status of genes in regions of interest. Pink: U-2932 R1; blue: U-2932 R2. Normal cell line NC-NC was used as a diploid reference and *ABL1* as an endogenous control. Error bars indicate the standard deviation (SD) (n=3). Benjamini-Hochberg corrected t-test *P*-values: *P*<0.05 (*), <0.01 (**), <0.001 (***). In addition to *CHMP2B* and *CGGBP1*, *C3orf38* and *ZNF654* were also affected by chr 3(p11.1-2) amplification; further targets of chr 13(q14.2) deletion besides *ITM2B* and *RB1* were *CAB39L*, *CDADC1*, and *FNDC3A* (*Online Supplementary Table S3*).

MEF2B: a transcriptional regulator of BCL6

One target of the AhR antagonist was *MEF2B*, member of the 'myocyte enhancer-binding factor 2' family of transcription factors (Figure 3B). Confirming that *MEF2B* lies downstream of AhR/ARNT, *ARNT* knockdown reduced *MEF2B* expression in U-2932 R1 cells (Figure 3C). *MEF2B* is a transcriptional regulator that cooperates with co-repressors and histone-modifying enzymes.[24-27] One target of *MEF2B* is *BCL6*, a proto-oncogene selectively expressed in GC B-cells.[28] Thus, it was not surprising that AhR/ARNT inhibition not only affected *MEF2B* but also *BCL6* (Figure 3B). Confirming previously published data, knockdown of *MEF2B* down-regulated *BCL6* (Figure 3D).[28] This, together with the U-2932 subclone-restricted expression pattern of *AhR*, *MEF2B* and *BCL6*, indicated that overexpression of *AhR* might well explain deregulation of the GC oncogene *BCL6* (Figure 2A,B).

To test whether *AhR*, *MEF2B* and *BCL6* showed coordinated regulation in DLBCL cell lines in general, we analyzed the expression of these genes and of *ARNT* in a panel of 23 DLBCL cell lines. *ARNT* was constitutively expressed in all cell lines. Expression levels of the other three genes varied, but in a highly correlated fashion (Figure 4, *Online Supplementary Figure S4*). A positive correlation between *AhR* and *MEF2B* (both positive with ΔΔCt values ≥0.15) could be shown (Pearson correlation coefficient 0.34). Values for sensitivity (0.61), specificity (1.0) and accuracy (0.78) were determined. With a *P*-value according to the Fisher exact test of 0.0026, independence of their expression levels could be rejected with a significance of 0.05 (*Online Supplementary Table S2*). Likewise, independence of *MEF2B* and *BCL6* expression levels (both positive with ΔΔCt values ≥0.15) could be rejected with a *P*-value according to the Fisher exact test of 0.0001 against a level of significance of 0.05, together with a Pearson correlation coefficient of 0.69, a sensitivity of 0.92, a specificity of 0.9, and an accuracy of 0.91 (*Online Supplementary Table S2*).

Until now, it has only been reported that mutant *MEF2B* enhances transcription of target genes, thereby contributing to the genesis of *BCL6*-positive DLBCL.[28] Mutations in *MEF2B* are frequent in DLBCL and in follicular lymphoma.[4,24,29,30] *MEF2B* point mutations are carried by 11% of GC and ABC DLBC lymphomas.[3] However, unmutated *MEF2B* triggered *BCL6* expression in U-2932 cells.[28] Results of sequencing analyses showed that *BCL6* levels in cell lines with *MEF2B* mutations were not generally high (Figure 4). We also found that cytogenetic translocations involving *BCL6* were uncorrelated with *BCL6* expression (Figure 4). Where *BCL6* translocations were present in DLBCL cell lines, none involved canonical immunoglobulin gene partners reported in patients with *BCL6* overexpression. Whole exome sequencing showed that the U-2932 subclones did not carry missense mutations in the open reading frame of *IRF4* or *STAT5*, negative regulators of BCL6 (*data not shown*).[31,32] Exon 1 of *BCL6* – important as a binding region for repressive *BCL6* and *STAT5* – was also unmutated (*data not shown*).[33,34] Thus, our results suggested that the AhR/ARNT-induced expression of wild-type *MEF2B* might be an independent regulator of *BCL6* expression, besides canonical *BCL6* translocations, *BCL6* promoter hypermutation and *MEF2B* mutations.

