B-cell lymphoma 6 (BCL6) is a 95-kDa nuclear phosphoprotein and member of the Pox virus zinc finger/bric-a-brac, tramtrack, broad complex (POZ/BTB) family of transcription factors. BCL6 is a transcriptional repressor required for germinal center formation, and the gene encoding it is frequently altered in diffuse large B-cell and follicular lymphomas. The dysregulation of BCL6 has therefore been implicated in lymphomagenesis. A limited number of proteins is known to interact with BCL6 and modulate its activity or participate in its role in transcriptional regulation. Identification of additional BCL6-binding proteins could reveal potential signaling targets and previously undescribed functional roles for BCL6. We used a functional proteomic approach to determine the identity of proteins that interact with BCL6. Proteins were isolated by co-immunoprecipitation with an anti-BCL6 antibody and identified using MS/MS. We identified 61 proteins in the BCL6 immunocomplex from the following Gene Ontology categories: transcription regulator activity (n = 18), binding activity (n = 11), signal transducer activity (n = 10), catalytic activity (n = 8), structural molecule activity (n = 3), enzyme regulator activity (n = 3), transporter activity (n = 2), motor activity (n = 2), chaperone activity (n = 1), and unknown function (n = 3). Importantly, we identified BCL6 and several previously reported BCL6-interacting proteins in the BCL6 immunocomplex. The remaining proteins have not been shown previously to be associated with BCL6. MS/MS results were validated on four proteins using immunoprecipitation and Western blotting. Two of these protein interactions were further confirmed by reciprocal immunoprecipitation. This study demonstrates the utility of antibody immunoprecipitation and subsequent peptide identification by MS/MS for the elucidation of BCL6-binding proteins. Many of the novel proteins identified in this study suggest additional functional roles for BCL6 beyond transcriptional repression.

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finger domains are likely to bind additional proteins that may further define and expand the role of BCL6 in the cell. Furthermore there are additional proteins that complex with BCL6 via indirect or secondary interactions. Identification of the complete interactome of BCL6 would provide additional information regarding the function and regulation of this oncogene.

In this study, we report the identification of multiple BCL6-interacting proteins via immunoprecipitation followed by LC-MS/MS peptide sequencing (Fig. 2). In addition to confirmation of previously described BCL6-binding proteins, we expand the list of BCL6-interacting proteins to include additional transcription regulation factors. Furthermore we identified potential BCL6-interacting proteins from other functional activity groups including binding, signal transduction, catalysis, structural molecule activity, enzyme regulation, transport, motor, and chaperone activity.

**EXPERIMENTAL PROCEDURES**

*Cell Cultures*—The SUDHL-4 (BCL6-expressing) and SUDHL-9 (non-BCL6-expressing) cell lines were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (Novatech, Inc., Brampto, Ontario, Canada) supplemented with 10% heat-inactivated fetal calf serum and antibiotic mixture (Invitrogen). The cell lines were grown to confluency, and samples (2 × 10⁷ cells each) were pelleted and stored at −80 °C.

*Cell Lysis, Immunoprecipitation, and Western Blot Analysis*—Immediately upon thawing, cells were lysed using 0.5 ml of lysis buffer (25 mM Tris-HCl, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA) per pellet. Before each use, 0.1% protease inhibitor mixture (Sigma) was added to the required volume of lysis buffer. Protein concentrations were estimated using the Bradford colorimetric method against known concentrations of bovine serum albumin.

For immunoprecipitation, lysate containing 800 µg of total protein was mixed with either 2 µg of primary anti-BCL6 (C-19) polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit IgG in a total volume of 1 ml. The mixtures were incubated with gentle shaking for 1 h at 4 °C. Protein G-agarose beads were then added to each tube, and the samples were incubated in a similar fashion. The immunocomplex was washed five times with cold lysis buffer, and the antibody-selected proteins were eluted from the agarose beads by boiling in SDS loading buffer (0.1 M Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol).

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0.05% bromphenol blue, and 0.1 M DTT) for 10 min. Each sample was divided equally, and each half was resolved on separate 8% SDS-polyacrylamide gels. On one gel, the proteins were visualized with mass spectrometry-compatible silver staining (Invitrogen). Separated proteins were transferred from the other gel to a nitrocellulose membrane (Millipore, Billerica, MA) for immunoblotting using semidry transfer. After blocking overnight at 4 °C with 5% milk, the membrane was incubated at room temperature with a 1:200 dilution of monoclonal anti-BCL6 antibody (DakoCytomation, Carpinteria, CA) for 1 h, then washed, and incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Western blot targets were visualized using chemiluminescence (ECL Plus kit, Amersham Biosciences).

