Original Article

Gene Regulation by BCL3 in a Cervical Cancer Cell Line

(NF-κB / signal transduction / STAT3)

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Abstract: *BCL3* is a putative proto-oncogene deregulated in haematopoieitic and solid tumours. It has been suggested that its oncogenic effects could be mediated, at least in part, by inducing proliferation and inhibiting cell death. To provide more insight into the mediators of these effects, we used an unbiased approach to analyse the mRNA expression changes after knocking-down *BCL3* using specific shRNAs. One hundred eighty genes were up-regulated and sixty-nine genes were down-regulated after knocking down *BCL3*. Function analyses showed enrichment in genes associated with cellular growth and proliferation, cell death and gene expression. We found that *STAT3*, an important oncogene in human cancer, was the central node of one of the most significant networks. We validated STAT3 as a bona fide target of BCL3 by additional interference RNA and *in silico* analyses of previously reported lymphoma patients.

Introduction

NF-κB is a pleiotropic transcription factor that responds to cytokine signalling and cellular stress translocating to the nucleus and binding to a common DNA sequence motif, the kB box (Aggarwal, 2004). This factor is composed of several homo- or heterodimers of Rel-domain-containing proteins, which are kept inactive in the cytoplasm by a member of a family of anchorin-domain-containing proteins termed I-κB proteins. After a stimulus, the inhibitory protein is phosphorylated, ubiquitinated and degraded, allowing the release of the NF-κB complex. The protein encoded by proto-oncogene *BCL3* is atypical among the inhibitory proteins, since it is also able to act as transcriptional co-activator (Fujita et al., 1993). This protein, together with NFKB1, constitutes an alternative NF-κB pathway, variously called "non-canonical" (Cristofanon et al., 2009), "atypical" (Perkins, 2007) or "pathway 3" (Gilmore, 2006), in which no upstream receptor or activator is known. *BCL3* was initially identified in a chromosome translocation from chronic lymphocytic leukaemia, although it has also been found over-expressed and/or activated in diverse solid tumours (Cogswell et al., 2000; Thornburg et al., 2003). Although not completely understood, the oncogenic potential of *BCL3* could be derived from its effects in cell proliferation and death. It has been shown that this proto-oncogene, in conjunction with *NFKB2 (p52)*, activates the *CCND1* (Cyclin D1) promoter (Rocha et al., 2003), and also mediates the effects of the tumour suppressor gene *CYLD* in proliferation (Massoumi et al., 2006). Additionally, BCL3 also suppresses p53 activation following DNA damage, and thus, is able to inhibit p53-dependent apoptosis (Kashatus et al., 2006). To gain more insight in the mechanisms of the oncogenic effect of *BCL3*, in the present paper we analysed the mRNA expression changes induced by knocking down the expression of this proto-oncogene by a specific short-hairpin RNA (shRNA).

Material and Methods

Cell culture

HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO) with 5% foetal bovine serum in a humidified incubator at 37 °C with 5% CO_2.

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Abbreviations: IPA – Ingenuity Analysis Pathway, SDS-PAGE – sodium dodecyl sulphate-polyacrylamide gels, STAT3 – signal transducer and activator of transcription 3.

BCL3 knockdown

In order to obtain cell lines defective in Bcl3 expression, we transfected AmphoPack 293 cells (Clontech, San Diego, CA) with a plasmid (pSIREN, Clontech) containing the sequence for producing a double stranded 21 base pair siRNA directed against *BCL3* mRNA, under the control of an U6 promoter (Mendoza et al., 2006). The target sequences were: CGGTGTCTGCCGTAGGTTGTT; GCTCGACATCTACAACAACTT; GGCACGGATCACGATGTAAAT. After selection and screening, retroviral supernatants were collected, titrated and stored in liquid nitrogen. To generate the HeBcl3- cell lines, ten million HeLa cells were infected at a multiplicity of infection of 1, selected for four weeks with G418, cloned, and five clones were pooled as described previously (Mendoza et al., 2006). For validation, independent transfections using additional shRNAs were used, and a different method for shRNA transduction employed. Aligned oligonucleotides were cloned into pSIREN-RetroQ-ZsGreen vector (Clontech) following the manufacturer's instructions. HeLa cells were then transfected with these plasmids using Escort reagent (Sigma-Aldrich) and sorted with a Becton-Dickinson FACSAria cytometer (BD, Franklin Lakes, NJ), taking advantage of the modified GFP reporter of the plasmid vector.

RNA isolation and RT-PCR

RNA extraction and RT-PCR analysis were performed as described previously (Bandala et al., 2001). Briefly, total RNA was extracted from cultured cells with Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. RNA purity was confirmed by the 260/280 nm absorbance ratio and its integrity was established with agarose gels. Total RNA (5 µg) was reverse-transcribed using ThermoScript reverse transcriptase. The following primers were used:

BCL3: CCCCTATACCCCATGATGTGC; ATAATTGCGGGCCTCCAGGTC. *GAPDH:* CCCCTTCATTGACCTCAACT; TTGTCATGGATGACCTTGGC. *STAT3* (Konnikova et al., 2005): ACCTGCAGCAATACCATTGAC; AAGGTGAGGGACTCAAACTGC. *EGFR* (Ji et al., 2006): CCACCAAATTAGCCTGGACA; CGCGACCCTTAGGTATTCTG. *PI3K*: CATTGCCCCGCCTCTTCTTAT; GAAAGCGTCAGCCAAAACGTG. *NRP1*; GCTCCGGACCCATACCAGAGAA; TGCATTCAAGGCTGTTGGGA.

