RNA Interference Knockdown of hU2AF³⁵ Impairs Cell Cycle Progression and Modulates Alternative Splicing of Cdc25 Transcripts[□]

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Submitted January 13, 2006; Revised July 10, 2006; Accepted July 11, 2006

Monitoring Editor: Karsten Weis

U2AF is a heterodimeric splicing factor composed of a large (U2AF⁶⁵) and a small (U2AF³⁵) subunit. In humans, alternative splicing generates two U2AF³⁵ variants, U2AF^{35a} and U2AF^{35b}. Here, we used RNA interference to specifically ablate the expression of each isoform in HeLa cells. Our results show that knockdown of the major U2AF^{35a} isoform reduced cell viability and impaired mitotic progression, leading to accumulation of cells in prometaphase. Microarray analysis revealed that knockdown of U2AF^{35a} affected the expression level of ~500 mRNAs, from which >90% were underrepresented relative to the control. Among mRNAs underrepresented in U2AF^{35a}-depleted cells we identified an essential cell cycle gene, *Cdc27*, for which there was an increase in the ratio between unspliced and spliced RNA and a significant reduction in protein level. Furthermore, we show that depletion of either U2AF^{35a} or U2AF^{35b} altered the ratios of alternatively spliced isoforms of *Cdc25B* and *Cdc25C* transcripts. Taken together our results demonstrate that U2AF^{35a} is essential for HeLa cell division and suggest a novel role for both U2AF³⁵ protein isoforms as regulators of alternative splicing of a specific subset of genes.

INTRODUCTION

Higher eukaryotes generate functional mRNAs by accurately removing noncoding sequences (introns) from pre-mRNAs—a process termed RNA splicing. This intron excision is carried out by an assembly of small nuclear ribonucleoprotein particles (snRNPs) and other, non-snRNP, splicing factors that are collectively recruited to pre-mRNAs to form the spliceosome (reviewed in Jurica and Moore, 2003).

The initial events of spliceosome assembly require recognition of specific sequences at the 5' and 3' splice junctions, as well as other sequences in the pre-mRNA. In both yeast and mammals, the 5' splice site is initially recognized by U1 snRNP, whereas the 3' end of introns in higher eukaryotes is recognized by the U2 auxiliary factor (U2AF). U2AF is a heterodimer composed of a large (U2AF⁶⁵) and a small (U2AF³⁵) subunit. U2AF⁶⁵ binds to the pyrimidine-rich (Py) tract upstream of the 3' splice junction (Zamore *et al.*, 1992), and U2AF³⁵ binds to the AG dinucleotide at the 3' splice site (Merendino *et al.*, 1999; Wu *et al.*, 1999; Zorio and Blumenthal, 1999a).

In contrast to U2AF⁶⁵, which is essential for splicing, U2AF³⁵ is dispensable for in vitro splicing of some model pre-mRNAs

containing strong Py tracts (i.e., a stretch of pyrimidines beginning at position-5 relative to the 3' splice site and extending 10 or more nucleotides upstream into the intron (Burge *et al.*, 1999). The presence of U2AF³⁵ and its interaction with U2AF⁶⁵ was however found essential for in vitro splicing of a pre-mRNA substrate with a Py tract that deviates from the consensus (Guth *et al.*, 1999). Introns with nonconsensual or weak Py tracts were previously called “AG-dependent” (Reed, 1989). Biochemical complementation experiments performed with extracts depleted of endogenous U2AF demonstrated that splicing of AG-dependent introns was rescued only when both U2AF subunits were added and not with U2AF⁶⁵ alone (Zuo and Maniatis, 1996; Guth *et al.*, 1999; Wu *et al.*, 1999).

