Differential Functions for the Transcription Factor E2A in Positive and Negative Gene Regulation in Pre-B Lymphocytes[*]

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Abstract

The transcription factors encoded by the *E2A* gene have been shown to play essential roles in the initiation and progression of lymphocyte development. However, there is still a lack of comprehensive understanding of E2A downstream genes in B-cell development. We previously developed a gene tagging-based chromatin immunoprecipitation (ChIP) system to directly evaluate E2A target genes in B-cell development. Here, we have improved this ChIP strategy and used it in conjunction with microarray analysis on *E2A*-deficient pre-B-cell lines to determine E2A target genes in lymphocyte development. Both microarray data and ChIP studies confirmed that E2A directly controls IgH gene expression. The microarray assay also revealed genes that were significantly up-regulated after E2A disruption. ChIP analysis showed that E2A was most likely to be directly involved in repression of some of these target genes such as *Nfil3* and *FGFR2*. An inducible E2A reconstitution system further demonstrated that E2A-mediated repression of *Nfil3* and *FGFR2* was reversible. Collectively, these findings indicate that E2A is a positive regulator for one set of genes and a negative regulator for another set of genes in developing B lymphocytes.

Lymphocytes develop from multipotent hematopoietic stem cells to provide cell-mediated and humoral immunological protection against foreign antigens. Hematopoietic stem cells give rise to mature B- and T-lymphocytes through a series of well defined and highly ordered differentiation stages in the bone marrow and thymus, respectively (1). These stages are functionally defined by the progressive assembly and expression of the lymphocyte antigen receptors. These antigen receptors are generated through tightly regulated genomic recombination events at the B- and T-lymphocyte antigen receptor loci to establish a diverse pool of receptor specificities (2).

Antigen receptor gene recombination and thus lymphocyte differentiation requires the activity of numerous broadly expressed and lineage-specific transcription factors. The basic helix-loop-helix transcription factors encoded by the *E2A* gene have been identified as essential regulators of gene expression during lymphocyte development (3). The E2A proteins were initially characterized as immunoglobulin heavy (IgH)[1] and light chain enhancer-binding factors and have since been shown to bind consensus E-box motifs (CANNTG) in the promoters and enhancers of numerous lymphoid lineage-specific genes (4,5). E2A proteins are highly expressed in lymphoid progenitor populations (6) and are required for the initiation of B-lymphocyte development in the bone marrow. Mice deficient for *E2A* show a complete and

[1]The abbreviations used are: IgH, immunoglobulin heavy; ChIP, chromatin immunoprecipitation; RT, reverse transcription; FACS, fluorescence-activated cell sorting; GFP, green fluorescent protein.

persistent block at the earliest stage of B-cell development prior to the initiation of immunoglobulin heavy chain rearrangements (7,8).

E2A has been implicated in regulating the expression of multiple BCR complex components, including the surrogate light chain $\lambda 5$, the BCR signaling molecule mb-1, and the immunoglobulin heavy chain (IgH) and k light chain genes (9-15). Whereas early ectopic expression and gene targeting studies indicated that these genes were downstream of E2A function, it was not known whether E2A directly regulated their transcription. A gene tagging-based chromatin immunoprecipitation system was recently used to confirm that E2A is physically associated with many of these B-cell-specific genes (16). The ChIP-based approach was also applied to identification of novel E2A targets in B-cell development. However, the total number of E2A target genes recovered in this study is still limited due to low cloning efficiency and binding specificity.

Previous investigations of E2A-deficient mice clearly demonstrated the importance of E2A in lymphocyte development (7,8). However, the strong block in B-lymphocyte development, complex thymocyte phenotype, and poor postnatal survival seen in these mice precluded more detailed studies on functions for E2A in lymphocyte differentiation and lymphoid gene regulation. A conditional *E2A* deletion model was developed in our laboratory to evaluate roles for E2A specifically in developing lymphocytes (17). Mice carrying the *loxp*-flanked *E2A* allele (*E2A^{loxp}*) enabled lineage-restricted deletion of the *E2A* gene based upon ectopic expression of the Cre recombinase. Here we describe a conditional *E2A* deletion system based upon the *E2A^{loxp}* mouse model for studying *E2A*-mediated gene regulation in B-lymphocyte development. We have utilized this conditional *E2A* knockout system in conjunction with our gene tagging-based ChIP strategy to investigate novel functions for E2A proteins in gene regulation during early B-lymphocyte development. Microarray analysis on *E2A*-deficient pre-B-cell lines has revealed numerous genes with E2A-dependent expression patterns. We have further evaluated a subset of these genes that appear to be directly suppressed by E2A in pre-B-cells. Chromatin immunoprecipitation analysis on the regulatory elements of these genes provides the first physiological evidence for direct gene repression by E2A proteins. These studies demonstrate novel functions for E2A proteins in both positive and negative gene regulation during lymphocyte development.

MATERIALS AND METHODS

Pre-B-cell Lines

The Abelson murine leukemia virus-transformed pre-B-cell lines E2A^{FH}1B and E2A^{GFP} were previously derived from bone marrow of mice carrying the dual affinity-tagged *E2A* allele (*E2A^{FH/FH}*) (16) or from *E2A^{GFP/GFP}* mice (18). For gene array studies, pre-B-cell lines were derived from bone marrow of a mouse carrying the *loxp*-flanked *E2A* allele (*E2A^{loxp/loxp}*) (17). Transformed cells were then transduced with a retrovirus encoding Cre recombinase as well as a puromycin resistance cassette. A control cell line was established in parallel by infecting the *E2A^{loxp/loxp}* pre-B-cells with a retrovirus encoding Cre recombinase in the antisense orientation. Transduced cells were selected by puromycin treatment and three different clonal *E2A*-deficient populations were established by limiting dilution plating. All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 55 μ_M 2-mercaptoethanol.