The primers were derived from the references shown. Those without references were obtained from the primer bank (http://pga.mgh.harvard.edu/primerbank/index.html) (Spandidos et al., 2008). Reactions without cDNA were performed as negative controls. Excised bands were sequenced to verify identity. Amplification products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Western blot

Standardized amounts of total protein were separated by electrophoresis in 15% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) and electroblotted to PVDF membranes. Blots were incubated with the antibodies indicated and the signal was visualized by chemiluminescence (ECL II; Millipore, Billerica, MA). The following antibodies were used: Cyclin D1 (Cell Signaling, Danvers, MA), Cdk4 (Cell Signaling), pERK (New England Biolabs, Ipswich, MA), tubulin (Chemicon, Temecula, CA), anti-mouse IgG horseradish peroxidase conjugated (Promega, Fitchburg, WI). pERK antibody recognizes both phosphorylated forms of ERK1 and 2, which can be discerned by molecular weight.

Microarray analyses

Total RNA (15 µg) derived from HeLa *BCL3* RNAi and a control (HeLa cells infected with a retrovirus containing an empty vector) were labelled by reverse transcription using 500 U of SuperScript II (Invitrogen). Integrity was verified by calculating the ratio of 28S and 18S ribosomal RNAs. Only RNAs with a ratio higher than 2.5 were used in the experiments. RT reaction was primed with 1 µg of oligo-dT plus 1 µg of hexamer random primers in the presence of 0.1 mM Cy3 (or Cy5)-dUTP, 0.5 mM of each dATP, dGTP, dCTP, 0.2 mM TTP, 2 U/µl RNAsin, and the SS-II buffer. The reaction was further incubated for 2.5 h at 42 °C and halted with EDTA. Template RNA was hydrolysed with NaOH for 15 min at 65 °C and then neutralized with 1 M Tris-HCl, pH 7.5 buffer. Labelled cDNA was purified with Microcon YM30 membranes and mixed with labelled control cDNA obtained by the same protocol. It was denatured at 99 °C for 3 min, cooled at room temperature, and deposited onto the microarray surface.

DNA microarrays were spotted on poly-lysine-coated slides at the Laboratory of Molecular Technology (NCI SAIC-Frederick, Inc., Frederick, MD) and consisted of 9,961 distinct PCR-amplified human cDNAs prepared from the IMAGE-EST clone collection. Hybridization was performed at 65 °C during 18 h. Washes in 2X SSC-0.1% SDS, 1X SSC, 0.2X SSC, and 0.05X SSC were sequentially performed, 1 min each. Microarrays were quickly dried and scanned at 10-µm resolution in a ScanArray-Lite scanner (Perkin-Elmer, Boston, MA). The scanner photomultiplicator was adjusted to obtain maximal signal intensities with minimal saturation. Microarray images were saved in TIFF format and extracted as GenePix result (gpr) files using GenePix Pro III software (Molecular Devices, Sunnyvale, CA). The signal was calculated by using the mean intensity minus median background. Data were subsequently normalized by the pre-calculated 50th percentile method, which is equivalent to the median (50th percentile) method but based on all spots with signals greater than 100 fluorescence units in both channels. Clones with over 30 % of missing values in all microarrays were excluded from the analysis, while those with less than 30 % of missing values were imputed with the KNN-impute tool available at the GEPAS server (http://www.gepas.org).

Bioinformatic analyses

To mine the database and provide significant associations of gene expression profiles with biofunctions and networks we used the Ingenuity Analysis Pathway (IPA) software (Ingenuity© Systems, Redwood City, CA). Cut-off values of ≤ 0.5-fold expression level for down-regulated genes and ≥ 1.5-fold expression level for the up-regulated ones were used to identify genes whose expression was significantly modulated. These differentially expressed genes were overlaid onto a global network developed from the IPA database. Networks of these genes were then generated based on their connectivity using proprietary algorithms. A score of 2 indicates that there is a 1 in 100 chance that the focus genes are together in a network due to random chance. Therefore, scores of 2 or higher have at least a 99 % confidence of not being due to random chance. In the present study, we only present networks with a score higher than 35 ($p \leq 1 \times 10^{-35}$). Functional analysis identified the biological functions and diseases that were most significant to the data set and networks. Fisher's exact test was used to calculate the P value determining the probability that each biological function and disease assigned to the data set under study is due to chance alone. In the final graphical representation, all edges were supported by at least one reference from the literature, or from canonical information stored in the IPA database. The intensity of the node colour indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes, provided by the software, representing the functional class of the specific gene product.