The importance of the small subunit of U2AF in vivo was first shown by the finding that the fruit fly *Drosophila melanogaster* ortholog of human U2AF³⁵ (dU2AF³⁸) is essential for viability (Rudner *et al.*, 1996). Orthologues of U2AF³⁵ are also essential for the viability of the fission yeast *Schizosaccharomyces pombe* (Wentz-Hunter and Potashkin, 1996) and the nematode *Caenorhabditis elegans* (Zorio and Blumenthal, 1999b) and for the early development of zebrafish (Golling *et al.*, 2002). Two more recent studies in *Drosophila* further provided hints of a role for dU2AF³⁸ in splicing regulation. First, loss-of-function mutations in dU2AF³⁸ affected splicing of the pre-mRNA encoding the female-specific RNA-binding protein Sex-lethal (Nagengast *et al.*, 2003). Second, depletion of the small subunit of U2AF by RNA interference (RNAi) affected alternative splicing of the *Dscam* gene transcript (Park *et al.*, 2004).

To determine whether U2AF³⁵ plays any vital role in mammalian cells, we used RNAi to specifically ablate its expression

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06-01-0036>) on July 19, 2006.

[□] The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

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in HeLa cells. Because in higher vertebrates primary transcripts encoding U2AF³⁵ can themselves be alternatively spliced to generate two protein isoforms termed U2AF^{35a} and U2AF^{35b} (Pacheco *et al.*, 2004), we designed small inhibitory RNA (siRNA) duplexes specifically to knockdown expression of each isoform. Our results reveal an essential function of the major U2AF^{35a} isoform in cell division and suggest that both U2AF³⁵ protein isoforms act as regulators of alternative splicing of a specific subset of genes.

MATERIALS AND METHODS

Cell Culture and siRNA Transfection

HeLa cells (ECACC 93021013) were grown as monolayers in minimum essential medium with Earle's salts supplemented with 10% (vol/vol) fetal calf serum and 1% (vol/vol) nonessential amino acids (Invitrogen, Paisley, Scotland). siRNA duplexes designed to specifically silence the expression of U2AF^{35a} and b isoforms were synthesized as 21-mers with 3' dTdT overhangs (Elbashir *et al.*, 2001b). The sequences used to target each U2AF³⁵ isoform were as follows: h35a: 5'-CCA UUG CCC UCU UGA ACA UdTdT-3' and h35a': 5'-CCC UCA AAA CUC UUC CCA GdTdT-3', which target the U2AF^{35a} isoform-specific exon 3 (GenBank accession number NM_006758, nt172–190 and nt200–218, respectively), and h35b: 5'-CCA UCU UGA UUC AAA ACA UdTdT-3' and h35b': 5'-UCC CCA AAA CAG UGC ACA GdAdC-3', which target the U2AF^{35b} isoform-specific exon Ab (GenBank accession number AJ627978, nt164–182 and nt192–210, respectively). siRNA targeting Ski-interacting protein (SKIP) sequence was 5'-AGG GUA UGG ACA GUG GAU UdTdT-3' (GenBank accession number NM_012245, nt1274–1292). Synthetic sense and antisense oligonucleotides (BioSpring, Frankfurt, Germany) were annealed in 100 mM potassium acetate, 30 mM HEPES-KOH (pH 7.4), 2 mM magnesium acetate for 1 min at 95°C and 1 h at 37°C and frozen. In a typical siRNA transient transfection, 35-mm-diameter Petri dishes were seeded with 6×10^4 cells on the day before transfection. Twenty-four hours later, siRNA duplexes were transfected using 5 μ l Lipofectin (Invitrogen) according to the supplier's recommendations (150 nM duplex siRNA per well).