Validation by Western Blot and Reciprocal Immunoprecipitation—For validation experiments, immunoprecipitations were performed using SUDHL-4 lysates containing 800 μg of total protein, anti-BCL6 polyclonal antibody, and normal rabbit IgG control. Immunoprecipitated proteins were resolved and transferred, and Western blots were performed using antibodies against HDAC4, SMRT, PKC, and WNK4 (Santa Cruz Biotechnology). Reciprocal immunoprecipitations were performed using SUDHL-4 lysates (800 μg of total protein) and antibodies against HDAC4 and PKC. Immunoprecipitated proteins were resolved by SDS-PAGE, and BCL6 was detected by immunoblotting.

Digestion, Peptide Extraction, and Mass Spectrometric Analysis—Each lane of interest on the silver-stained gel was isolated and divided into 12 equal slices. The proteins in each slice were digested, and the peptides were extracted using the Invitrogen protocol. Briefly each gel slice was destained, washed, crushed, dried, and then rehydrated in ammonium bicarbonate buffer. Freshly prepared sequencing grade lysine-C endopeptidase (Princeton Separations, Ad-
elphia, NJ) was added (1:50) to each tube, and the tubes were incubated at 37 °C for 4 h. Sequencing grade, modified trypsin (Princeton Separations) was then added (1:50), and the tubes were incubated at 37 °C overnight.

The digested peptides were extracted from the gel pieces using 50% acetonitrile with 0.1% TFA and evaporated to a final volume of 30 µl. Duplicate 15-µl aliquots of each sample were analyzed by automated nanoflow reverse-phase LC-MS/MS using the LCQ Deca XP ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Digested peptides were injected by an autosampler using an acetonitrile gradient (100% Buffer A to 40% Buffer A, 60% Buffer B in 90 min; Buffer A = 5% acetonitrile with 0.4% acetic acid and 0.005% heptafluorobutyric acid; Buffer B = 95% acetonitrile with 0.4% acetic acid and 0.005% heptafluorobutyric acid) through a reverse-phase column (75-µm inner diameter fused silica packed with 12 cm of 5-µm C18 particles) to elute the peptides at a flow rate of 200 nl/min into the mass spectrometer. An electrospray voltage of 1.8 kV was used with the ion transfer tube temperature set to 200 °C. Peptide analysis was performed using data-dependent acquisition of one MS scan (600–2000 m/z) followed by MS/MS scans of the three most abundant ions in each MS scan. Dynamic exclusion was used to acquire a more complete survey of the peptides contained in the BCL6 antibody pull-down complex by automatic recognition and temporary exclusion of ions from which definitive mass spectral data
had been acquired previously. Dynamic exclusion was set to a repeat count of two with the exclusion duration of 3 min.

Data Analysis—The MS/MS-acquired data were searched using the SEQUEST algorithm in Bioworks 3.1 (ThermoFinnigan) against amino acid sequences in the UniProt protein database (September 27, 2004 download; 190,183 entries). Protein search parameters included a precursor peptide mass tolerance of ±0.7 amu, fragment mass tolerance of ±0.1 amu, and carboxymethylation for cysteine residues. The search was constrained to tryptic peptides with one missed enzyme cleavage allowed. The peptide matching criteria of a cross correlation score (Xcorr) >1.2 for +1 peptides, >2.2 for +2 peptides, and >3.2 for +3 peptides and a Δ correlation score (ΔCn) >0.100 were used as a threshold of acceptance. Other factors were also taken into consideration such as the number of unique peptides found per protein, the total number of matching y and b series ions, the Δ correlation score from the next nearest hit, any known conserved domain protein interactions, and feasible molecular signal transduction interactions.

Finally the SEQUEST data (.dta) and output (.out) files from duplicate aliquots were combined and analyzed using INTERACT and ProteinProphet (Institute for Systems Biology, Seattle, WA) (31, 32). Data analysis using INTERACT and ProteinProphet improved the confidence of protein identification by best fit distribution of probability scores specific to each data set and reduced the risk of false positive protein identifications. All proteins passing a threshold of less than 5% predicted error (false positive rate) were summarized.
In Silico Analysis—We used several search methodologies in publicly available protein interaction databases (Online Mendelian Inheritance in Man (OMIM) and Human Protein Reference Database (HPRD)) to create a list of primary (direct) and secondary (indirect) protein-protein interactions centered on BCL6. The OMIM is hosted by the National Center for Biotechnology Information (NCBI) and contains information on 10,281 genes specifically associated with diseases in man as summarized from published literature. The OMIM search page is found at www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM. The HPRD (33) is a joint project combining the efforts of Bangalore’s Institute of Bioinformatics and PandyLab. As of August 2005, the database contained 18,288 human protein entries and 27,330 documented protein-protein interactions with nearly 171,677 links to PubMed literature. HPRD can be found at www.hprd.org.

By searching each database first for direct BCL6 protein interactions then for any secondary BCL6 interactions, a summary of the BCL6 protein “interactome” was compiled. In addition, primary and secondary protein interactions with BCL6 were further screened by a literature survey using PubMed. Finally these in silico findings were compared with the protein interactions identified by BCL6 pull-down purification and tandem mass spectrometry.