Results and Discussion

Gene expression changes induced by BCL3 depletion

In order to identify genes regulated by *BCL3*, we produced a cell line with an integrated retroviral vector

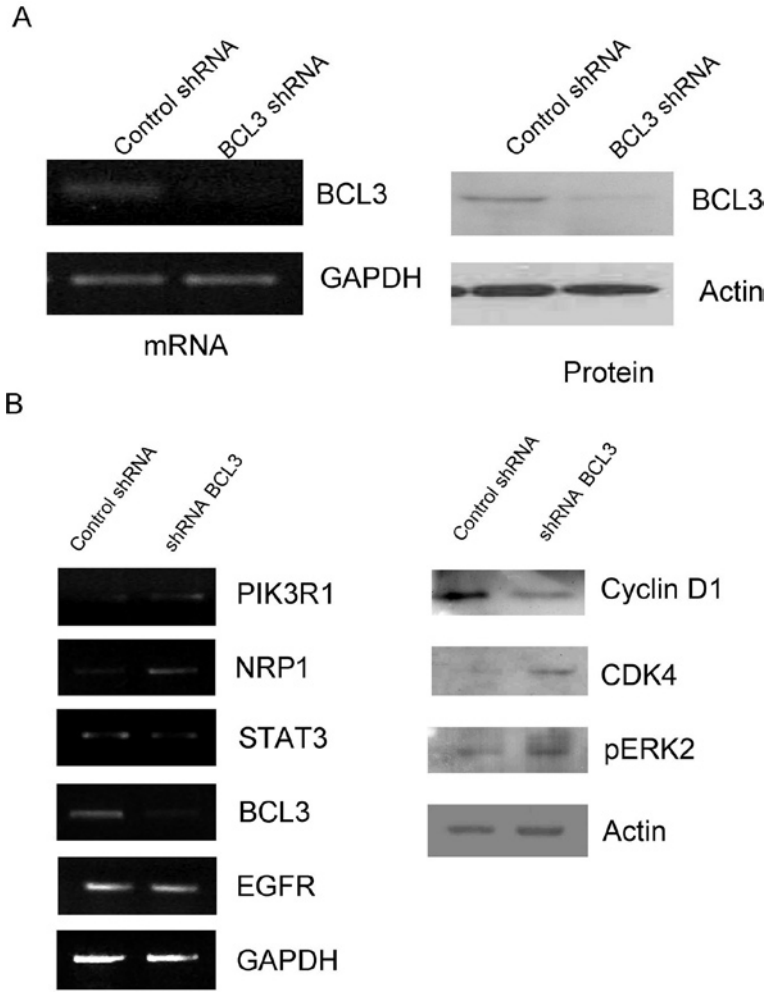

Fig. 1. Production of *BCL3* knock-down cells. HeLa cells were infected with a retrovirus containing a sequence that codifies for an shRNA directed toward *BCL3* mRNA. After a selection of four weeks, clones were isolated by limiting dilution. To create the knock-down *BCL3* cell line, five clones were pooled. A) BCL3 mRNA and protein level. B) Gene expression validation using semi-quantitative RT-PCR or Western blot analyses. These experiments were performed in sorted HeLa cells transfected with a plasmid containing a different shRNA directed to *BCL3*, cloned in pSIREN-RetroQ-ZsGreen vector. CDK4: Cyclin-dependent kinase-4, pERK1/2: extracellular signal-regulated kinase-1/2, phosphorylated.

Table 1. Genes regulated in Bcl3-deficient cells. Columns describe Unigene record, HUGO-approved name and fold change (log2 ratio between Bcl3 -knockdown cells and cells transfected with an empty vector).