Microscopic Analysis

To analyze cell viability, cells were trypsinized, washed in phosphate-buffered saline (PBS), and resuspended in 0.2% trypan blue. Apoptosis was assayed using the In situ Cell Death detection kit (Roche Diagnostics, Indianapolis, IN). To determine the mitotic index, cells were trypsinized, fixed, and then made to adhere to coverslips. For immunofluorescence microscopy, cells grown on coverslips were rinsed briefly in PBS, fixed in 3.7% formaldehyde: PBS for 10 min at room temperature, and washed with PBS. Cells were then permeabilized with 0.5% (wt/vol) Triton X-100:PBS for 10 min at room temperature, and washed with PBS. DNA was either stained with the vital dye Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) or with TO-PRO-3 iodide (Molecular Probes, Eugene, OR). Immunofluorescence and confocal microscopy were performed as previously described (Custodio *et al.*, 2004). The following primary antibodies were used: mouse monoclonal antibodies directed against α -tubulin (clone B-5-1-2; Sigma), cyclin B and Cdc27 (clone 18 and clone 35, respectively; BD Biosciences Pharmingen, San Diego, CA), and a human serum directed against the kinetochore proteins CENP-A/C (kindly provided by Professor W. van Venrooij, Nijmegen, The Netherlands). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

Flow Cytometry

Cell cycle analysis was performed by flow cytometry in accordance with standard protocols. Cells were harvested and fixed in ice-cold 70% (vol/vol) ethanol for 2 h at 4°C. Before flow cytometry, fixed cells were washed in PBS, resuspended in DNA staining solution (propidium iodide [PI, 20 μ g/ml]; RNase A [1 mg/ml]; 0.1% [vol/vol] Triton X-100 in PBS), and incubated at room temperature for 30 min. DNA content was measured using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using the CellQuest software (BD Biosciences). Cell aggregates were gated out of the analysis based on the width of the PI fluorescence signal and each profile was obtained from ~20,000 gated events. The percentage of G1, S, and G2/M phase populations were calculated with ModFitLT cell cycle analysis software package (Verity Software House, Topsham, ME).

Immunoblotting

Total cell protein extracts were prepared by scraping cells into SDS-PAGE buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β -mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) with 200 U/ml benzonase (Sigma-Aldrich), incubating for 10 min at room temperature, and then boiling for 5 min. For Western blot analysis, 15 μ g of total protein extract was separated on

8, 10, or 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Western blotting was carried out following standard procedures. The following primary antibodies were used: mouse monoclonal antibodies directed against α -tubulin (clone B-5-1-2; Sigma), Cdc27 and Cdc25B (clone 35 and clone 23, respectively; BD Biosciences Pharmingen), and rabbit polyclonal serums directed against α -actin (provided by Prof. Ira Herman, Tufts University, Boston, MA) and U2AF³⁵ (kindly provided by Prof. Tom Maniatis, Harvard University, Cambridge, MA). Immunoblots were developed using horseradish peroxidase-coupled secondary antibodies and detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Microarray Analysis

At 48 h after siRNA treatment, 10^7 cells were harvested, and total RNA was extracted. Hybridization and scanning of human U133A GeneChip Arrays (Affymetrix, Santa Clara, CA) were performed as recommended by the manufacturer. The complete list of genes induced or repressed by each siRNA treatment is presented in Supplementary Table 1.

RT-PCR and Real-Time Quantitative PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen), and treated with RNase-free DNase I (Roche Diagnostics). RT-PCR reactions were random primed and cDNA was produced using Superscript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. PCR products were separated by gel electrophoresis and detected by ethidium bromide staining. All primer sequences are presented in Supplementary Table 2. qRT-PCR reactions were performed in the ABI7000 Sequence Detector (Applied Biosystems, Foster City, CA). The relative abundance of each gene was calculated from standard curves of CT versus quantity of mRNA obtained from a serial dilution of cDNA produced from human universal RNA (Applied Biosystems, Bulletin 2) and using U6 small nuclear RNA as an endogenous reference. The relative expression of different isoforms of the same gene was calculated using a derivative of the $2^{-\Delta\Delta C_t}$ method as described (Schmittgen *et al.*, 2003). qRT-PCR analysis of U2AF^{35a} and U2AF^{35b} expression was performed as previously described (Pacheco *et al.*, 2004) using GADPH as an endogenous reference (TaqMan gene expression assay 4333764T, Applied Biosystems, Foster City, CA) and the noninterfered sample as the calibrator.