RT-PCR Assay

Total RNA was purified using Trizol reagent (Invitrogen) from cultured Abelson transformed pre-B-cell lines, and cDNA was synthesized with random hexamers. The following oligonucleotide primers were used in PCR analysis of serial 3-fold cDNA dilutions: *E2A*-

E47, *E8* (5′-AGG TCC CAC GCA CGC GCA CC-3′) and *E20* (5′-CGC CTG CTG CAG GAT GAG CA-3′); *EBF*, *EBF for* (5′-TCC AGG AAA GCA TCC AAC GG-3′) and *EBF rev* (5′-TCG TGT GTG AGC AAT ACT CGG C-3′); *Igα*, *mb-1 for* (5′-CCT CTC CTC CTC TTC TTG TCA TAC G-3′) and *mb-1 rev* (5′-CCC CTG TGT TCT TGT TTA CTT CGG-33); *Igβ*, *B29 for* (5′-TGT TCC TGC TGC TGC TCT TCT C-3′) and *B29 rev* (5′-TCG GTG ACA TTA TGG TTG GCG-3′); *Pax-5*, *Pax-5 for* (5′-CCG CCA AAG GAT AGT GGA AAC TTG-3′) and *Pax-5 rev* (5′-CAC AGT GTC ATT GTC ACA GAC TCG C-3′); *EF1α*, *EF1α-for* (5′-AGT TTG AGA AGG AGG CTG CT-3′) and *EF1α-rev* (5′-CAA CAA TCA GGA CAG CAC AGT C-3′); *FGFR2*, *FGFR2A* (5′-GCG CTT CAT CTG CCT GGT CTT GG-3′) and *FGFR2B* (5′-TCC AAC TGA TCA CGG CGG CAT CT-3′); *NFIL3*, *NFIL3A* (5′-AGC GCC GCC TCA ATG ACC TGG TT-3′) and *NFIL3B* (5′-GCT CAC GGC AGC CTT GGA TGT CT-3′).

Flow Cytometry Assay

FACS analysis of the parental *E2A* wild type cells and the *E2A*-deficient lines was performed on a FACSCalibur (BD Biosciences) using the following fluorescent antibody conjugates: CD19-PE and CD43-PE (Pharmingen).

Electrophoretic Mobility Shift Assay

Abelson transformed pre-B-cells were cultured and treated with 1 μ_M tamoxifen dissolved in Me_2SO or Me_2SO alone as indicated. Nuclei were isolated, and nuclear extracts were prepared as previously described (19). Extracts were incubated with ^{32}P-labeled $\mu E5$ oligonucleotide probe with or without anti-E2A monoclonal antibody YAE (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and resolved on a 5% polyacrylamide gel containing Walsh TBE buffer. Gels were dried and exposed to a phosphor screen (Amersham Biosciences) for analysis.

Microarray Analysis

RNA was isolated from three independent *E2A*-deficient pre-B-cell lines and the antisense Cre-infected control cell line using Trizol solution followed by isopropyl alcohol precipitation. RNA was then resuspended in diethyl pyrocarbonate-treated distilled H_2O for global differential gene expression analysis using the Affymetrix murine microarray (U74Av2). All samples were reverse-transcribed, analyzed, and processed by the Duke University Microarray Core Facility. Subsequent data analysis was conducted with the R statistical package using the Bioconductor library (20). Briefly, gene expression levels were quantified by the gcRMA model ($n = 3$). The p values from the t test were adjusted by false discovery rate (21) for multiple comparisons.

Chromatin Extracts and Immunoprecipitations

Chromatin extracts were prepared using a modified version of the protocol described by Fernandez *et al.* (22). Approximately 5×10^7 pre-B-cells (E2A[FH]1B and E2A[GFP]) were harvested and fixed by adding one-tenth culture volume of fixing buffer (11% formaldehyde, 100 m$_M$ NaCl, 50 m$_M$ Tris-HCl, pH 7.9). After fixing for 15 min rocking gently at room temperature, one-twentieth volume of 2.5 $_M$ glycine quenching buffer was added, and the cells were incubated for 5 min rocking at room temperature (all subsequent steps were performed at 4 °C). Fixed cells were harvested by centrifugation, washed with PBS, resuspended in 15 ml of Triton buffer (10 m$_M$ Tris-HCl, pH 8.0, 0.25% Triton X-100), and incubated on ice for 15 min. Cells were then harvested, resuspended in 15 ml of NaCl buffer (10 m$_M$ Tris-HCl, pH 8.0, 200 m$_M$ NaCl) and incubated for an additional 15 min on ice. Washed cells were harvested, resuspended in 1 ml of sonication buffer (10 m$_M$ Tris-HCl, pH 8.0, 1% SDS; supplemented with phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin), and sonicated (Fisher Scientific 550 tapered microtip probe, setting 4.5) for 14 cycles of 25 s on a cold block with

15 s of cooling between each cycle. Sonicates were centrifuged at $14,000 \times g$ for 10 min, and bulk chromatin extracts were harvested as supernatants and stored at $-80\,°C$.

E2A-bound DNA fragments were isolated from chromatin extracts using either the previously described anti-FLAG one-step purification protocol (16) or the new dual epitope isolation protocol. For dual epitope purifications, bulk chromatin extracts (2 mg) from the E2A^{FH}1B pre-B-cell line and the E2A^{GFP} control cell line were thawed on ice, and one-tenth volume of $5\times$ extraction/wash buffer (250 m$_M$ NaH$_2$PO$_4$, pH 7.0, 1.5 $_M$ NaCl) was added to each. Samples were then incubated with $100\,\mu$l of washed Talon cobalt affinity resin for 30 min rotating slowly at room temperature. Bound resin was then harvested by centrifugation, washed twice for 10 min each with $1\times$ extraction/wash buffer (50 m$_M$ NaH$_2$PO$_4$, pH 7.0, 300 m$_M$ NaCl), and transferred to gravity flow chromatography columns (Bio-Rad polyprep 0.8×4.0 cm). After the resins settled, the column end caps were removed, and the resins were washed with 3 ml of $1\times$ extraction/wash buffer. Bound protein-DNA complexes were then eluted three times with $500\,\mu$l of 100 m$_M$ EDTA, pH 8.0, and the eluted materials were collected into 15-ml conical tubes.

Secondary anti-FLAG immunoprecipitations were then performed on the eluted fractions from the Cobalt affinity resin (or on bulk chromatin extracts for single-epitope purifications). Eluted solutions were diluted 1:10 with ChIP buffer (140 m$_M$ NaCl, 100 μg/ml bovine serum albumin, 100 μg/ml yeast tRNA, 1% Triton X-100, 1 m$_M$ phenylmethylsulfonyl fluoride) and incubated with $50\,\mu$l of washed anti-FLAG-conjugated M2-agarose (Sigma) for 2 h at $4\,°C$ rotating slowly. Bound agarose was harvested by centrifugation, washed once with 10 ml of ChIP buffer, and then transferred to microcentrifuge tubes. The bound agarose was washed twice with 1 ml of ChIP buffer, twice with 1 ml of ChIP buffer containing 500 m$_M$ NaCl, twice with 1 ml of wash buffer (10 m$_M$ Tris-HCl, pH 8.0, 250 m$_M$ LiCl, 1 m$_M$ EDTA), and twice with 1 ml of TE buffer (10 m$_M$ Tris-HCl, pH 8.0, 1 m$_M$ EDTA). Bound DNA was eluted for 15 min at 65 °C in $250\,\mu$l of elution buffer (50 m$_M$ Tris-HCl, pH 8.0, 10 m$_M$ EDTA, 1% SDS), and both bound and input DNA samples were adjusted to 0.5% SDS in $500\,\mu$l. Samples were incubated at $65\,°C$ overnight to reverse cross-links and then RNase-treated, deproteinized, and precipitated as described by Fernandez *et al.* (22). Processed DNA fragments were resuspended in $50\,\mu$l of distilled water.