UPREGULATED GENES			DOWNREGULATED GENES		
Unigene	**Name**	**Fold change**	**Unigene**	**Name**	**Fold change**
Hs.46296	NHLH2	1.009	Hs.440899	TTYH3	-6.569
Hs.253726	PAPOLA	0.996	Hs.113614	ADD2	-1.689
Hs.418192	ZNF440	0.985	Hs.408903	C2	-1.161
Hs.508738	ARHGEF7	0.868	Hs.415342	TCF25	-1.138
Hs.485729	RIMS1	0.851	Hs.509008	G2E3	-1.048
Hs.464184	SEC14L1	0.834	Hs.524910	FTH1	-0.988
Hs.212088	EPHX2	0.803	Hs.486489	ENPP3	-0.977
Hs.95120	CYGB	0.796	Hs.136309	SH3GLB1	-0.960
Hs.431498	FOXP1	0.783	Hs.12956	TAX1BP3	-0.902
Hs.558450	SORBS1	0.782	Hs.425769	OMA1	-0.900
Hs.440967	PON3	0.777	Hs.199593	AP3B2	-0.888
Hs.202238	KIAA1429	0.774	Hs.493275	TRIM31	-0.882
Hs.440049	ZXDC	0.769	Hs.49407	REG1A	-0.881
Hs.389277	ARFRP1	0.762	Hs.533738	C11ORF60	-0.867
Hs.169718	CNN2	0.753	Hs.169348	BLM	-0.813
Hs.417710	C16ORF14	0.752	Hs.295923	SIAH1	-0.797
Hs.422466	BTBD16	0.742	Hs.440263	TP53RK	-0.741
Hs.76753	ENG	0.734	Hs.355722	ITGB8	-0.732
Hs.436803	VBP1	0.720	Hs.23119	TMEM187	-0.711
Hs.89584	INSM1	0.713	Hs.408614	ST8SIA1	-0.703
Hs.171939	APBA1	0.704	Hs.463059	STAT3	-0.693
Hs.533831	USPL1	0.689	Hs.484068	RAB40B	-0.686
Hs.567322	PRKG1	0.689	Hs.494192	OSTF1	-0.684
Hs.63335	TERF2	0.680	Hs.166539	ITGB3BP	-0.671
Hs.472213	RRBP1	0.678	Hs.429666	CEBPG	-0.656
Hs.288809	BIVM	0.676	Hs.380027	C1ORF61	-0.642
Hs.511754	SPHAR	0.668	Hs.198072	PDE4B	-0.637
Hs.523936	PRCP	0.667	Hs.175905	AUH	-0.632
Hs.377090	ZHX2	0.667	Hs.151413	GMFB	-0.627
Hs.407152	MUC15	0.666	Hs.38516	FAM89A	-0.623
Hs.37706	ZCCHC8	0.665	Hs.75139	ARFIP2	-0.623
Hs.21388	ZDHHC21	0.658	Hs.515542	AKT1S1	-0.622
Hs.515696	ZNF737	0.656	Hs.387995	GLT25D2	-0.618
Hs.454790	LOC388692	0.656	Hs.250581	SMARCD2	-0.613
Hs.45127	CSPG5	0.656	Hs.287797	ITGB1	-0.612
Hs.459652	TMEM204	0.653	Hs.509637	PLEKHG3	-0.593
Hs.482497	TNPO1	0.647	Hs.1420	FGFR3	-0.592
Hs.124047	ZNF829	0.645	Hs.518773	UBE2D3	-0.586
Hs.435342	SLU7	0.644	Hs.518197	IQCB1	-0.569
Hs.432616	IGF2BP3	0.644	Hs.445356	C9ORF150	-0.569
Hs.444986	METAP2	0.644	Hs.404056	EIF3J	-0.568
Hs.363572	CEPT1	0.642	Hs.1765	LCK	-0.568
Hs.82793	PSMB3	0.642	Hs.426312	AMOTL2	-0.567
Hs.532755	C16ORF80	0.638	Hs.269560	CDK5RAP2	-0.566
Hs.43834	ZNF280B	0.635	Hs.350268	LOC729397	-0.563
Hs.109225	VCAM1	0.635	Hs.301048	SEH1L	-0.555
Hs.517761	LBA1	0.631	Hs.164384	PKP2	-0.553
Hs.428360	ZNF721	0.631	Hs.567479	TRA@	-0.551
Hs.200716	MECP2	0.626	Hs.36959	ZFAND3	-0.551
Hs.271940	ELF4	0.626	Hs.507681	MAP3K7IP1	-0.549
Hs.315177	IFRD2	0.625	Hs.499620	GEMIN4	-0.549
Hs.485449	GTPBP2	0.622	Hs.555902	ASAP2	-0.544

UPREGULATED GENES			DOWNREGULATED GENES		
Hs.1976	PDGFB	0.621	Hs.188553	RBBP6	-0.541
Hs.315137	AARS	0.618	Hs.526425	ATXN3	-0.540
Hs.167741	BTN3A3	0.618	Hs.487774	HNRNPA2B1	-0.536
Hs.95577	CDK4	0.618	Hs.47166	C3ORF14	-0.535
Hs.433300	FCER1G	0.618	Hs.406861	HSD17B4	-0.533
Hs.513491	YPEL3	0.615	Hs.95351	LIPE	-0.532
Hs.520182	TRAM2	0.615	Hs.435228	SYNPO	-0.529
Hs.474206	HIRA	0.614	Hs.93338	MGAT2	-0.529
Hs.278839	RPRD1B	0.612	Hs.433701	RPL37A	-0.527
Hs.483873	GM2A	0.611	Hs.504670	RIMKLB	-0.525
Hs.348387	GSTM4	0.609	Hs.12723	CNTN3	-0.520
Hs.134816	ZSCAN12	0.609	Hs.401316	IGFBP1	-0.518
Hs.489051	STEAP2	0.608	Hs.55452	ZNF676	-0.514
Hs.253903	STOM	0.606	Hs.362817	NUP85	-0.511
Hs.105269	SC4MOL	0.605	Hs.23585	CAMSAP1L1	-0.510
Hs.171825	BHLHE40	0.605	Hs.1600	CCT5	-0.506
Hs.78482	PALM	0.601	Hs.171280	HSD17B10	-0.505
Hs.486520	ALDH8A1	0.599			
Hs.504136	TBCEL	0.599			
Hs.109514	RYR2	0.596			
Hs.551713	MBP	0.594			
Hs.190495	GPNMB	0.594			
Hs.472716	FAM83D	0.592			
Hs.152385	KIAA1370	0.591			
Hs.149957	RPS6KA1	0.590			
Hs.474018	ADARB1	0.585			
Hs.469386	INPP4A	0.584			
Hs.370834	ATAD2	0.584			
Hs.515169	TRMT1	0.584			
Hs.131704	NRP1	0.582			
Hs.460298	POLR3E	0.582			
Hs.532793	KPNB1	0.578			
Hs.503510	EED	0.577			
Hs.106674	BAP1	0.576			
Hs.458973	ZFHX4	0.575			
Hs.193268	MTAP	0.574			
Hs.551068	CMBL	0.573			
Hs.196384	PTGS2	0.572			
Hs.225661	FLJ36644	0.572			
Hs.109514	RYR2	0.572			
Hs.167741	BTN3A3	0.571			
Hs.356285	HMGN1	0.570			
Hs.7189	CYTH4	0.569			
Hs.2913	EPHB3	0.569			
Hs.311100	C3ORF75	0.568			
Hs.303676	G3BP2	0.566			
Hs.118127	ACTC1	0.564			
Hs.425144	MTMR11	0.563			
Hs.509414	KTN1	0.563			
Hs.437941	C14ORF106	0.562			
Hs.488293	EGFR	0.561			
Hs.124299	FAM167A	0.559			
Hs.533597	PYGO2	0.558			
Hs.511251	SQRDL	0.555			