RESULTS

Identification of BCL6-interacting Proteins—To determine the identity of proteins associated with BCL6, we performed immunoprecipitation of total cell lysates from the BCL6-expressing cell line SUDHL-4 using anti-BCL6 polyclonal antibody. Immunoprecipitation reactions using the non-BCL6-expressing SUDHL-9 cell line and normal rabbit IgG served as separate negative controls. A band at 95 kDa corresponding to BCL6 was identified in the SUDHL-4 BCL6 pull-down lane but not in the SUDHL-9 or IgG control lanes on a silver-stained gel (Fig. 3A) and a Western blot (Fig. 3B).

Duplicate MS/MS analysis of the SDS-PAGE lanes obtained from BCL6 immunoprecipitation resulted in a total of 1183 peptides, which were matched to 726 known database entries and 139 proteins. The average size of identified peptides was 12 amino acid residues (range, 7–29). The MS full scan and MS/MS scan are shown for peptides identifying BCL6, BCL11A (previously reported to interact with BCL6), and PKC-ι (previously unreported interaction), although the predicted error rate for PKC-ι was above the 5% cut-off (Fig. 4). The peptide sequence identified in each scan is underlined in the complete amino acid sequence of the protein.

To select for proteins that interact specifically with the BCL6 protein and not those that associate with the IgG in a nonspecific manner, we subtracted those proteins that were common between the IgG control immunoprecipitation lane (Supplemental Table 1) and the BCL6 immunocomplex lanes. One protein was identified as unique to the SUDHL-9 anti-BCL6 immunocomplex (Supplemental Table 2) and was also subtracted from the final summary of BCL6-interacting proteins. Thus, a total of 139 unique proteins were identified by BCL6 antibody pull-down and tandem mass spectrometry. After subtraction of proteins identified in control lanes, 61 BCL6-interacting proteins were identified with less than 5% error (Table I). These 61 proteins, specific to the BCL6 immunocomplex, are from the following Gene Ontology functional groups: transcription regulator activity (n = 18), binding activity (n = 11), signal transducer activity (n = 10), catalytic activity (n = 8), structural molecule activity (n = 3), enzyme regulator activity (n = 3), transporter activity (n = 2), motor activity (n = 2), chaperone activity (n = 1), and unknown function (n = 3) (Table I). Importantly BCL6 was identified among these proteins along with the previously reported BCL6-interacting proteins BCL11A (29), HDAC5, (15), HDAC9, N-CoR2/SMRT (18–20), and mSin3A (19, 22). The remaining 55 proteins have not been reported previously in the literature to interact with BCL6. A complete listing of identified peptides and corresponding Xcorr scores is provided in Supplemental Table 3. Interestingly several additional proteins known to interact with BCL6 were identified (HDAC2 (23), HDAC4, HDAC7 (15), and N-CoR1 (18)), but the predicted error rates for their identifications exceed the 5% false positive rate threshold.

Validation of MS/MS Data by Western Blotting and Reciprocal Immunoprecipitation—To further validate the authenticity of the proteins identified by MS/MS, we confirmed the presence of some selected proteins within the BCL6 immunocomplex using Western blotting analysis. After anti-BCL6 immunoprecipitation of SUDHL-4 lysates, Western blotting demonstrated bands correlating with two proteins reported previously to interact with BCL6 (HDAC4 and N-CoR2/SMRT) and two novel interactions (PKC-ι and WNK4) (Fig. 5A). The bands were similar to those present in total cell lysates but were not seen in IgG immunoprecipitation controls. In addition, we performed reciprocal immunoprecipitations using SUDHL-4 lysates and polyclonal antibodies to HDAC4 and PKC. Immunoprecipitated proteins were resolved using SDS-PAGE, and bands correlating to BCL6 were identified by Western blotting in both pull-downs (Fig. 5B). Although HDAC4, PKC-ι, and WNK4 are below the error rate cut-off, these validation experiments suggest that they do interact with BCL6, and they are therefore included in Table I.

In Silico Analysis of BCL6-interacting Proteins—To validate potential BCL6-interacting proteins, we carried out in silico proteomic analysis of proteins that have been documented to interact, either directly or indirectly, with BCL6. Using a combination of the publicly available databases OMIM, HPRD, and PubMed, a total of 196 proteins were identified that have been reported to interact either directly or indirectly with BCL6 (Table II). These in silico protein interactions included 19 primary, or direct, interactions with BCL6. The remainder represent indirect interactions with BCL6. For example, HDAC4 is known to interact directly with BCL6, whereas ERK2 has not been reported to interact with BCL6 but does have interactions with HDAC4. Thus, both HDAC4 and ERK2 were summarized as potentially belonging to the BCL6 interactome as primary and secondary interactions, respectively.