PCR Analysis of Immunoprecipitated DNA

PCR screening for enrichment of target regulatory regions was performed using a slightly modified version of the original protocol (16). Serial 3-fold dilutions of input chromatin and immunoprecipitated DNA were PCR-amplified for 32–34 cycles (94 °C for 45 s, 57 °C for 45 s, 72 °C for 45 s, with a 2-min final extension at 72 °C) in $20\,\mu$l of PCR buffer containing 1.5 m$_M$ MgCl$_2$ and *Platinum Taq* polymerase (Invitrogen). PCR samples were then resolved on 1% agarose gels and visualized by ethidium bromide staining. Previously published oligonucleotide sequences were used in PCR screening of IgH enhancers and *λ5* and *mb-1* promoters (16). The following primer sequences were used to screen for E2A binding to novel target loci in ChIP-PCR analysis: *FGFR2-A* (5′-TGG TGG CTT GCG GCT GTC CAC TT-3′) and *FGFR2-B* (5′-CAG AGA GGC TGT GCC TCC AGA GC-3′); *pNfil3-A* (5′-GCC ACT GAA GTA GAA GGG TTT GT-3′) and *pNfil3-B* (5′-GTC CCT TGG GCA GCT GTG TGC TA-3′).

Inducible E2A Reconstitution System

293T cells were cotransfected with the GFP-expressing E47-estrogen receptor fusion construct MigR1-E47R (23) along with the vesicular stomatitis virus glycoprotein coat protein and gag/pol. Retroviral supernatant was harvested, concentrated by ultracentrifugation, and used to transduce an *E2A*-deficient clonal pre-B-cell line. Transduced cells were selected by FACS

based upon GFP expression to establish a stable bulk population of E47R-reconstituted cells. For induction of E47R activity, cells were cultured in the presence of 1 μ_M tamoxifen (resuspended in Me_2SO) for 5 h.

RESULTS

A conditional *E2A* deletion mouse model was previously established in our laboratory by introducing the *loxp*-flanked *E2A* allele (*E2A^{loxp}*) into the genomic *E2A* locus (17). This *E2A^{loxp}* model was adapted to facilitate studies on E2A-mediated gene regulation in B-lymphocyte development. Pre-B-cell lines were first derived by transforming *E2A$^{loxp/loxp}$* bone marrow with the Abelson murine leukemia virus. Abelson-transformed pre-B-cells were then transduced with a retrovirus encoding a puromycin resistance cassette along with the Cre recombinase in either the sense (*E2A*-deleted cell lines) or antisense (control line) orientation. Transduced cells were selected by puromycin treatment, and three independent clonal *E2A*-deficient populations were subsequently established. Complete deletion of *E2A* in each clone was confirmed by genomic PCR specific for either the deleted or undeleted alleles (Fig. 1*A*). The absence of E2A expression in each *E2A*-deficient cell line was confirmed by RT-PCR analysis (Fig. 1*B*). FACS analysis demonstrates that *E2A*-deficient B-cells maintain expression of the surface antigens CD43 and CD19, indicating that these cells retain a phenotype indicative of pro- or early pre-B-cells (Fig. 1*C*). In order to confirm that *E2A$^{-/-}$* Abelson B-cells maintain their global B-cell gene expression profile, we evaluated the relative transcript levels of important B-cell-specific genes. Transcript levels of the genes *mb-1* and *B29*, which encode the B-cell receptor-associated signaling molecules Igα and Igβ, respectively, were not significantly altered by *E2A* deletion (Fig. 1*D*). Expression levels of the *Pax-5* gene, which encodes the B-cell-specific transcription factor BSAP and EBF, another important B-cell transcription factor, were also unaffected by *E2A* deletion. These data strongly indicate that *E2A* deficiency in pre-B-cells does not cause an overall shift in cell identity away from the B-cell lineage.

Total RNA was isolated from three independent *E2A*-deficient pre-B-cell lines, and gene expression levels were compared with that of the antisense Cre-transduced control cell line using Affymetrix microarrays. These microarray experiments revealed numerous genes with E2A-dependent expression patterns in the deficient pre-B-cell lines. For the initial analysis, we chose to use these relatively high stringent criteria: 1) average -fold change larger than 3; 2) average difference larger then 48 intensity units; 3) adjusted *p* value smaller than 0.01; and 4) detectable hybridization signal in at least one of the four hybridizations. This analysis yielded 32 independent probe sets that are down-regulated and 35 that are up-regulated due to *E2A* disruption (Table I). The previously characterized E2A target gene immunoglobulin heavy chain and a number of novel punitive E2A target genes were significantly down-regulated in the absence of *E2A* (Table I, upper section).

The expression of another subset of genes was found to be dramatically up-regulated in the absence of E2A (Table I, lower section). These up-regulated genes represented nearly one-half of the genes with significant and consistent changes in E2A-dependent expression in the pre-B-cell lines. Although E2A is generally considered a positive regulator of transcription, these findings suggested that E2A proteins might be involved in the lineage-specific repression of certain genes as well.

E2A-dependent gene expression profiling was combined with chromatin immunoprecipitation analysis to further characterize E2A target genes in B-lymphocyte development. We previously utilized a gene tagging-based ChIP system (E2A^{FH}) to investigate physiological E2A interaction within numerous genomic regulatory regions in pre-B-cells (16). However, the anti-FLAG purification procedure used in our original ChIP study incorporated only one of the two

affinity tags expressed on the E2A^{FH} fusion protein. To further improve the specificity of the E2A ChIP, a dual epitope purification methodology was developed for more selective isolation of E2A-bound DNA fragments from the pre-B-cell line E2A^{FH}1B (this line carries dual affinity-tagged E2A alleles) (16). The His and FLAG tags were sequentially used in the affinity purification of E2A bound chromatin. We first evaluated this revised protocol for E2A interaction with the regulatory regions of the $\lambda5$ surrogate light chain and the *mb-1* genes (13,14). Physiological E2A interactions were previously demonstrated at the promoters for both *$\lambda5$* and *mb-1* by ChIP-PCR screening (16). As expected, these regulatory regions immunoprecipitated with E2A with high specificity in the dual epitope ChIP assay (Fig. 2*B*).