RESULTS

RNAi Knockdown of Human U2AF³⁵ Isoforms

Previously, we have shown that primary transcripts encoding splicing factor U2AF³⁵ in higher vertebrates are alternatively spliced (Pacheco *et al.*, 2004). At least two different protein isoforms are derived from the human U2AF1 gene: U2AF^{35a}, the translation product of a primary transcript containing exon 3, and U2AF^{35b}, which arises from an alternatively spliced transcript in which exon Ab replaces exon 3 with no alteration of the reading frame (Figure 1A). The U2AF^{35a} isoform is 9–18-fold more abundant than U2AF^{35b}, depending on the tissue analyzed (Pacheco *et al.*, 2004). In this study, we designed siRNA duplexes specifically to knockdown expression of either the U2AF^{35a} isoform containing exon 3 (h35a) or the U2AF^{35b} isoform containing exon Ab (h35b; Figure 1A). Analysis by BLAST search confirmed that the selected sequences were unique to U2AF³⁵. Two different oligonucleotides were used to target each isoform (see *Materials and Methods*), and similar results were obtained with both. As a negative control, we used the GL2 dsRNA, which targets the firefly luciferase gene (Elbashir *et al.*, 2001a).

To confirm the efficiency and specificity of the RNAi knockdown experiments, we performed RT-PCR on total cellular RNA extracted 24, 48, and 72 h after transfection with the oligonucleotides. Reverse transcriptase reactions were random-primed, and PCR was carried out using primers flanking the alternatively spliced U2AF³⁵ regions. The amplified DNA was digested with HinfI, which specifically cleaves inside exon Ab of isoform b, thus generating a smaller fragment that can be resolved from isoform a. The results obtained 72 h after transfection are shown in Figure 1B. When cells were treated with h35a siRNA alone, the levels of U2AF^{35a} mRNA significantly decreased, whereas

Figure 1. Isoform-specific U2AF³⁵ RNAi. (A) Schematic representation of the human *U2AF1* gene and the alternative splicing patterns that give rise to isoforms U2AF^{35a} and U2AF^{35b} (a and b, in the figure); exons are represented by boxes and introns by lines. The regions targeted by the siRNAs are indicated. (B) RT-PCR analysis. Total RNA was extracted from cells treated for 72 h with the siRNAs against isoforms U2AF^{35a} and/or U2AF^{35b} (lanes 3–5) or with no siRNA (lane 2). RT-PCR amplification was carried using primers flanking the alternatively spliced regions. The amplification products were then digested with *HinfI* to resolve isoforms a and b. The exon structure of each product is illustrated on the right. GADPH amplification was used as an internal control. Molecular weight markers are indicated on the left (lane 1). (C) Expression of U2AF^{35a} and U2AF^{35b} mRNAs determined by quantitative real time PCR. Results were normalized to GADPH mRNA and are expressed relative to the mRNA levels in mock-transfected cells; results are presented as means $\pm$ SEM; the data were validated using the Student's *t* test ($p < 0.05$). A schematic representation of gene-specific PCR primers (arrows) and TaqMan probes (thick lines) used to amplify and detect each isoform is indicated below; boxes represent exons. (D) Western blot analysis of HeLa cell lysates prepared 72 h after mock transfection (lane 1) or transfection with siRNAs against isoforms U2AF^{35a} and/or U2AF^{35b} (lanes 2–4). The blot was probed with antibodies against U2AF³⁵ and α -actin.