Combining In Vitro and in Silico Strategies—A total of five
## Analysis of BCL6-interacting Proteins

### Table I

**Identification of BCL6-interacting proteins by tandem mass spectrometry**

SUMO, small ubiquitin-like modifier; JNK, c-Jun amino-terminal kinase; CLL, chronic lymphocytic leukemia.

| UniProt accession number | Protein | Top Xcorr (charge) | Peptides | Predicted error |
|--------------------------|---------|--------------------|----------|-----------------|
| **Transcriptional regulator activity** | | | | |
| Q9UQL6 | Histone deacetylase 5 | 5.41 (+3) | 3 | 0.0 |
| Q9Y618 | Nuclear receptor co-repressor 2 (SMRT) | 2.87 (+2) | 4 | 0.0 |
| Q9UH73 | Transcription factor COE1 (OE-1) (early B-cell factor) | 1.92 (+1) | 4 | 0.1 |
| Q9UKV0 | Histone deacetylase 9 | 3.42 (+2) | 3 | 0.1 |
| Q9UBK2 | Peroxisome proliferator-activated receptor γ coactivator | 3.06 (+2) | 3 | 0.4 |
| P32519 | ETS-related transcription factor Elf-1 (E74-like factor 1) | 3.03 (+2) | 3 | 1.1 |
| P20823 | Hepatocyte nuclear factor 1 α (HNF-1A) | 3.00 (+3) | 3 | 1.3 |
| P25490 | Transcription factor YY1 | 3.00 (+2) | 3 | 1.3 |
| O00716 | E2F transcription factor 3 | 2.98 (+2) | 3 | 1.3 |
| Q9U182 | B-cell lymphoma 6 protein | 2.96 (+2) | 3 | 1.3 |
| Q9H165 | B-cell CLL/lymphoma 11A | 3.10 (+2) | 3 | 1.7 |
| P17544 | Cyclic-AMP-dependent transcription factor ATF-7 | 2.89 (+2) | 3 | 1.7 |
| Q9UK9 | POU domain, class 2, transcription factor 3 | 2.90 (+2) | 2 | 1.7 |
| Q9497 | SWI/SNF-related (actin-dependent regulator of chromatin subfamily) | 3.14 (+2) | 3 | 1.7 |
| Q9BYK8 | Peroxisomal proliferator-activated receptor A-interacting complex | 1.92 (+3) | 2 | 2.2 |
| Q9BZK7 | Nuclear receptor co-repressor-HDAC3 complex subunit TBLR1 | 3.42 (+3) | 3 | 2.2 |
| Q96T58 | Max2-interacting protein (SMART/HDAC1-associated repressor protein) | 2.12 (+1) | 2 | 3.8 |
| **Q96ST3** | Transcriptional co-repressor Sin3A | 3.03 (+2) | 2 | 4.1 |
| P56524 | Histone deacetylase 4 | 1.92 (+1) | 1 | 17.9 |
| **Binding activity** | | | | |
| Q9Y2K7 | F-box/LRR repeat protein 11 (F-box and leucine-rich repeat protein 11) (trithorax homolog 2) | 3.48 (+2) | 3 | 0.0 |
| Q9UMN6 | Myeloid/lymphoid or mixed lineage leukemia protein 4 (trithorax homolog 2) | 3.17 (+2) | 2 | 0.0 |
| Q9Y228 | TRAF3-interacting JNK-activating modulator | 2.86 (+2) | 3 | 0.0 |
| Q9Y2A4 | Zinc finger protein 443 (Kruppel-type zinc finger protein ZK1) | 2.45 (+1) | 3 | 0.0 |
| Q9ULU4 | Protein kinase C-binding protein 1 (Rack7) | 1.90 (+1) | 6 | 0.1 |
| Q9UBE0 | Ubiquitin-like 1 (sentrin)-activating enzyme E1A | 3.13 (+2) | 5 | 0.4 |
| P26196 | Probable ATP-dependent RNA helicase p54 (oncogene RCK) | 2.87 (+2) | 3 | 1.1 |
| P28715 | DNA repair protein complementing XP-G cells | 3.28 (+2) | 3 | 1.1 |
| Q9H0J9 | Zinc finger CCCH type domain-containing protein 1 | 1.87 (+1) | 3 | 1.7 |
| O95116 | U6 snRNA-associated Sm-like protein LSm1 (small nuclear ribonuclear CaSm) | 3.06 (+2) | 3 | 2.2 |
| Q9BXW6 | Oxysterol-binding protein-related protein 1 (OSBP-related protein 1) | 3.13 (+2) | 3 | 2.7 |
| **Signal transducer activity** | | | | |
| Q9UPT6 | JNK-interacting protein 3 | 3.36 (+2) | 2 | 0.0 |
| Q9ULK4 | Cofactor required for Sp1 transcriptional activation subunit 3 | 2.87 (+2) | 3 | 0.1 |
| Q9UBV4 | Wnt-16 protein | 3.85 (+3) | 3 | 0.1 |
| P28908 | Tumor necrosis factor receptor superfamily member 8 precursor (CD30L receptor) | 2.91 (+2) | 3 | 1.1 |
| Q9H2Z2 | Aryl hydrocarbon receptor nuclear translocator 2 (ARNT protein 2) | 3.03 (+2) | 3 | 1.3 |
| Q9HBW0 | Lysoosphatidic acid receptor Edg-4 (LPA-2) | 3.01 (+2) | 3 | 1.3 |
| O15197 | Ephrin type-B receptor 6 precursor | 3.85 (+3) | 2 | 2.2 |
| O15226 | NF-κB-repressing factor (NFKB-repressing factor) | 3.04 (+2) | 3 | 3.1 |
| Q9H835 | Smoothened homolog precursor (SMO) (Gx protein) | 2.89 (+2) | 3 | 3.1 |
| Q9H67 | P2Y purinoceptor 9 (P2Y9) (purinergic receptor 9) | 2.86 (+2) | 2 | 3.8 |
| Q94806 | Protein kinase C, μ type (EPK2) | 2.43 (+2) | 2 | 12.4 |
| **Catalytic activity** | | | | |
| Q9Y27 | FVYE finger-containing phosphoinositide kinase | 2.94 (+2) | 5 | 0.0 |
| Q9NZJ5 | Eukaryotic translation initiation factor 2-α kinase 3 precursor | 2.93 (+2) | 3 | 0.4 |
| Q9NR4 | Ribonuclease III (RNase III) (p241) | 2.94 (+2) | 3 | 1.2 |
| Q9HB1 | Histone-lysine N-methyltransferase, H3 lysine-9-specific 5 | 2.94 (+2) | 3 | 1.7 |
| Q9CH62 | Smoothened homolog precursor 2 (sentrin/SUMO-specific protease SENP2) | 2.87 (+2) | 3 | 2.0 |
| Q9BXS1 | Isopentenyl-diphosphate Δ-isomerase 2 (IPP isomerase 2) | 3.39 (+2) | 3 | 2.7 |
| Q96RU7 | Neuronal cell death-inducible putative kinase (SKIP3) | 2.85 (+2) | 3 | 4.1 |
| O15270 | Serine palmitoyltransferase 2 | 3.42 (+2) | 2 | 4.6 |
proteins identified by BCL6 antibody pull-down also had reported direct or indirect interactions with BCL6. The previously reported direct BCL6 protein interactions identified in our experiment and BCL6 are bolded in Table I. The remaining 55 proteins not found in the database entries or reported literature may represent novel direct or indirect BCL6 protein interactions.