Although E2A was previously shown to regulate transcription and rearrangement within the IgH locus our original ChIP study generated ambiguous results regarding E2A interaction with the IgH enhancers (16). It was therefore important to clarify the potential for E2A interaction with these regulatory regions using the two-step purification strategy. One intronic enhancer (*Eμ*) and several 3′ enhancers, including *hs3a*, *hs3b*, and *hs4*, contribute to the regulation of the IgH locus during B-lymphocyte development (Fig. 2*A*) (24-26). We first screened for E2A binding within the IgH intronic enhancer, which had previously shown only slight enrichment due to a high background signal (16). Significant enrichment of *Eμ* was observed after dual epitope purification, whereas immunoprecipitated E2A^{GFP} chromatin extract generated minimal background signal across this regulatory region (Fig. 2*C*). The new two-step purification procedure dramatically improved the purity of the isolated DNA fragments, thereby enhancing our ability to discern E2A association with target regulatory regions. Similar results were obtained for the IgH 3′ enhancer *hs3b*, which was also significantly enriched in sequentially purified E2A^{FH}1B DNA (Fig. 2*C*).

Consistent with the results obtained in the original ChIP study, the IgH 3′ enhancer *hs3a* was not enriched after dual epitope purification (Fig. 2*D*). However, the two-step purification approach eliminated the high background signal observed in the first study and therefore established with greater certainty the lack of detectable E2A binding at this enhancer. This finding indicated that either E2A interaction with the *hs3a* enhancer was below the threshold of sensitivity for our dualepitope ChIP assay or that E2A does not interact with this IgH enhancer region in the pre-B-cell lines. Interestingly, the IgH 3′ enhancer *hs4* also showed no detectable enrichment after dual epitope purification of E2A-bound DNA fragments (Fig. 2*D*). Although this enhancer appeared to be slightly enriched in the original ChIP study, the high background signal again raised doubt as to whether this minimal enrichment was actually indicative of association with E2A (16). The lack of detectable *hs4* sequence enrichment and clean background further emphasized the utility of the dual purification strategy in elucidating true genomic targets for physiological E2A interaction.

Few studies thus far have investigated the role of E2A in suppression of gene transcription. Once we established the efficacy of the dual-epitope ChIP, we used this method to evaluate the role of E2A in mediating gene repression. ChIP analysis was performed on several genes shown to be up-regulated in E2A-deficient pre-B-cells in order to determine whether they were direct targets for physiological regulation by E2A proteins. Nfil3 (also known as E4BP4) and FGFR2 (fibroblast growth factor receptor 2) were chosen for further characterization based on their functional relevance to B-cell development. Transcription of the basic region/leucine zipper transcription factor Nfil3 was up-regulated in each of three E2A-deficient pre-B-cell lines, although this probe set on the microarray failed to meet the stringent criteria used to create Table I. The *Nfil3* promoter region contains five canonical E-box sequences (CANNTG), which could represent potential E2A-binding sites, including two adjacent E-boxes within 100 bp of the transcription start site (Fig. 3*A*). We therefore sought to determine whether E2A was directly involved in Nfil3 repression in pre-B-cells. Single or dual epitope purifications were carried out on E2A^{FH}1B and E2A^{GFP} pre-B-cell chromatin extracts to evaluate the potential

for E2A association with the *Nfil3* locus. Significant E2A interaction with the *Nfil3* promoter region was detected by dual epitope ChIP analysis (Fig. 3*B*). As seen with the IgH enhancers, the dual epitope purification significantly reduced the background signals seen after only one round of purification. In fact, significant enrichment was only detectable at the *Nfil3* promoter after the dual epitope purification.

We also investigated the potential for E2A interaction with the regulatory elements of *FGFR2*. Microarray analysis indicated consistent up-regulation of *FGFR2* in the absence of E2A expression (Table I). Sequence analysis identified 13 E-box motifs within the 1-kb region upstream of the FGFR2 gene transcription start site (Fig. 3*A*). Significant E2A interaction was detected within the *FGFR2* promoter region by dual epitope ChIP analysis, suggesting that E2A may be directly involved in *FGFR2* repression in the pre-B-cell lines (Fig. 3*B*).

Gene expression studies using $E2A^{-/-}$ pre-B-cells reconstituted with an inducible human E47-estrogen receptor ligand binding domain fusion protein (E47R) were used to complement the ChIP assays in order to gain a clearer picture of which genes are actively suppressed by E2A (23). A retroviral vector encoding E47R-IRES-GFP was stably transduced into one of our *E2A*-deficient pre-B-cell clones (Fig. 4*A*). The E47R fusion protein exhibits very low activity due to the fused estrogen repressor domain, but E2A DNA binding activity can be strongly induced in the presence of the drug tamoxifen (Fig. 4*B*). Semiquantitative RT-PCR was conducted on RNA isolated from cells reconstituted with E47R and cultured in the presence or absence of tamoxifen. These samples were compared with control cells ($E2A^{+/+}$) and the untransduced *E2A*-deficient ($E2A^{-/-}$) pre-B-cell clone to determine whether E2A activity influences repression of the putative target genes analyzed by ChIP. E47R mediated the transcriptional suppression of the *Nfil3* and *FGFR2* genes in a tamoxifen-dependent manner (Fig. 4*C*, E47R(+) and E47R(−) compared with $E2A^{-/-}$). Tamoxifen alone is not sufficient to suppress the expression of Nfil3 and FGFR2, since the treatment of $E2A^{-/-}$ cells with tamoxifen does not decrease the expression of these genes (Fig. 4*D*). *FGFR2* and *Nfil3* each exhibited E2A-dependent suppression of transcript levels and were shown to have physiologic interaction of E2A with their promoter regions, suggesting that these genes may be true targets of E2A-mediated gene suppression. We have also identified additional genes that exhibit physiologic E2A interaction with their promoter regions and increased expression in $E2A^{-/-}$ cells but are not suppressed by activated E47R (data not shown). This illustrates that expression profiling or ChIP alone may not be completely reliable for predicting true targets of E2A. However, when these two assays are combined together, we believe that the probability of identifying true E2A target genes is dramatically improved.