UPREGULATED GENES			DOWNREGULATED GENES
Hs.496811	PLAC1	0.554	
Hs.159481	SLC26A10	0.553	
Hs.445387	RPS6KA3	0.552	
Hs.464416	USP14	0.552	
Hs.105700	SFRP4	0.550	
Hs.473087	CTPS	0.549	
Hs.466766	LTBP4	0.548	
Hs.187284	PAPPA2	0.548	
Hs.192586	CMBL	0.548	
Hs.165904	EPN3	0.546	
Hs.258209	RAB3IP	0.545	
Hs.283869	TTPAL	0.545	
Hs.512963	ALG11	0.544	
Hs.117853	LOC100129674	0.541	
Hs.150136	MAPK7	0.541	
Hs.369056	SP100	0.541	
Hs.445890	CNIH4	0.540	
Hs.468048	LCLAT1	0.540	
Hs.5301	PRPF38A	0.540	
Hs.491611	SLC20A2	0.539	
Hs.160871	PTPRO	0.538	
Hs.408241	NUPL2	0.537	
Hs.72885	AZU1	0.536	
Hs.132225	PIK3R1	0.536	
Hs.237825	SRP72	0.533	
Hs.369762	ENOSF1	0.533	
Hs.459927	PTMA	0.532	
Hs.23642	SAC3D1	0.532	
Hs.143766	ATN1	0.532	
Hs.161000	ARID4A	0.530	
Hs.492280	ATP7B	0.530	
Hs.166011	CTNND1	0.529	
Hs.546557	SFT2D2	0.529	
Hs.502302	CAT	0.529	
Hs.519313	AFF4	0.528	
Hs.170310	CECR1	0.528	
Hs.135406	CEBPZ	0.527	
Hs.522684	ZMYM3	0.526	
Hs.371139	KRT4	0.526	
Hs.532968	HJURP	0.526	
Hs.132868	USP32	0.525	
Hs.157441	SPI1	0.525	
Hs.471156	ABI2	0.525	
Hs.162963	ANTXR2	0.522	
Hs.492740	ATF6	0.522	
Hs.390420	FUT4	0.521	
Hs.128060	FIT1	0.521	
Hs.30385	MLLT10	0.520	
Hs.284414	HSD17B13	0.518	
Hs.517172	DIDO1	0.517	
Hs.556258	NCRNA00084	0.517	
Hs.547544	IGKC	0.514	
Hs.310453	TSPAN14	0.514	
Hs.149387	MYO6	0.512	

UPREGULATED GENES			DOWNREGULATED GENES
Hs.561846	GFRA2	0.512	
Hs.522605	MID1IP1	0.511	
Hs.461727	ACSF3	0.510	
Hs.473877	FAM3B	0.510	
Hs.432424	TPP2	0.510	
Hs.507333	GOLGA3	0.509	
Hs.1584	COMP	0.508	
Hs.529901	XIST	0.507	
Hs.161008	KPNA1	0.506	
Hs.444467	EEF1G	0.506	
Hs.534032	TARP	0.506	
Hs.76873	HYAL2	0.506	
Hs.280342	PRKAR1A	0.505	
Hs.334534	GNS	0.504	
Hs.533336	BAMBI	0.504	
Hs.7258	CORO7	0.504	
Hs.516090	EMX1	0.503	
Hs.76253	ATXN2	0.503	
Hs.91816	RBM7	0.502	
Hs.558396	SCD	0.500	

codifying for an shRNA directed toward the mRNA of this oncogene, as described previously (Mendoza et al., 2006). Fig. 1a shows that this shRNA depleted more than 80 % of the BCL3 mRNA and protein levels in HeLa cells. We then performed an expression profiling assay using microarrays. We selected an up-regulation cut-off point of 1.5 or more or a down-regulation point of less than 0.5, which gave 180 up-regulated and 69 down-regulated genes (Table 1). We then validated several candidates including both up- and down-regulated genes by means of an end-point RT-PCR or Western blot assays (Fig. 1b). Consistent with previous reports, we found down-regulation of Cyclin D1 (Massoumi et al., 2006; Park et al., 2006), and most interestingly, a divergent increase in Cyclin-dependent kinase 4, Cyclin D1's major catalytic partner (Fig. 1). We could not validate the *EGFR* up-regulation, perhaps due to the reported (Blobel, 2005) variety of splicing forms of this gene. Nevertheless, we validated the expression changes of phosphoinositide 3 kinase, regulatory subunit 1 (*PIK3R1*) and neuropilin 1 (*NRP1*).