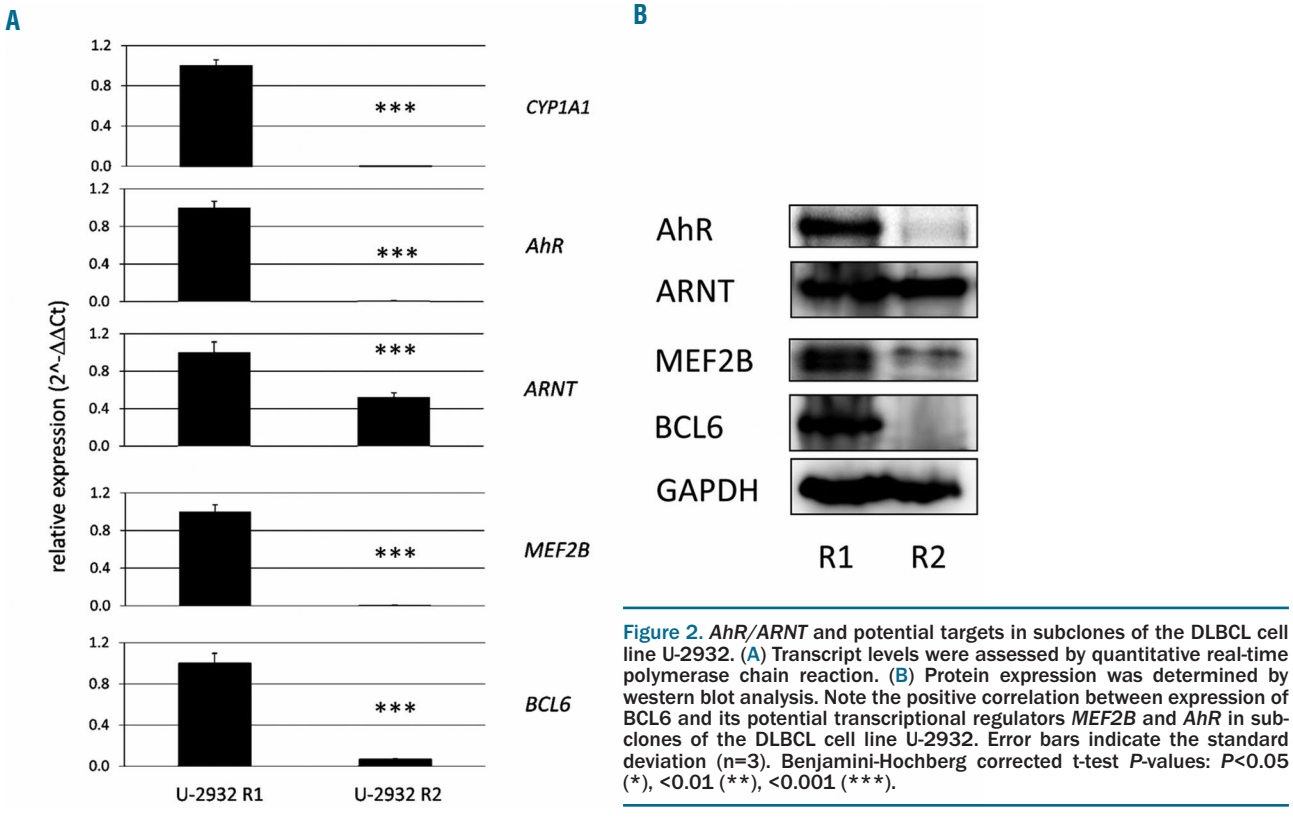

Figure 2. ***AhR/ARNT*** **and potential targets in subclones of the DLBCL cell line U-2932. (A) Transcript levels were assessed by quantitative real-time polymerase chain reaction. (B) Protein expression was determined by western blot analysis. Note the positive correlation between expression of BCL6 and its potential transcriptional regulators *MEF2B* and *AhR* in subclones of the DLBCL cell line U-2932. Error bars indicate the standard deviation (n=3). Benjamini-Hochberg corrected t-test *P*-values: *P*<0.05 (*), <0.01 (**), <0.001 (***).**

BCL6: *regulator of gene expression*

More than 200 genes were differentially expressed in the two subclones of the U-2932 cell line (*Online Supplementary Table S3*). Having shown that copy number aberrations and AhR/ARNT pathway activation were responsible for a substantial proportion of these differences, for most genes the underlying molecular cause for subclone-specific expression remained to be elucidated. To determine the extent to which activation of the transcriptional regulator *BCL6* in the R1 clone (Figure 2) contributed to this phenomenon, we ectopically expressed this oncogene in the *BCL6*-negative subclone R2. As previously reported, we infected R2 cells with a retroviral *BCL6* construct (MSCV-BCL6-IRES-GFP) or with empty vector (MSCV-IRES-GFP).[12] Treated cells were single-cell sorted to isolate clones with defined levels of *BCL6*. According to fluorescence microscopy, all MSCV-BCL6-IRES-GFP clones carried the construct. However, only a minority (5/25) expressed *BCL6* mRNA, of which only two expressed BCL6 protein (*Online Supplementary Figure S5*).