DISCUSSION

We have successfully generated a stable E2A-deficient pre-B-cell line to allow us for the first time to directly evaluate the function of E2A-mediated gene regulation in the context of developing B lymphocytes. Previous work has identified genes such as *EBF*, *Pax-5*, *mb-1*, *TdT*, *RAG1*, immunoglobulin heavy chain, and immunoglobulin light chain as likely downstream targets of E2A regulation (8,16). Each of these genes plays an important role either to regulate B lymphocyte development or produce a functional B-cell antigen receptor. Microarray analysis helped confirm that immunoglobulin gene expression was indeed perturbed by E2A deficiency. Surprisingly, many previously defined E2A target genes, although slightly decreased, were still expressed at relatively high levels compared with wild type controls, indicating that sustained expression of these genes is not dependent on E2A. These results suggest that E2A is not absolutely required for maintaining the expression of these B-lineage-specific genes once B-cell development has progressed to pre-B-cell stage.

The two-tiered approach of gene expression analysis and chromatin immunoprecipitation analysis provides a highly effective system for characterizing numerous E2A target genes. Here we have verified E2A-mediated regulation of the immunoglobulin heavy chain locus by combining gene expression analysis with ChIP-based chromatin binding studies. The dual epitope purification approach significantly enhances our ability to determine target sequence enrichment and thus E2A interaction with the IgH locus as well as numerous other lymphoid regulatory regions. Clarification of E2A association with the IgH enhancers will facilitate future studies on the regulation of immunoglobulin gene transcription and rearrangement by E2A and other transcription factors. E2A protein association with these enhancers might contribute to the stage-specific activation of IgH locus accessibility, transcription, and rearrangement during B-lymphocyte differentiation. The absence of even the earliest IgH gene rearrangements in bone marrow E2A-deficient mice may be explained in part by the lack of E2A binding within the IgH enhancers. These findings may also have mechanistic significance in the context of earlier observations regarding E2A-induced IgH transcription and rearrangement in non-B-cells (9,15).

The study identified a novel group of genes whose expression is negatively affected by E2A expression. Few studies thus far have evaluated the potential for E2A-mediated gene repression. One such study has demonstrated the potential for E2A proteins to function as repressors of E-cadherin expression through direct interactions with E-box-containing promoter sequences (27). E-cadherin down-regulation is thought to be a critical event during embryonic development and in the acquisition of invasive properties in carcinoma tumor progression. E2A was isolated from a yeast one-hybrid screen to identify factors directly involved in mediating E-cadherin repression. Subsequent studies on E2A and the zinc finger protein Snail suggested that these two transcription factors might coordinate E-cadherin down-regulation both during embryonic epithelial-mesenchymal transitions and at the onset of tumor metastasis. E2A has also been implicated in regulating the activity of a cell type-specific repressor within the CR2/CD21 proximal promoter (28). CR2 functions as the receptor for complement C3 activation fragments and is expressed on immature and mature B-cells, epithelial cells, and select subsets of thymocytes, peripheral T cells, and dendritic cells. Mutagenesis experiments have shown that the CR2 proximal promoter contains a cell type-specific repressor element involved in controlling CR2 expression. Interestingly, the promoter contains two functional E-box sites: one of these (E box 1) is required for transcriptional activation, whereas the other (E box 2) is essential for transcriptional repression (28). E2A has been shown to bind E box 2 within the repressor element of the CR2 promoter, suggesting that E2A might be involved in CR2 repression during early B-lymphocyte development. The expression patterns of E2A and CR2 correlate well with this model, since E2A protein activity is high in pro- and pre-B-cells and is then down-regulated at the onset of CR2 expression in immature B-cells (18).

Here we have established a potential direct role for E2A-mediated suppression of *Nfil3* and *FGFR2*. The observation that E2A interacts with genomic sequences within these loci is intriguing; however, it is still unclear how these interactions may influence gene repression. The E47R reconstitution system further demonstrates that E2A-mediated gene repression is reversible for *Nfil3* and *FGFR2*. Previously published studies on the functions of proteins encoded by these genes may provide clues as to the significance of the gene expression and ChIP data. Nfil3 has been implicated in the cytokine-mediated survival of pro-B lymphocytes, and IL-3 signaling has been shown to control Nfil3 expression in murine pro-B-cell lines (29-31). Interestingly, Nfil3 has also been identified as one of four basic region/leucine zipper transcription factors that can competitively bind the consensus sequence recognized by the E2A-HLF oncoprotein. It is likely that the v-Abl oncogene provides sufficient survival and proliferation signals to preclude the need for significant Nfil3 expression in Abelson pre-B-cell lines. However, E2A has been previously shown to be a potent proapoptotic transcription

factor (32). It is temping to speculate that active suppression of the *Nfil3* gene by E2A may be one mechanism through which E2A mediates its proapoptotic activity.

FGFR2 has been implicated in the differentiation and activation of multiple hematopoietic cell lineages, including B-lymphocytes and myeloid cells (33-35). Basic fibroblast growth factor functions as the ligand for FGFR2 and is highly expressed in the bone marrow (33). Interestingly, *FGFR2* expression is not detectable on bone marrow stem cells but is significantly up-regulated in myeloid progenitor populations (33,34). Thus *FGFR2* induction may occur as a consequence of myeloid lineage commitment to regulate subsequent differentiation events. FGFR2 up-regulation in E2A-deficient pre-B-cells may provide insight into how E2A controls B-cell lineage commitment not only through the activation of B-cell specific genes but possibly through the suppression of alternate developmental programs. Interestingly, E2A-deficient hematopoietic progenitors retain the ability to differentiate into multiple lineages that include myeloid cells, raising the possibility that suppression of alternate cell fate decisions may be a mechanism by which E2A mediates B-cell lineage commitment (36).

E2A is likely to play complex roles in the stage- and lineage-specific repression of numerous other genes during cell differentiation. Previous studies on E2F transcription factors have demonstrated the utility of combining ChIP-based studies with microarray-based gene expression analysis (37). We now show that this is also an effective approach to define E2A targets. E2A-mediated repression of certain genes is likely to be required for lineage restriction during lymphocyte differentiation. Other genes are probably repressed by E2A in a stage-specific manner to coordinate dynamic protein expression patterns at different stages of lymphocyte development and activation. Thus, a detailed and comprehensive characterization of E2A-repressed genes should contribute to a more complete understanding of E-protein function in lymphocyte development.