Function analysis of regulated genes

We then performed an unbiased molecular and cellular function analysis using the IPA software, which showed, among other processes, an enrichment in genes associated with cellular growth and proliferation, cell death and gene expression ($P = 5 \times 10^6$ to 8.5×10^6 by Fisher's exact test) (Fig. 2). These results agree with the known effects of BCL3 on proliferation (Westerheide et al., 2001), cell death (Bauer et al., 2006; Kashatus et al., 2006) and transcription regulation (Park et al., 2006). As expected, genes regulated by BCL3 were also enriched for cancer pathogenesis ($P = 9.6 \times 10^5$) (Fig. 2a), and several signalling pathways associated with cancer, including, NF-κB (Fig. 2b). Using a more systematic approach, we performed an *in silico* analysis of gene networks significantly associated with the profile. Two functional networks presented a score higher than 35 (Fig. 3 and not shown, available at request). The most significant network was associated with tissue development, whereas the second one was associated with cellular and molecular processes, so we chose to focus on it.

BCL3 regulates a common oncogenic pathway

One of the networks found presented functions associated with cellular development, growth and proliferation (Fig. 3). The main nodes on this network were part of the EGFR signalling cascade, including connections with STAT3, ITGB1 and PIK3R1. As mentioned earlier, we could not validate the *EGFR* up-regulation, perhaps due to the presence of multiple splice variants (Blobel, 2005), although the regulation of *STAT3* and *PIK3R1* was reproducible (Fig. 1). In addition, we found that the phosphorylation of the downstream target ERK2 was increased by BCL3 depletion (Fig. 1B), supporting the role of BCL3 in this signalling cascade. It is interesting to note that Brocke-Heidrich et al. have shown that STAT3 directly regulates *BCL3* transcription (Brocke-Heidrich et al., 2006). Our results add an additional level of modulation in which BCL3 also regulates STAT3 expression, creating a STAT3-BCL3 positive amplification feedback loop. STAT3 is an important node in the signalling pathways of multiple cytokines and tyrosine kinase oncoproteins, even being indispensable for the transformation effects of Ras (Gough et al., 2009). To assess whether these results could be relevant to human cancer, we performed an *in silico* analysis of