Ectopic expression of *BCL6* in subclone R2 affected 48 of the 221 (22%) genes that were differentially expressed 10-fold or more (Figure 5A). Interestingly, 28 of these 48 genes were up-regulated by *BCL6*, although *BCL6* is believed to act as a transcriptional repressor (Figure 5A,B).[35] This also held true at the protein level. Under the influence of *BCL6*, the B-cell marker *CD24* was repressed and, at the same time, *CD20*, *CD22*, *CD27*, *CD38* and *CD59* were significantly induced (Figure 5C). Confirming that *BCL6* positively regulates the expression of target genes, the majority of the *BCL6*-stimulated genes of Figure 5 were positively correlated with *BCL6* in a series of B-cell non-Hodgkin lymphoma cell lines (*Online Supplementary Table S4*).

Table 1. Effect of 5-Aza-2' deoxycytidine and trichostatin A on *AhR* expression.

		AhR-positive		AhR-negative	
		SU-DHL6	U-2932 R1	U-2932 R2	OCI-LY19
Aza	exp. # 1	1.1	1.2	6.2	93
	exp. # 2	n.d.	0.8	3.9	221
TSA	exp. # 1	1.3	1.6	17.5	42
	exp. # 2	1.6	1.9	17.9	49

Transcript levels were assessed by quantitative real-time polymerase chain reaction; n.d.: not done. Results show effect of reagents compared to DMSO (0.3%) controls as x-fold. Aza (5 μM, 72 h) is an inhibitor of DNA methylation, trichostatin A (TSA) (2 μM, 16 h) is a histone deacetylase inhibitor.