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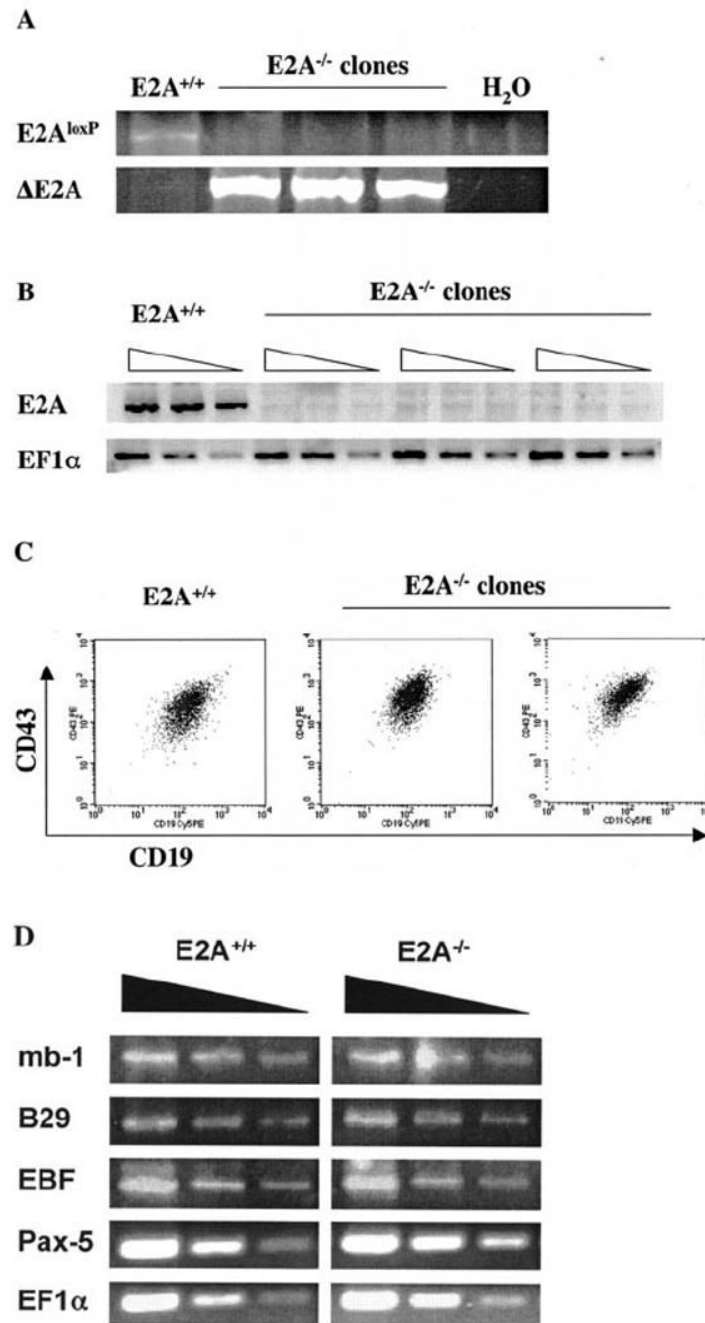
FIG. 1. Analysis of E2A-deficient Abelson pre-B-cells
A, genomic PCR of control and Cre-transduced Abelson B-cells to detect the unrearranged
(*E2A^{loxp}*) and deleted (Δ*E2A*) conditional E2A allele (17). *B*, RT-PCR analysis of total RNA
from control Abelson pre-B-cells and three E2A$^{-/-}$ pre-B-cell lines. PCR for E2A (E47) and
EF1α expression was performed on serial 3-fold dilutions of cDNA from each cell line. *C*,
FACS analysis of E2A$^{+/+}$ and E2A$^{-/-}$ pre-B-cell lines. Cells were analyzed for surface
expression of the common pro-/pre-B-cell markers CD19 and CD43. *D*, expression of B-cell-
specific genes in E2A$^{-/-}$ cell lines. Semiquantitative PCR was used to assess the relative
expression levels of *mb-1*/Igα, *B29*/Igβ, *EBF*, and *Pax-5*/BSAP in control and E2A$^{-/-}$ cell lines.
EF1α expression was used to verify equivalent sample loading.
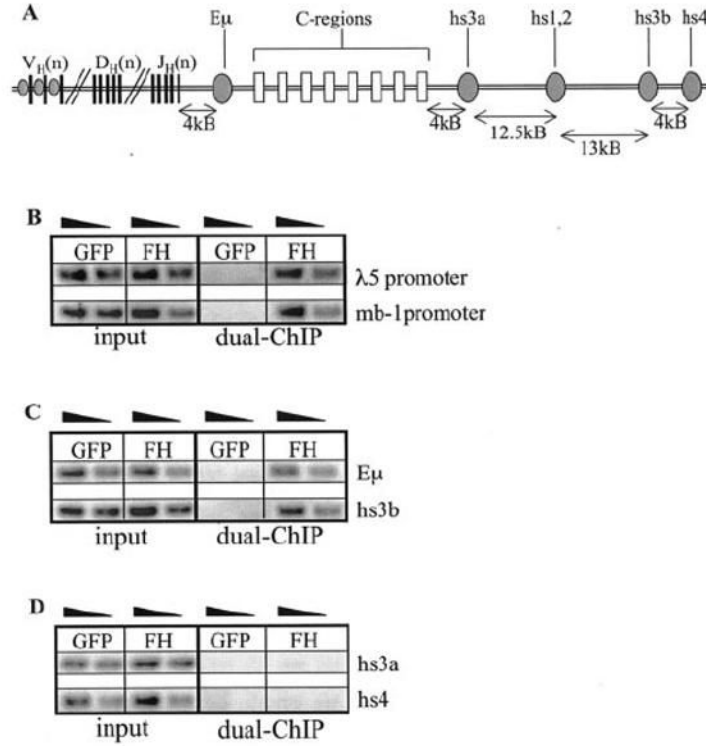
FIG. 2.
A, schematic representation of the immunoglobulin heavy chain locus. IgH variable (*V*), diversity (*D*), and joining (*J*) exon clusters are shown as *vertical boxes*. V-region promoters are indicated by *small ovals*. Constant (*C*) region exons are shown as *filled boxes*. IgH intronic (*Eμ*) and 3′ enhancer regions are represented by *large ovals. B–D*, dual epitope ChIP-PCR screen for E2A interaction with target regulatory regions in pre-B-cells. Serial 3-fold dilutions of input chromatin and dual ChIP DNA were PCR-amplified using primers covering potential E2A binding sites within the indicated regulatory regions. *B*, E2A association with the *lambda-5* and *mb-1* promoters. *C*, E2A association with the IgH intronic (*Eμ*) and *hs3b* enhancers. *D*, no detectable E2A interaction with the 3′ IgH enhancers *hs3a* and *hs4*.
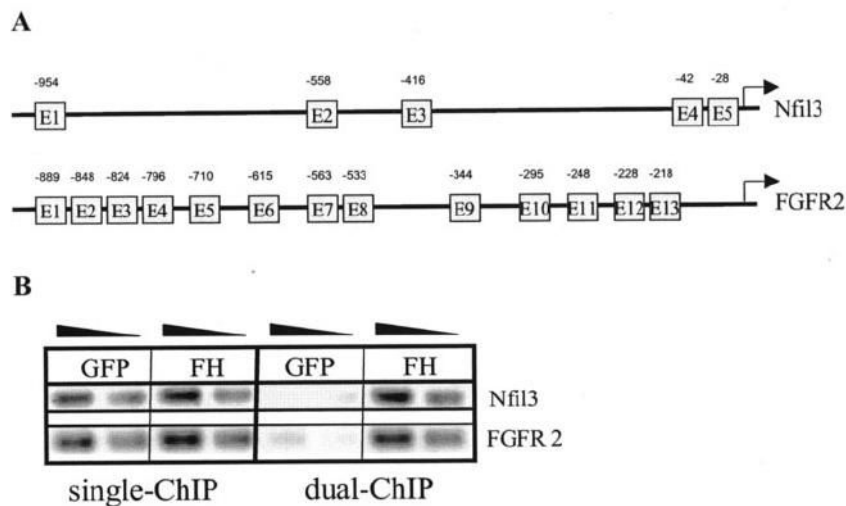
A