**DISCUSSION**

This study represents the first global, high throughput functional proteomic approach to determine the identity of proteins that may interact directly or indirectly with BCL6 in multimeric protein complexes. Our analysis of the anti-BCL6 pull-down revealed a total of 61 proteins that were not seen in pull-downs from non-BCL6-expressing cells (SUDHL-9) or using rabbit isotype antibody controls. Importantly BCL6 was identified along with several proteins reported to bind to BCL6, including BCL11A (29), HDAC5 (15), HDAC9, mSin3A (19, 22), and SMRT/N-CoR2 (18, 20). In addition, numerous proteins not known previously to be associated with BCL6 were identified.

Although we report a large number of proteins, we did identify several proteins whose interaction with BCL6 has already been described. Interestingly these known BCL6-interacting proteins were distributed throughout our list, not just among the proteins with the lowest predicted error. In addition, we confirmed using Western blotting, immunoprecipitation, and reciprocal immunoprecipitation our findings of BCL6 interactions with three proteins that fall below the 5% cut-off.

![Western blot and reciprocal immunoprecipitation validation](image)

**Fig. 5.** Western blot and reciprocal immunoprecipitation validation. A, proteins immunoprecipitated from SUDHL-4 lysates using anti-BCL6 were subjected to Western blotting using antibodies to the previously reported BCL6-interacting proteins HDAC4 and N-CoR2/SMRT and the novel interacting proteins PKC-\(\nu\) and WNK4. The bands were similar to those present in total cell lysates but were not seen in IgG immunoprecipitation controls. B, proteins immunoprecipitated from SUDHL-4 lysates using polyclonal antibodies to HDAC4 (previously reported BCL6-interacting protein) and PKC (novel interacting protein) were subjected to Western blotting using anti-BCL6. Bands correlating to BCL6 were identified by Western blotting in both pull-downs but not in the IgG control. **IP**, Immunoprecipitation; **WB**, Western blot.