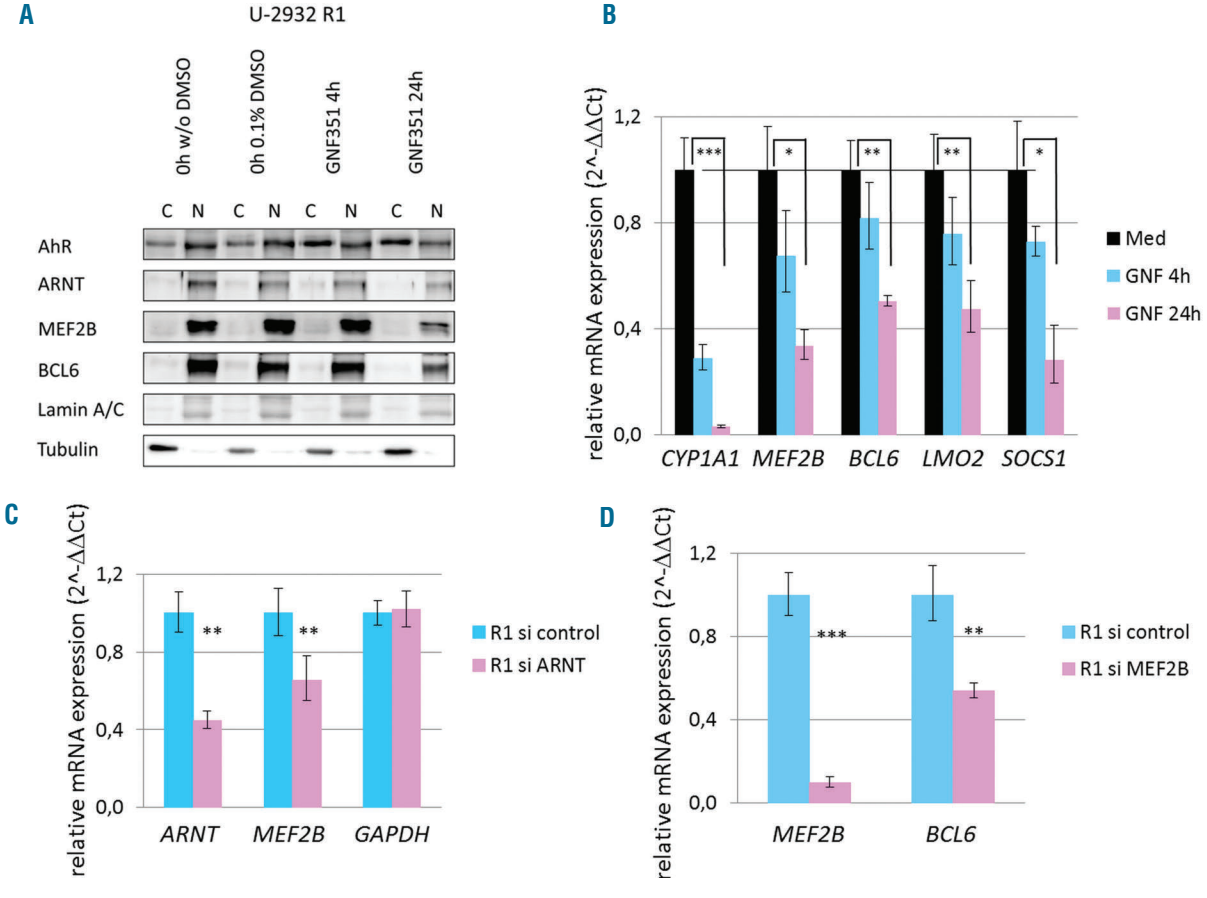

Figure 3. Effect of the AhR/ARNT inhibitor GNF351 on U-2932 R1 cells. (A) Translocation of AhR into the cytoplasm after stimulation with GNF351 (1 μM). C: cytoplasm; N: nucleus. Protein expression was determined by western blot analysis. (B) Inhibitory effect of GNF351 (1 μM) on expression of target genes including *MEF2B*. (C) Knockdown of *ARNT* led to reduced expression of *MEF2B*. (D) Knockdown of *MEF2B* led to reduced expression of *BCL6*. (B-D) RNA expression levels were determined by quantitative real-time polymerase chain reaction. Error bars indicate the standard deviation (n=3). Benjamini-Hochberg corrected t-test *P*-values: *P*<0.05 (*), <0.01 (**), <0.001 (***).

Thus, our data demonstrated that differences in *BCL6* expression resulted not only in down-regulation but also in up-regulation of a panel of genes, begging the question of how a transcriptional repressor might induce gene expression.

LMO2: a mediator for BCL6-initiated gene induction?

To assess whether the *BCL6*-triggered induction of gene expression was brought about by inhibition of a transcriptional repressor we analyzed which transcription factor binding sites (TFBS) were over-represented in *BCL6*-regulated genes. Putative TFBS were analyzed in the promoter region (±1000 bp relative to the transcriptional start site) of 160 genes equally up- and down-regulated by *BCL6* (by at least a factor of 2). Confounding our original prediction, *BCL6*-up-regulated genes did not show enrichment for cognate repressors. It was notable that 68% of the *BCL6*-up-regulated genes contained binding sites for *LMO2* complexes which were totally absent near down-regulated genes.

LMO2 forms complexes with distinct sets of partners in different types of cell. In contrast to hematopoietic stem cells and erythroid cells, the *LMO2* complex in DLBCL cells excludes *TAL1* and *GATA* proteins.[36] Accordingly, neither *TAL1* nor *GATA1* nor *GATA2* was expressed in the U-2932 subclones (Figure 6A). In contrast, other common *LMO2* partners, such as *E2A* (*TCF3*) and *SP1*, but also DLBCL-specific partners such as *ELK1*, *LEF1* and *NFATc1* were expressed (Figure 6A). *LMO2* was the only transcription factor complex gene expressed at different levels in the two subclones, suggesting that its presence might be crucial for the activity of the whole complex (Figure 6A). The expression of *LMO2* in U-2932 cells was under the control of *BCL6*: cells expressing *BCL6* were *LMO2*-positive, cells without *BCL6* were *LMO2*-negative (Figures 5B and 6B). This and the fact that 68% of the *BCL6*-up-regulated genes contained TFBS for *LMO2* complexes highlighted *LMO2* as a plausible mediator for the inductive effects of *BCL6*. The observation that *BCL6* stimulated expression of *LMO2* was noteworthy in another context. On the basis of expression array data, U-2932 had been categorized as an ABC DLBCL cell line.[11] However, *LMO2* is one of the genes whose expression is indicative of the GC type of DLBCL.[1,37] Applying a short list of five ABC marker genes and five GC markers, the U-2932 subclone R1 differed from R2 by elevated expression of four out of five GC markers (Figure 6C).[37] Besides *LMO2*, *BCL6*, *MYBL1* and *NEK6* are also GC markers highly expressed in subclone R1 (Figure 6C). Ectopic expression of one of these genes (*BCL6*) led to the induction of two other GC markers (*LMO2* and *MYBL1*) (Figure 5B).