U2AF^{35b} mRNA levels were unaffected (Figure 1B, compare lanes 2 and 3). Similarly, after treatment of HeLa cells with h35b siRNA, only the levels of U2AF^{35b} mRNA decreased compared with mock-transfected cells (Figure 1B, lanes 2 and 4). As expected, simultaneous transfection with h35a

and h35b siRNAs caused a significant decrease of both U2AF³⁵ isoforms (Figure 1B, lane 5). We also analyzed U2AF³⁵ mRNA expression by quantitative RT-PCR using primers and TaqMan probes designed to target specifically each isoform (Pacheco *et al.*, 2004). This confirmed the effi-

Figure 2. U2AF³⁵-depletion inhibits cell proliferation, induces cell death by apoptosis, and causes a mitotic delay or arrest. Cells were treated with either control siRNA (GL2) or siRNAs against U2AF^{35a} and/or U2AF^{35b} and analyzed at the indicated time points. (A) Effects on cell proliferation; the total number of viable cells is depicted. (B) Effects on cell viability. The proportion of viable cells relative to the total cell number is indicated. (C) Effects on apoptosis (n ≥ 400 cells per experiment). (D) Cell cycle analysis by FACS. (E) Proportion of mitotic cells (n ≥ 1500 cells per experiment). (F) Proportion of living cells that remain in mitosis after a 45-min period (n ≥ 150 mitotic cells per experiment). Results are presented as means ± SD for at least three independent experiments; * p < 0.05 relative to control (Student's *t* test).

cient and specific reduction of each mRNA isoform relative to the levels in mock-transfected cells (Figure 1C).

Western blot analysis of control mock-transfected cells showed a single band corresponding to U2AF³⁵ (Figure 1D, lane 1). After treatment with siRNAs, however, two closely migrating bands were detected (Figure 1D, lanes 2–4): the major, lower band corresponded to the most abundant protein isoform a, whereas the minor, upper band corresponded to the less abundant protein isoform b. Because isoform a is expressed at much higher level, treatment with siRNAs directed against U2AF^{35a} caused a significant decrease in the lower band intensity (Figure 1D, lanes 2 and 4), whereas siRNAs directed against U2AF^{35b} almost abolished expression of the minor, upper band (Figure 1D, lanes 3 and 4).

From these data we conclude that siRNA can specifically repress the expression of U2AF^{35a} and U2AF^{35b} isoforms at both the mRNA and protein levels.

Depletion of U2AF^{35a} Inhibits Cell Proliferation and Causes Cell Death by Apoptosis

To determine whether U2AF³⁵ proteins are essential, we transfected HeLa cells with GL2 (negative control), h35a and h35b siRNAs and analyzed them 24, 48, and 72 h after transfection. The number of viable and nonviable cells was counted after staining with trypan blue. The results show that knockdown of either U2AF^{35a} or U2AF^{35b} impaired cell proliferation (Figure 2A); however, knockdown of U2AF^{35a}, alone or in conjunction with U2AF^{35b}, had a more drastic effect on cell number than

knockdown of U2AF^{35b} alone. We further observed that at 72 h after transfection, knockdown of U2AF^{35a} reduced the proportion of viable cells to ~64%, whereas knockdown of U2AF^{35b} had no significant effect when compared with treatment with the control siRNA (Figure 2B). We further observed that the fraction of apoptotic cells increased about threefold in U2AF^{35a}-depleted cells, whereas depletion of U2AF^{35b} had no significant effect on apoptosis (Figure 2C). Interestingly, simultaneous knockdown of U2AF^{35a} and U2AF^{35b} induced more cell death than knockdown of U2AF^{35a} alone (Figure 2C), arguing that U2AF^{35b} may play an additional accessory role important for cell survival.

Cells Depleted of U2AF^{35a} Accumulate in G2/M Phase

As knockdown of U2AF^{35a} impaired cell proliferation, we asked whether this splicing factor is involved in cell cycle progression. To address this question, exponentially growing HeLa cells were treated with GL2, h35a, and h35b siRNAs and analyzed by flow cytometry 24, 48, and 72 h after transfection. As depicted in Figure 2D, knockdown of U2AF^{35a} (either alone or in conjunction with U2AF^{35b}) induced a significant increase the proportion of G2/M phase cells ($22.0 \pm 1.1\%$ of cells transfected for 72 h with h35a siRNA compared with $14.1 \pm 1.1\%$ of control cells). This was paralleled by a decrease in the proportion of G0/G1 phase cells in the population ($53.7 \pm 1.4\%$ of cells transfected with h35a siRNA compared with $61.6 \pm 0.4\%$ of control cells), whereas the proportion of S cells was unaltered. Although knockdown of U2AF^{35b} alone had no significant effect on the distribution of cells through the cell cycle, the simultaneous repression of both isoforms caused an even greater increase in the proportion of G2/M phase cells when compared with knockdown of U2AF^{35a} alone (Figure 2D).