For example, HDAC4 has been shown previously to interact with BCL6, and we identified it within the BCL6 interactome in our studies albeit at a false positive rate of 17.1%. We further validated the presence of HDAC4 in the BCL6 pull-down by

| UniProt accession number | Protein | Top \(X_{corr}\) (charge) | Peptides | Predicted error % |
|--------------------------|---------|--------------------------|---------|-----------------|
| Structural molecule activity |
| Q9HC56 | Protocadherin 9 precursor | 3.07 (+2) | 3 | 1.2 |
| Q96QZ0 | Pannexin 3 | 1.71 (+1) | 2 | 4.1 |
| Q96PZ7 | CUB and sushi multiple domains protein 1 precursor | 2.87 (+2) | 2 | 4.6 |
| Enzyme regulator activity |
| Q9NRY4 | Glucocorticoid receptor DNA binding factor 1 | 2.20 (+1) | 3 | 0.9 |
| O14495 | Lipid phosphate phosphohydrolase 3 | 2.88 (+2) | 3 | 1.7 |
| Q9BOF6 | Sentrin-specific protease 7 (sentrin/SUMO-specific protease SENP7) | 2.93 (+2) | 3 | 3.1 |
| Transporter activity |
| Q9NR8 | Eukaryotic translation initiation factor 4E transporter (eIF4E transporter) | 3.21 (+2) | 3 | 1.2 |
| O00555 | Voltage-dependent P/Q-type calcium channel \(\alpha\)-1A subunit | 2.97 (+2) | 2 | 1.3 |
| Q9J92b | Serine/threonine protein kinase WNK4 | 1.92 (+1) | 2 | 5.4 |
| Motor activity |
| Q9NYC9 | Ciliary dynein heavy chain 9 (axonomal \(\beta\) dynein heavy chain 9) | 3.04 (+2) | 4 | 0.9 |
| Q9QTC8 | Kinesin-like protein KIF13B (kinesin-like protein GAKIN) | 3.09 (+2) | 4 | 1.2 |
| Chaperone activity |
| Q9UL5 | Heat shock factor protein 4 (HSF 4) | 3.14 (+2) | 3 | 0.1 |
| Unknown function |
| Q9P2L0 | WD repeat protein 35 | 3.39 (+2) | 3 | 0.4 |
| Q9NYH9 | Hepatocellular carcinoma-associated antigen 66 | 2.89 (+2) | 4 | 0.4 |
| Q9NTK5 | Putative GTP-binding protein PTD004 (PRO2455) | 2.86 (+2) | 3 | 0.9 |

* Bold entries indicate previously reported BCL6 interactions.
* Protein identifications confirmed by immunoblotting.

![Analysis of BCL6-interacting Proteins](image)
TABLE II
Identification of BCL6-interacting proteins by in silico proteomics