B

FIG. 3. Chromatin immunoprecipitation analysis of genomic regulatory elements within potential E2A-repressed genes

A, promoters and 5′ regulatory regions of *Nfil3* and *FGFR2*. E-box motifs (CANNTG) representing potential E2A binding sites are indicated, including approximate positions with respect to transcription start sites or the 5′ end of published cDNA. *B*, ChIP-PCR screen on 3-fold dilutions of E2A-bound DNA fragments from single (FLAG only) and dual epitope purifications. E2A interacts with the *Nfil3* promoter and the 5′ upstream regions of the *FGFR-2*.
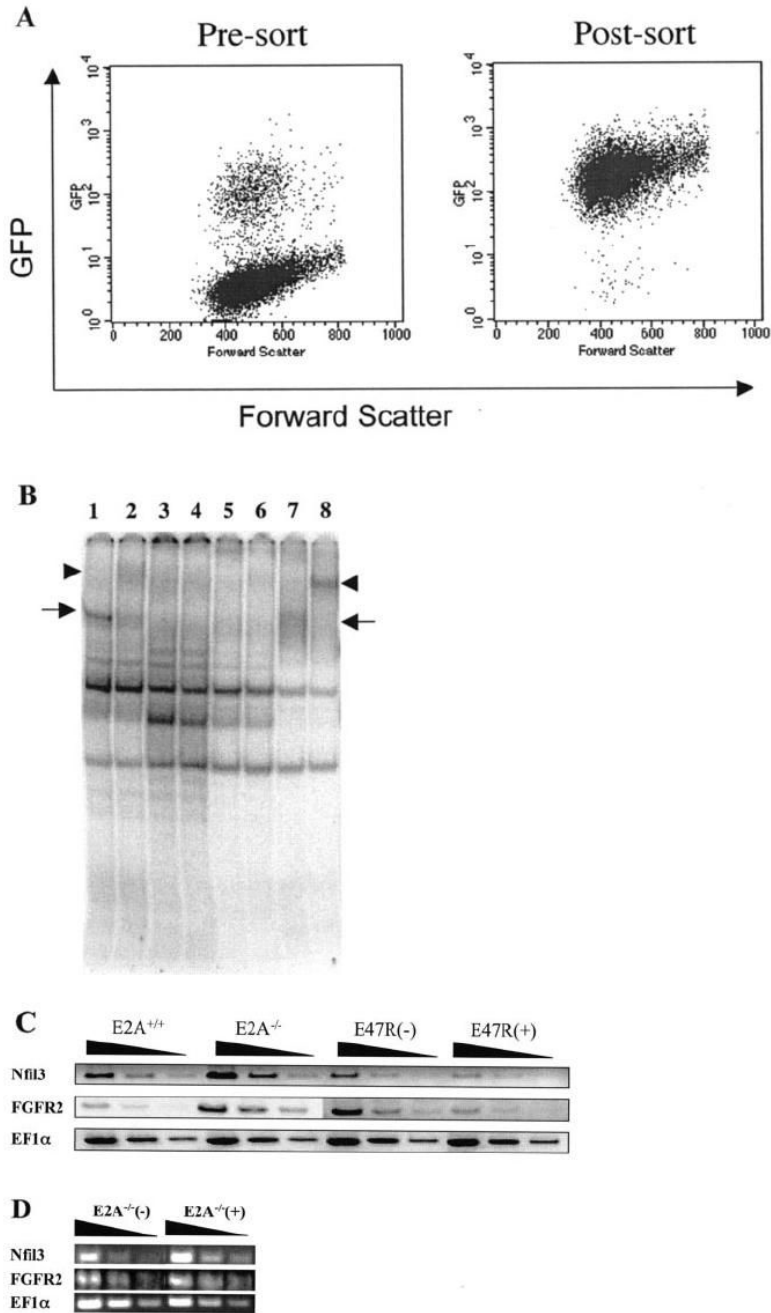
FIG. 4. Modulated expression of E2A-repressed genes in an inducible E2A reconstitution system
A, E2A$^{-/-}$ pre-B-cells were infected with E47R-IRES-GFP retrovirus (*left plot*), and GFP$^+$ cells were sorted and cultured (*right plot*). *B*, tamoxifen treatment of E47R reconstituted B-cells restores inducible E2A DNA binding activity. Nuclear extracts from E2A$^{+/+}$, E2A$^{-/-}$, and E47R reconstituted pre-B-cells were incubated with labeled μE5 oligonucleotide probe in the presence (*even-numbered lanes*) or absence (*odd-numbered lanes*) of a pan-E2A monoclonal antibody (YAE). Specific DNA binding is indicated by the *arrows*, and the supershifted band is shown by *arrowheads*. E2A DNA binding activity was detected in extracts from E2A$^{+/+}$ (*lanes 1* and *2*) and E47R reconstituted cells treated with 1 μM tamoxifen (*lanes 7* and *8*) but not E2A$^{-/-}$ (*lanes 3* and *4*) or 0.1% Me$_2$SO-treated E47R-reconstituted cells

(*lanes 5* and *6*). *C*, RT-PCR analysis of total RNA isolated from pre-B-cell lines with either functional E2A (E2A$^{+/+}$), deleted E2A (E2A$^{-/-}$), or E2A$^{-/-}$ cell line reconstituted with E47R fusion protein. Reconstituted cells were treated with either 0.1% Me$_2$SO (*E47R*(−)) or 1 μM tamoxifen in Me$_2$SO (*E47R*(+)) for 5 h to activate the E47R protein. Samples are presented as a series of serial 3-fold dilutions. *D*, tamoxifen treatment in the absence of E47R does not suppress gene expression. RT-PCR analysis of total RNA from E2A$^{-/-}$ pre-B-cells mock-treated with 0.1% Me$_2$SO (−) or treated with 1 μM tamoxifen in Me$_2$SO (+) for 5 h. Each sample is presented as a series of 3-fold serial dilutions.