Discussion

Tumors often show heterogeneity at the chromosomal and genetic levels explaining why analysis of the clonal architecture of tumors is such an important topic in cancer research. A disadvantage of studies with primary tumor cells is that they rarely allow direct conclusions to be drawn on the cellular consequences of a specific mutation. Functional studies are, therefore, often conducted with

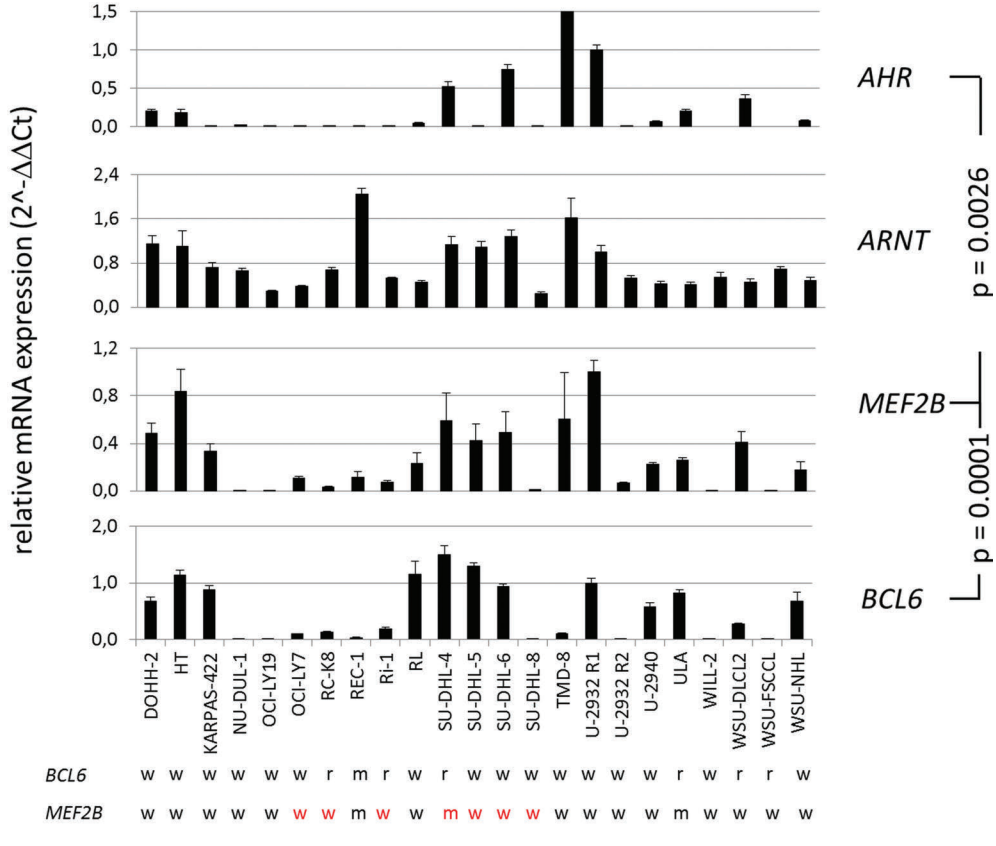

Figure 4. Expression of *AhR*, *ARNT*, *MEF2B* and *BCL6* in DLBCL cell lines. Transcript levels were assessed by quantitative real-time polymerase chain reaction and normalized against U-2932 subclone R1. TBP was used as the endogenous control. The mutational status of *MEF2B* shown had been assessed by Pasqualucci[4] (red) or by us (black). We sequenced *MEF2B* from AA1 to AA141 including the mutational hot spot only; w, wild-type; m, missense mutation. *BCL6* translocations were analyzed by three-color FISH using straddling (211G3) and flanking centromeric/telomeric (88P6/732M13) RP11-library BAC clones; w, wild-type, r; rearrangements involving the *BCL6* site. Error bars indicate the standard deviation (n=3). The positive correlations between *AhR-MEF2B* (*P*=0.0026) and *MEF2B-BCL6* (*P*=0.0001) according to the Fisher exact test are shown.

cells that ectopically express the mutated gene, e.g. cytokine-dependent cell lines or knock-in mice. However, in most cases these systems do not truly represent the histological origin of the studied tumor. Immortalized cell lines have often proven suitable for elucidating the function of a mutated gene. However, originating from a single cell, a cell line usually does not represent more than one clone. Exceptionally, the DLBCL cell line U-2932 consists of two populations representing two tumor clones, as evidenced by immunoglobulin hypermutation analysis.[12] The two U-2932 populations overexpress common (*BCL2*) and subclone-specific oncogenes (*BCL6 versus MYC*).[12]