| Primary | BCL6 | BCL6 co-repressor; BCL-11A; CREB-binding protein (p300); histone deacetylases 1, 2, 4, 5, 6, and 9; interferon-regulatory factor 4; JunB; c-Jun; JunD; mSin3A; N-CoR1; SMRT/N-CoR2; promyelocytic leukemia zinc finger; transcription factor Sp1 |
|---------|------|---------------------------------------------------|
| BCL6 co-repressor | BCL6; copines 1, 2, and 4; histone deacetylase (1, 3, 4, 5, 6, C, and D); MLLT3 |
| Secondary | Nuclear receptor subfamily 2, group F, members 1 and 2 |
| CREB-binding protein | Nuclear receptor coactivator 6, NUP98, p35, p73, E2F, PPARG coactivator 1, proline glutamic acid leucine-rich protein 1, prothymosin α, Prox 1, RBP4, RSK3, -3, -6, Sam68, SEI1, SMAD1, SRCAP, SRC1, SREBP2, STAT1, -2, -6, thymine DNA glycosylase, thyroid hormone receptor coactivator 4, transcriptional coactivator 4, transcription factor 3, TRAP100, TRAP220 |
| HDAC1 | Androgen receptor; ASH2-like; BAZ2A; BCL6 co-repressor; BCH80; brain factor 1; BCL11A; breast cancer metastasis suppressor 1; calcineurin-binding protein 1; CAP bp-interacting 1; CD8 antigen; chromodomain helicase DNA-binding proteins 1, 3, and 4; CREBBP/EP300 inhibitor 2; CTBP1; damage-specific DNA-binding protein 2; Daxx; DNA methyltransferases 1, 3; DNA methyltransferase 3-like protein; DNA topoisomerase II α; Dmmt3a; E1A-binding protein p300; E2F transcription factors 1, 4, and 5; ecotropic viral integration site1; EED; eukaryotic translation initiation factor 3, subunit 10; EZH2; FK506-binding protein 3; Hairless; HSP70; HEY2; HIC1; HMG 20B; HDAC2; -3, -9; histone H3 Lys-9 methyltransferase 1; HMG box transcription factor 1; host cell factor C1; HSP90A; HUS1; inhibitor of growth 1; MAD; MDS1; mesoderm induction early response 1; MTA1, -2, -3; methyl-CpG binding domain proteins 2 and 3; methyl-CpG binding domain protein 3-like 1; mortality factor 4-like 2; mSDS3; mSin3A-associated protein 130; MTG2; myogen differentiation antigen 1, NCOA3, Nedd4-binding protein 2, Nemo-like kinase, neurogenin 1, 4, MafG, Mastermind like 1, melanocyte-specific protein 1, methyl-CpG binding domain protein 2, MLLT3; NFKB1; NFKB3; nuclear receptor-interacting protein 1; nuclear receptor subfamily 2; Mdm2; p130; PBX1; prohibitin; PCNA; protein inhibitor of activated STAT3; prothymosin α; RAD9; RBP1-like protein; replication factors C1 and C4; REST co-repressor; Raf inhibitor; RB1; RBP1, -4, -7; retinoblastoma-like 1 and 2; retinol-binding protein 1; Sal-like protein 1; Sds3; Set1; SHARP; SIN3A; SIN3B; SMAD2; T-cell acute lymphocytic leukemia 1; TGF2; Topo II-α; Sp1; Sp3; WD repeat domain 5; Znf 145; Znf 1A |
| HDAC2 | ASH2-like, BHC110, BCH80; BRCA1; breast cancer metastasis suppressor 1; calcineurin-binding protein 1; CAP bp-interacting 1; CD8 antigen; chromodomain helicase DNA-binding proteins 1, 3, and 4; CREBBP/EP300 inhibitor 2; CTBP1; damage-specific DNA-binding protein 2; Daxx; DNA methyltransferases 1, 3; DNA methyltransferase 3-like protein; DNA topoisomerase II α; Dmmt3a; E1A-binding protein p300; E2F transcription factors 1, 4, and 5; ecotropic viral integration site1; EED; eukaryotic translation initiation factor 3, subunit 10; EZH2; FK506-binding protein 3; Hairless; HSP70; HEY2; HIC1; HMG 20B; HDAC2; -3, -9; histone H3 Lys-9 methyltransferase 1; HMG box transcription factor 1; host cell factor C1; HSP90A; HUS1; inhibitor of growth 1; MAD; MDS1; mesoderm induction early response 1; MTA1, -2, -3; methyl-CpG binding domain proteins 2 and 3; methyl-CpG binding domain protein 3-like 1; mortality factor 4-like 2; mSDS3; mSin3A-associated protein 130; MTG2; myogen differentiation antigen 1, NCOA3, Nedd4-binding protein 2, Nemo-like kinase, neurogenin 1, 4, MafG, Mastermind like 1, melanocyte-specific protein 1, methyl-CpG binding domain protein 2, MLLT3; NFKB1; NFKB3; nuclear receptor-interacting protein 1; nuclear receptor subfamily 2; Mdm2; p130; PBX1; prohibitin; PCNA; protein inhibitor of activated STAT3; prothymosin α; RAD9; RBP1-like protein; replication factors C1 and C4; REST co-repressor; Raf inhibitor; RB1; RBP1, -4, -7; retinoblastoma-like 1 and 2; retinol-binding protein 1; Sal-like protein 1; Sds3; Set1; SHARP; SIN3A; SIN3B; SMAD2; T-cell acute lymphocytic leukemia 1; TGF2; Topo II-α; Sp1; Sp3; WD repeat domain 5; Znf 145; Znf 1A |
| HDAC4 | 14-3-3 protein; BCL6; BCL6 co-repressor; ecotropic viral integration site 1; ERK1; ERK2; HDAC3; HDAC9; HP1α; MEFA2; -G; and -D; N-CoR1; N-CoR2; PLZF; TNPFR-14; TR2 |
| HDAC5 | 14-3-3 protein; calmodulin, estrogen receptor, GATA-1, HP1, MEF2, RIP-140, SMRTE |
| HDAC7 | 14-3-3 protein; calmodulin, estrogen receptor, GATA-1, HP1, MEFA2, N-CoR2, PLZF, TNPFR-14, TR2 |
| HDAC9 | BCL6, CBP, HDAC1, HDAC3, HDAC4, HP1α, mSin3A, MEF2A, N-CoR, PLZF, Smrtin 2, SUMO1, TEL |
| IRF4 | BCL6, BLIMP1, FKBP52, NFA1, SP1, STAT6, SPIB transcription factor |
| JunB | B-ATF, BCL6, BRCA1, fosB protein, c-Fos, FRA1, FRA2, JNK1, NFE2L1, SMAD3, SMAD4 |
| c-Jun | Androgen receptor, ATF1, ATF2, ATF4, BCL3, BCL6, C Ski, c-Fos, c-Maf, CAPER, CBF2, CHOP, CK2, COP9, CREBBP, DNA topoisomerase II, E1A 52R, E-7, early growth response protein 1, En-1, ERG, ERK, estrogen receptor, ETS1, ETS2, FRA1, FRA2, GATA b2, GILZ, glucocorticoid receptor, Hex, HMG AT hook 1, hnRNPI, I, hypoxia-inducible factor 1, IE2 86, JNK1, L18a, MYF3, NDI1II, NFT1, NFE2L1, NFBK3, NFT, nuclear receptor activator 2, nuclear receptor subfamily 5, octamer binding transcription factor 1, P160, p52/54, PKC, Pin1, P11, retinoblastoma 1, Runt 2, SMAD2, SMAD3, SMAD4, SMARC1, SP1, SRC1, STAT1, STAT3, STAT4, TATA box-binding protein 1, T antigen, TFIB, TFIID, TFIIF β, TGIF, transcription factor 20, transcription factor Sp1, Ubcd9, vitamin D receptor, VP16 |
interactions with BCL6. For example, TBLR1 (transducin-like protein 1-related protein) was identified in the BCL6 immunocomplex. Similarly SMRT, WNK4, and PKC-α were identified and validated by Western blotting. Furthermore BCL6 was identified in reciprocal immunoprecipitations using anti-HDAC4 and anti-PKC. We thus chose to retain HDAC4, SMRT, and the two novel BCL6 interactors PKC-α and WNK4 identified in our study.