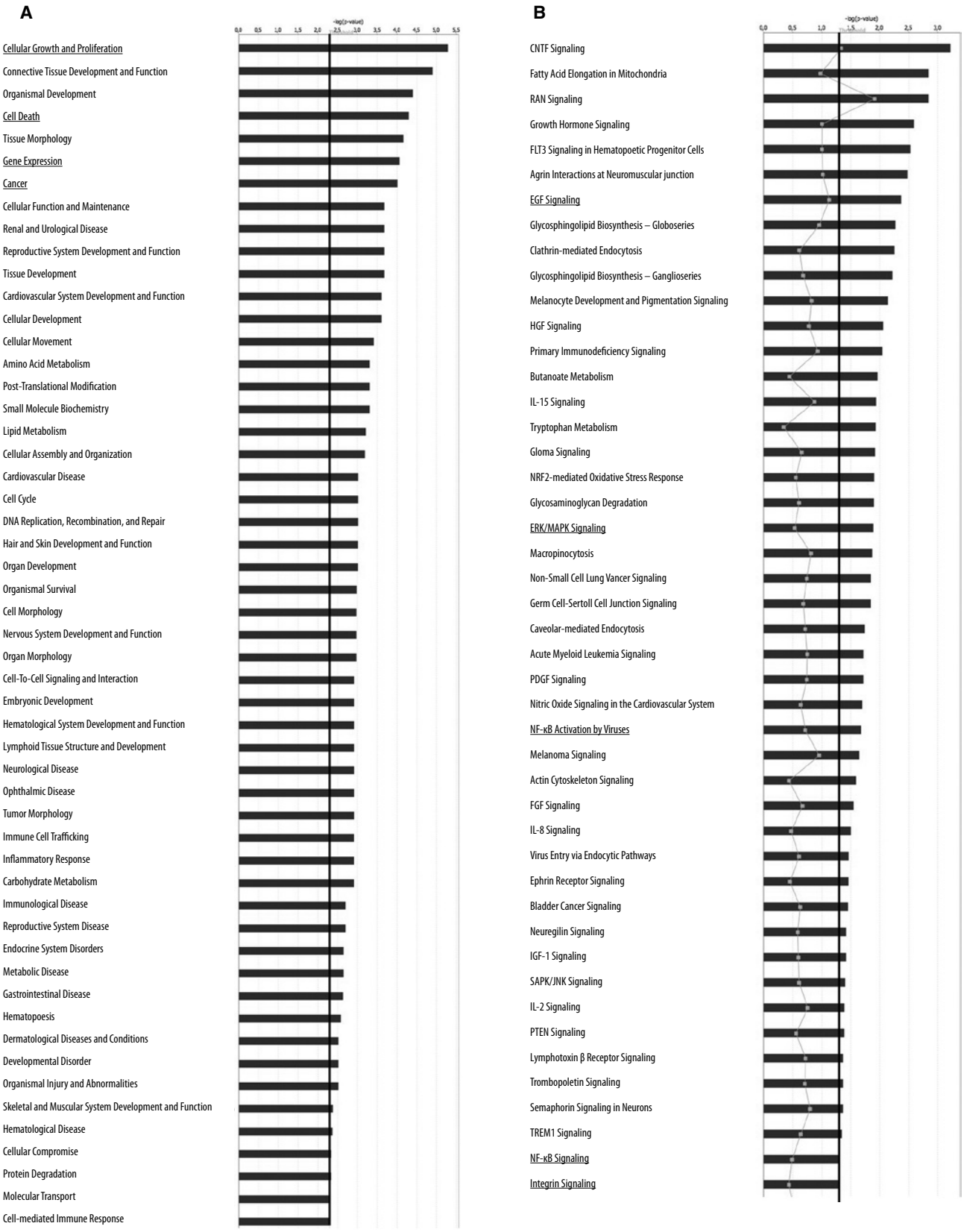

Fig. 2. Molecular and cellular function analysis of regulated genes. Genes up- or down-regulated were analysed for enrichment of biofunctions, diseases or canonical pathways using the IPA® software suite. A) Gene enrichment in specific biofunctions and diseases. Threshold: significative results using Fisher's exact test. B) Gene enrichment in canonical pathways. Threshold: significant results using Fisher's exact test**.** The score is generated using a P value calculation, and indicates the likelihood that the set of focus genes could be explained by random chance alone (score = -log(P.value). Networks with scores of 2 or higher have at least 99 % confidence of not being generated by random chance.

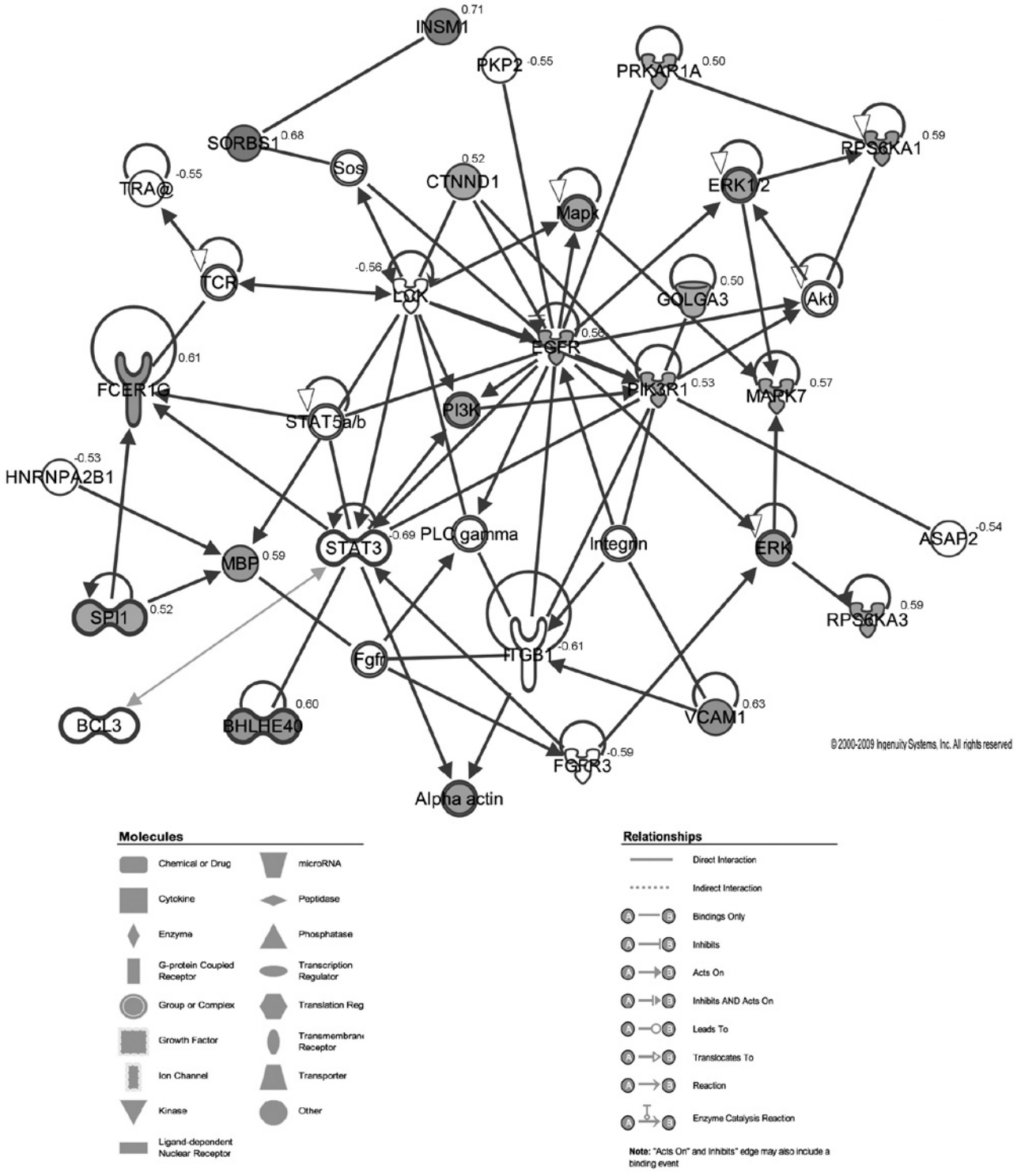

Fig. 3. Cellular development, growth and proliferation network enriched in *BCL3* knock-down cells. The intensity of the node colour indicates the degree of regulation. Nodes are displayed using various shapes, provided by the software, representing the functional class of the specific gene product. The network presented a score of 39. Functional class of node and relationships are shown at the bottom.