TABLE I

Summary of microarray analysis

Shown is a summary of microarray analysis on E2A-deficient pre-B cell lines. Global gene expression profiles from three independent E2A-deficient Abelson pre-B cell clones were analyzed using Affymetrix microarrays (U74Av2) and compared with the gene expression profile from E2A$^{+/+}$ (antisense Cre-transduced) pre-B cells. The table indicates transcripts whose expression was significantly altered (average 3-fold or greater) across three E2A-deficient pre-B cell clones compared with the E2A$^{+/+}$ pre-B cell line. Transcripts down-regulated 3-fold or greater in the E2A-deficient cell lines are shown in the upper section of the table. The lower section of the table includes transcripts up-regulated 3-fold or greater in the E2A-deficient cell lines.

Accession number	Gene name	Change	*p* value
		-fold	
AF099973	Schlafen 2	−261.7	0.000123
X04653	Lymphocyte antigen 6 complex, locus A	−222.1	5.58E-05
AA204265	B lymphoid kinase	−112.4	3.04E-05
AA795946	Immune associated nucleotide 1	−101.6	0.000143
X98471	Epithelial membrane protein 1	−81.1	8.09E-05
X02466	Unknown mRNA	−68.7	0.001694
X03453	Unknown mRNA	−65.5	0.000141
AI844939	CREBBP/EP300-inhibitory protein 1	−53.9	6.21E-05
X02463	Unknown mRNA	−48.9	0.002875
AI844839	RIKEN cDNA 6330442E10 gene	−48.2	0.000444
X00496	Ia-associated invariant chain	−26.5	0.002311
M30903	B lymphoid kinase	−24.7	0.000245
AF099973	Schlafen 2	−22.6	0.000535
U81453	Myosin VIIa	−22.4	0.000337
X67210	Unknown (protein for MGC:68300)	−22	0.002151
AI835274	Cytoplasmic FMR1-interacting protein 2	−19.3	0.000711
AF065324	Immunoglobulin heavy chain (J558 family)	−18.1	0.000273
AJ007970	Guanylate nucleotide-binding protein 2	−12.6	0.012706
L38444	T-cell-specific GTPase	−12	0.011238
U09816	GM2 ganglioside activator protein	−11.6	0.002324
AI553536	RIKEN cDNA E030024M05 gene	−6	0.007887
M38700	Thyroid autoantigen	−5.6	0.001055
L29441	Tumor differentially expressed 1	−5.3	0.004501
AW125333	Ceramide kinase	−5	0.003614
AI852553	Thymosin, β 10	−3.9	0.016597
J02980	Alkaline phosphatase 2, liver	−3.7	0.011843
M35244	Histocompatibility 2, T region locus 10	−3.3	0.000239
U72519	Ena-vasodilator-stimulated phosphoprotein	−3.2	0.005328
AI046826	Growth factor receptor-bound protein 2-associated protein 1	−3.1	0.006082
AW125526	RIKEN cDNA 0610038D11 gene	−3.1	0.004102
U10410	Recombinant antineuraminidase single chain Ig VH and VL domains	−3	0.002839
AI850401	DNA segment, Chr 15, Wayne State University 75, expressed	−3	0.009169

Accession number	Gene name	Change	*p* value
		-fold	
M34141	Prostaglandin-endoperoxide synthase 1	15.1	0.010314
U04354	Scinderin	11.8	0.004649
AF019385	Heparan sulfate (glucosamine) 3-*O*-sulfotransferase 1	8.2	0.000513
M13352	Thymidylate synthase	8.1	0.005353
U92454	WW domain binding protein 5	7.4	0.00198
D12646	Kinesin family member 4	7.2	0.000119
AA590345	RIKEN cDNA C330027C09 gene	7.2	0.008416
AW123907	Kelch domain containing 2	5.6	0.00714
U80932	Serine/threonine kinase 6	5.4	0.01059
AF002823	Budding uninhibited by benzimidazoles 1 homolog (*S. cerevisiae*)	5.1	0.006987
U19596	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	5.1	0.001504
AW123269	RIKEN cDNA 2900037I21 gene	4.6	8.83E-06
AA184423	Unknown mRNA	4.5	0.00087
U83902	MAD2 (mitotic arrest-deficient, homolog)-like 1 (yeast)	4.5	0.004558
X64713	Cyclin B1	4.4	0.008331
L29480	Serine/threonine kinase 18	4.3	0.003255
X75483	Cyclin A2	4.3	0.006751
AJ223293	Kinesin family member 11	4.1	0.001694
AA007891	Kinesin family member 2C	4	0.01095
AI853802	Phosphofructokinase, platelet	3.9	0.005044
U13174	Solute carrier family 12, member 2	3.9	0.004716
M86377	Ttk protein kinase	3.8	0.008107

Accession number	Gene name	Change	*p* value
M23362	Fibroblast growth factor receptor 2	3.8	0.001661
AW122347	Rac GTPase-activating protein 1	3.8	0.002574
L11316	*ect2* oncogene	3.8	0.015746
M15668	Phosphoglycerate kinase 1	3.7	0.016656
X82786	Antigen identified by monoclonal antibody Ki 67	3.7	0.004989
U01915	Topoisomerase (DNA) II alpha	3.5	0.003634
Z35401	H2A histone family, member X	3.4	0.003817
AA275196	RIKEN cDNA 2610201A12 gene	3.3	0.015762
X66032	Cyclin B2	3.3	0.011732
M25944	Carbonic anhydrase 2	3.2	0.008015
U28656	Eukaryotic translation initiation factor 4E-binding protein 1	3.1	0.007679
AB017189	Solute carrier family 7+B100, member 5	3	0.009212
X54327	Glutamyl-prolyl-tRNA synthetase	3	0.013704