Many of the proteins previously known to bind BCL6 are members of repressor complexes through which BCL6 participates in transcriptional control. The present study identified 18 proteins with transcriptional regulator activity. Of these proteins, only five were known previously to interact with BCL6. Although the remaining proteins could be novel direct BCL6-binding proteins, many of them represent indirect interactions with BCL6. For example, TBLR1 (transducin β-like protein 1-related protein) was identified in the BCL6 immunocomplex, and it is known, like BCL6, to interact with the SMRT-HDAC1 complex (34). Other known members of co-repressor complexes found in the BCL6 immunocomplex include retinoblastoma-binding protein 1 (RBP1), HDAC5, and HDAC9. Histone methyltransferase 1, which modifies chromatin and can mediate repression, was also identified.

BCL6 is known to associate with the transcription factors c-Jun, JunB, and JunD (27); Sp1 (28); and BCL11A (29), which was identified in the current study. Interestingly JunB was identified but with a predicted false positive rate exceeding 20%. Previously unreported BCL6-interacting transcription factors identified in this study include ATF-7, early B-cell factor, Elf-1, heat shock factor protein 4, hepatocyte nuclear factor-1α, and YY1. In addition to sequence-specific transcriptional repression, BCL6 can mediate repression at promoters that do not contain BCL6 binding sites via interactions with transcription factors that do bind the promoter. For example, the interaction of BCL6 with c-Jun, JunB, and JunD is believed to explain BCL6-mediated repression at AP-1 sites (27). Therefore, the identification of additional transcription factors associated with BCL6 may lead to identification of additional targets of BCL6 in transcriptional regulation.

Although BCL6 is known to interact with transcription factors and play a role in chromatin remodeling, this study identified proteins with more diverse cellular functions within the BCL6 immunocomplex (Fig. 6). These included proteins with signal transducer activity such as NF-κB-repressing factor, LP2A, P2Y9/LPA4, EphpB6 precursor, and Smoothened homolog precursor. The kinases identified here could potentially participate in regulation of BCL6 similar to mitogen-activated protein kinase, which phosphorylates and targets BCL6 for

![Fig. 6. Cellular location and molecular function of BCL6-interacting proteins. The relative proportions of identified proteins are shown for the subcellular localization (A) and functional classification (B).](image-url)
degradation (30). Because BCL6 is known to associate with proteins that modify chromatin, it was interesting to identify proteins that directly modify DNA (XP-G) as well as RNA (RNA helicase, ribonuclelease III, and LS1m).

Our proteomic analysis of BCL6-interacting proteins identified several proteins previously shown to interact with BCL6. Of additional significance, our study also identified a number of proteins that have not been reported previously or found by in silico analysis. These may represent novel proteins that interact with BCL6 and may be regulators of its function. Alternatively many of these proteins may not form direct interactions with BCL6 but may participate in multimeric complexes with BCL6. Many of the database interactions found by in silico analyses were compiled from yeast two-hybrid systems, which report interactions between pairs of proteins with no account for weaker interactions between proteins that can be stabilized by the presence of additional binding proteins in multimeric protein complexes. Our results indicate that a combinatorial approach involving immunoprecipitation-LC-MS/MS followed by validation using orthogonal and in silico methods is useful for identifying direct and indirect protein interactions.

In summary, we identified multiple novel protein interactions with BCL6. This should lead to a more complete understanding of transcriptional repression by BCL6. More importantly, we identified BCL6-interacting partners from diverse classes of proteins that may lead to an expansion of the known cellular roles and/or regulation of BCL6. This information will improve the understanding of BCL6 function and could lead to the identification of potential therapeutic targets in BCL6-associated lymphomas.

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