BCL3 and *STAT3* expression from lymphoma patients using data from two previous studies (Dave et al., 2006; Hummel et al., 2006) taking advantage of the Oncomine Database platform (Rhodes et al., 2004). Figure 4 shows a clear correlation between *BCL3* and *STAT3* expression (R = 0.656 to 0.821 in two studies).

These results warrant further analyses to establish the possible role of STAT3 as a mediator of the oncogenic effects of BCL3. It is noteworthy that we also found down-regulation of LCK, which has been shown to be a binding partner and a regulator of BCL3 activity (Zhao et al., 2005). Finally, it is important to note that

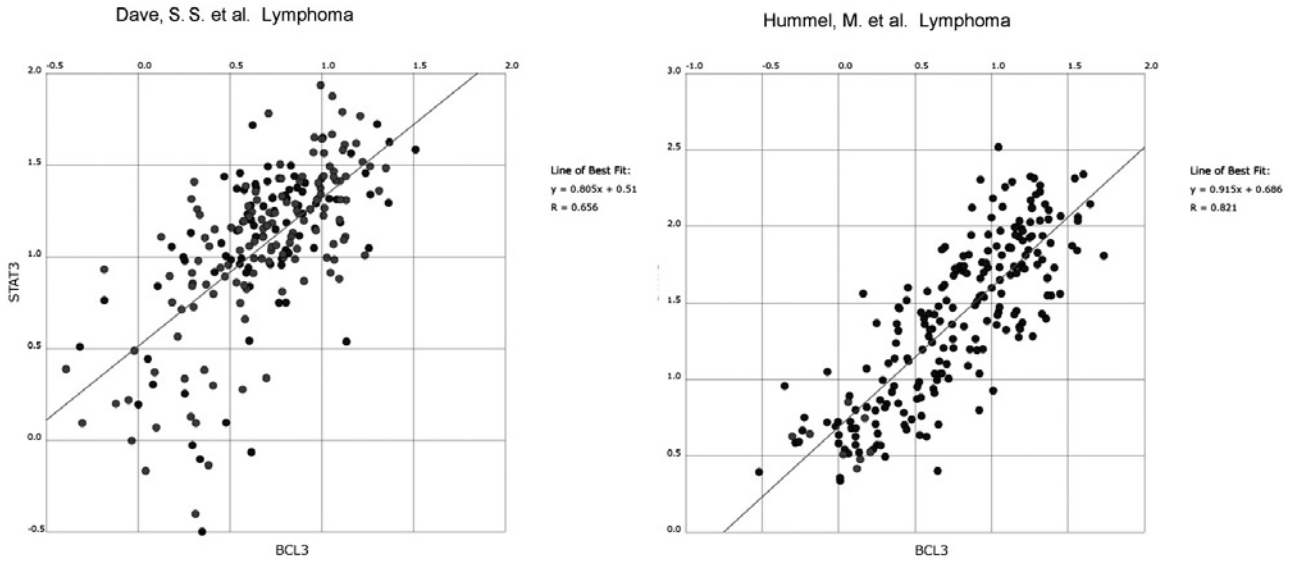

Fig. 4. A) Validation and relevance of the oncogenic pathway revealed from the microarray analysis. Scatter plot of normalized expression units showing the correlation between *BCL3* and STAT3 expression in two genome-wide expression studies (Dave et al., 2006; Hummel et al., 2006). Log^2-transformed expression values were used. The median was scaled to zero and the standard deviation of values was scaled to 1 to calculate the normalized expression units. Graphic derived from Oncomine database (Rhodes et al., 2004). B) Alternative (or Pathway 3) NF-κB pathway. From an unknown upstream signalling cascade p50 (or p52) homodimers are translocated to the nucleus after processing of the precursor p105 (or p100). Transcriptional activation results from the association of these homodimers with BCL3. Presumably, BCL3 can also be regulated by protein modifications (e.g. ubiquitination) to further modulate the pathway.

down-regulation of BCL3 in other cancer cell lines also decreased STAT3 mRNA levels (not shown), supporting a more generalized role of this network in other cancer cell types.

In conclusion, BCL3 regulates a diverse array of genes involved in signalling pathways associated with cancer, in addition to its known role as a part of the atypical NF-κB pathway. Among the key genes regulated by this oncogene is *STAT3*, which could provide a positive feedback mechanism leading to enhancing activities of both factors. Further studies analysing the importance of STAT3 in the oncogenic potential of *BCL3* and the specific mechanism of regulation are clearly needed.

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