To further investigate the effects of depleting U2AF³⁵ on the cell cycle, RNAi-treated cells were stained with a DNA dye. Cells with decondensed chromosomes were scored as interphase, whereas cells at any stage from prophase to telophase were scored as mitotic. As shown in Figure 2E, the proportion of mitotic cells increased from $6.0 \pm 0.6\%$ of control cells to $12.4 \pm 0.9\%$ of cells transfected with h35a and to $16.7 \pm 2.74\%$ of cells transfected with h35a and h35b siRNAs. Knockdown of U2AF^{35b} alone did not significantly affect the mitotic index (Figure 2E). To further study the effect of U2AF³⁵ on mitosis, we analyzed living cells growing on grided coverslips 24, 48, and 72 h after transfection with the same siRNAs. Rounded cells with condensed chromosomes and a smooth, uniform membrane were scored as mitotic, and their positions were noted on a coverslip replica grid. After 45 min, the same cells were reobserved. The total number of cells that had progressed through cytokinesis and those that were still in mitosis was estimated. As depicted in Figure 2F, knockdown of U2AF^{35a}, but not of U2AF^{35b}, induces an increase in the proportion of cells that do not progress through cytokinesis, suggesting a mitotic delay.

To determine more accurately at which phase of mitosis the cells depleted of U2AF^{35a} were delayed, double-labeling experiments were performed using a DNA dye to visualize the chromosomes and antibodies against α -tubulin to visualize spindle microtubules or against kinetochore protein CENP-A/C to visualize the site of attachment of the spindle microtubules to the chromosomes (Figure 3A). The proportion of cells in different mitotic stages was estimated (Figure 3B). In both control and U2AF^{35b}-depleted cells, most mitotic cells were in metaphase when all chromosomes and kinetochores are aligned in the center of a bipolar spindle. By contrast, most mitotic cells in populations depleted of U2AF^{35a} (either alone or in conjunction with U2AF^{35b}) have chromosomes and ki-

netochores distributed all along an abnormally elongated spindle. To identify this mitotic stage unambiguously, we performed immunofluorescence microscopy using antibodies to B-type mitotic cyclins (Figure 3A), which are degraded at the metaphase-anaphase transition (Murray, 2004). The mitotic cells depleted of U2AF^{35a} and with unaligned chromosomes still contained B-type cyclins, indicating that they were most probably still in prometaphase, when chromosomes shuffle back and forth between the poles and the center of the spindle. In parallel, knockdown of U2AF^{35a} alone or in conjunction with U2AF^{35b} induced a significant decrease in the proportion of mitotic cells in metaphase, anaphase, and telophase (Figure 3B), as expected if cells were delayed (or arrested) in prometaphase. Our data additionally show that simultaneous knockdown of both U2AF^{35a} and U2AF^{35b} induced the appearance of mitotic cells with a monopolar spindle (Figure 3A, d and h). These cells were only detected at late time points after siRNA treatment (60–72 h), when they represent up to 13% of the mitotic cells (Figure 3C).

Taken together, the data indicate that knockdown of U2AF^{35a} (but not U2AF^{35b}) causes an accumulation of mitotic cells at prometaphase, whereas simultaneous knockdown of U2AF^{35a} and U2AF^{35b} further induces the appearance of mitotic cells with abnormal spindle morphology.