Genomic amplification (*BCL2*; 6-7x in both clones) and a subclone-restricted translocation [t(8;14) affecting *MYC* in R2] explain overexpression of two key oncogenes. Here, we set out to determine why *BCL6* was exclusively expressed in subclone R1. *BCL6* was not subject to any of the canonical *BCL6* rearrangements in U-2932. The *BCL6* repressors *STAT5* and *IRF4* were unmutated, as was *BCL6* exon 1 – important for negative autoregulation of *BCL6*. The transcriptional activator *MEF2B*, mutated in 11% of DLBCL, was also unmutated. However, the wild-type form of *MEF2B* can also regulate *BCL6*.[28] *MEF2B* and *BCL6* levels were highly correlated in the U-2932 subclones and in a large panel of DLBCL cell lines. Our inhibitor and knock-down experiments demonstrated that *MEF2B* is a downstream target of the AhR/ARNT pathway. Consequently *AhR* and *MEF2B* levels were highly correlated in a series of DLBCL cell lines.

AhR has been described as an epigenetically regulated gene.[22,23] Accordingly, inhibitors of histone deacetylation and DNA methylation triggered expression of this gene in *AhR*-negative cell lines. Thus, results with the U-2932 subclones and other DLBCL cell lines indicate that AhR-regulated high expression of wild-type *MEF2B* might be an additional, independent cause of the aberrant expression of *BCL6* in DLBCL besides canonical *BCL6* translocations, *BCL6* hypermutation and *MEF2B* mutations.

That aberrant activation of signaling cascades can trigger the expression of a characteristic set of target genes is a well-described phenomenon. Inhibitor experiments showed that AhR/ARNT activation in subclone R1 was responsible for the expression of *BCL6* and ten additional genes. However, gross (>10x), subclone-specific expression differences were observed in 221 genes, not just 11. Genomic copy number aberrations correlated with and explained subclone specific expression differences for 58 genes. *BCL6* itself regulated 48 subclone-characterizing

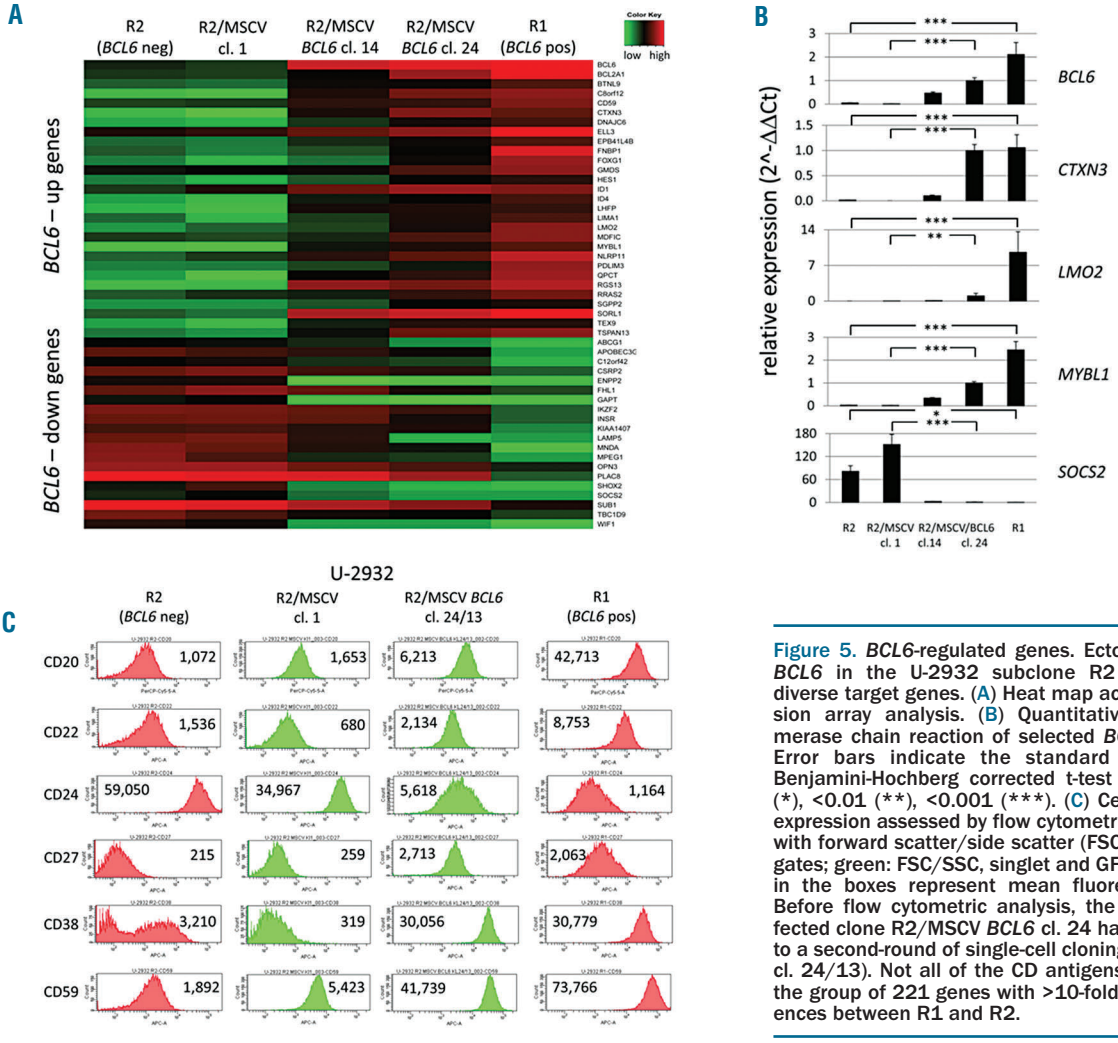

Figure 5. *BCL6*-regulated genes. Ectopic expression of *BCL6* in the U-2932 subclone R2 cells modulates diverse target genes. (A) Heat map according to expression array analysis. (B) Quantitative real-time polymerase chain reaction of selected *BCL6* target genes. Error bars indicate the standard deviation (n=3). Benjamini-Hochberg corrected t-test *P*-values: *P*<0.05 (*), <0.01 (**), <0.001 (***). (C) Cell surface marker expression assessed by flow cytometry. Red: cells gated with forward scatter/side scatter (FSC/SSC) and singlet gates; green: FSC/SSC, singlet and GFP gates. Numbers in the boxes represent mean fluorescence intensity. Before flow cytometric analysis, the retrovirally transfected clone R2/MSCV *BCL6* cl. 24 had been subjected to a second-round of single-cell cloning (R2/MSCV *BCL6* cl. 24/13). Not all of the CD antigens tested belong to the group of 221 genes with >10-fold expression differences between R1 and R2.

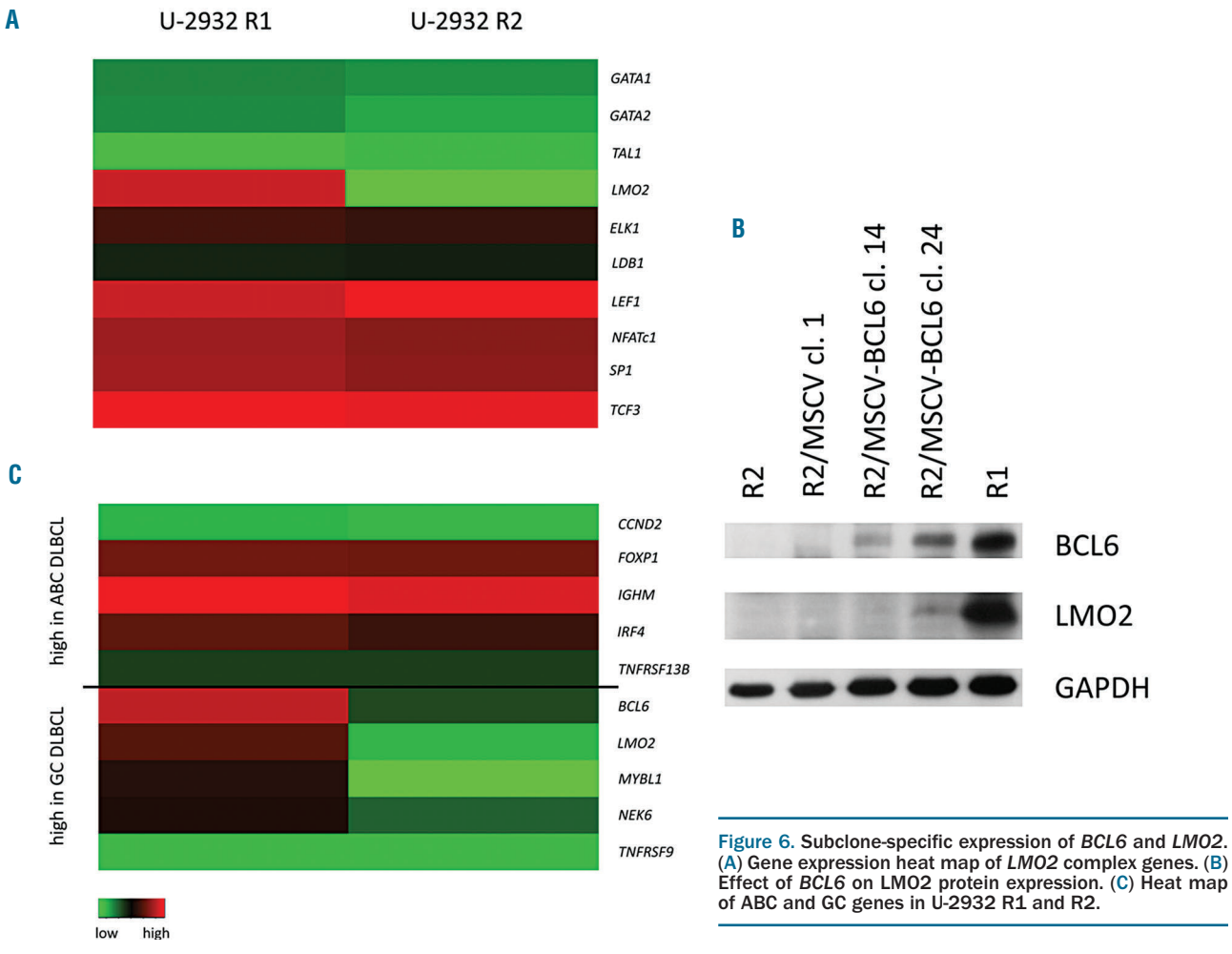

Figure 6. Subclone-specific expression of *BCL6* and *LMO2*. (A) Gene expression heat map of *LMO2* complex genes. (B) Effect of *BCL6* on LMO2 protein expression. (C) Heat map of ABC and GC genes in U-2932 R1 and R2.

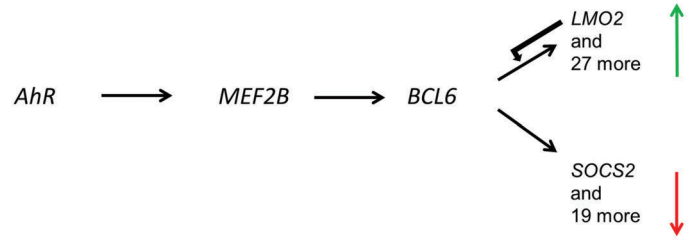

Figure 7. *BCL6* – regulated by AhR/ARNT and wild-type *MEF2B* – controls target genes including *SOCS2* and *LMO2*. Epigenetically regulated *AhR* controls expression of *MEF2B*, a transcription factor for *BCL6*. *BCL6* represses (e.g. *SOCS2*) and induces (e.g. *LMO2*) target genes. *LMO2* is involved in the positive regulatory process.

genes. Interestingly, 28/48 genes were induced when *BCL6* was ectopically expressed, suggesting a novel - most likely indirect - transcriptional role rather than the well-described suppressive role of *BCL6*. It is also a novel finding that the GC marker *BCL6* triggered expression of other GC markers, *MYBL1* and *LMO2*. The transcriptional *BCL6* target *LMO2* forms part of a transcriptional complex with cognate TFBS in 68% of the *BCL6*-up-regulated genes, which are absent in *BCL6*-repressed genes, suggesting that *LMO2* plays a role in the observed stimulatory effects of *BCL6*.

In summary, genomic copy number aberrations, activation of the AhR/ARNT complex, and overexpression of *BCL6* act together to regulate over 100 genes in subclones of the DLBCL cell line U-2932. The levels of *AhR*, *MEF2B* and *BCL6* were strongly correlated in a panel of DLBCL cell lines. Knock-down and inhibitor experiments confirmed that *AhR/ARNT* regulates *MEF2B*, a key transcription factor for the regulation of *BCL6*. Another new finding is that *BCL6* not only acts as a repressor, but is also capable of inducing expression of genes including the GC markers *LMO2* and *MYBL1*. Thus, *BCL6* – regulated by AhR/ARNT and wild-type *MEF2B* – may control expression of GC markers in DLBCL (Figure 7). Collectively our findings document an *in vitro* model for genetic heterogeneity in DLBCL to serve as a novel tool for investigating the pathology and therapeutics of a clonally diverse cancer.

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