Expression Profile Analysis of Cells Depleted of U2AF^{35a} and U2AF^{35b} Isoforms

A possible explanation for the cell division phenotypes observed is that depletion of U2AF³⁵ leads to impaired pre-mRNA processing of genes essential for cell division, in turn resulting in depletion of the corresponding mRNAs and proteins. To further explore this possibility, we performed a genome-wide analysis of the effect of depleting U2AF^{35a} and U2AF^{35b} on the expression of the HeLa cell transcriptome. Affymetrix oligonucleotide microarrays representing 14,500 well-characterized human genes were used. The relative abundance of each transcript was analyzed 48 h after transfection with siRNAs targeting U2AF^{35a}, U2AF^{35b}, or both. As reference samples, total RNAs were isolated from mock-transfected cells. Two independent RNAi experiments were performed and the efficiency of U2AF³⁵ depletion was confirmed by RT-PCR. When U2AF^{35a} was knocked down, the levels of 572 mRNAs were significantly different from the reference sample (i.e., at least 1.5-fold different in the two independent experiments). Most mRNAs ($n = 525$; 92%) were underrepresented, whereas 47 (8%) were overrepresented (Supplementary Table 1, A and B, respectively). In contrast, when U2AF^{35b} was knocked down, only 138 mRNAs were changed (73% underrepresented and 27% overrepresented; see Supplementary Table 1, C and D, respectively). Simultaneous depletion of U2AF^{35a} and U2AF^{35b} affected the levels of 378 mRNAs (92% underrepresented and 8% overrepresented; see Supplementary Table 1, E and F, respectively). To highlight the differences in mRNA expression patterns in cells depleted of each U2AF³⁵ isoform, we selected mRNAs that were at least twofold different in the cells depleted of one isoform when compared with the reference cells and analyzed their expression levels in the cells that were either depleted of the other isoform or simultaneously depleted of both isoforms (Figure 4, A and B). Thus, Figure 4, A and B, depicts subgroups of the mRNAs listed in Supplementary Table 1, for which more stringent cutoff values were used. The vast majority of mRNAs (>90%) altered in cells depleted of U2AF^{35a} were also affected in cells depleted of both U2AF^{35a} and U2AF^{35b}, but their levels remained largely unchanged in cells depleted of U2AF^{35b} (74%, <1.5-fold difference). From all mRNAs al-

Figure 3. U2AF³⁵-depleted cells are arrested at prometaphase. (A) Immunofluorescence analysis of U2AF³⁵-depleted cells. HeLa cells were transfected with control (GL2) or h35a and h35b siRNAs, as indicated. The cells were fixed 72 h after transfection and labeled with antibodies against α -tubulin (green staining) and TOPRO-3 iodide (red staining) for DNA (a–d); with antibodies against kinetochore proteins CENP-A/C (red staining) and α -tubulin (green staining; e–h), or with antibodies against cyclin B (green staining) and TOPRO-3 iodide (red staining) for DNA (i–l). Bar, 10 μ m. (B) Proportion of mitotic cells in each stage of mitosis 60 h after transfection ($n \geq 450$ mitotic cells per experiment). (C) Proportion of mitotic cells with a monopolar spindle 60 h after transfection ($n \geq 450$ mitotic cells per experiment). Results are presented as means $\pm$ SD for at least three independent experiments; * $p < 0.05$ relative to control (Student's t test).

tered in cells depleted of U2AF^{35b}, 25 (46%) were also affected in cells depleted of U2AF^{35a} (Figure 4B). Most of the mRNAs whose expression was altered specifically by either U2AF^{35a} or U2AF^{35b} knockdown and whose genes could be

identified corresponded to functional groups such as cell cycle and cell proliferation genes, signal transduction and transcription factors, DNA damage and stress response genes, or metabolic pathway components. However, neither

Figure 4. Depletion of U2AF³⁵ isoforms specifically affects expression of different subsets of genes. Hierarchical clustering of transcripts that were at least 2.0-fold overrepresented (red) or 2.5-fold underrepresented (green) in U2AF³⁵a-depleted cells (A) or U2AF³⁵b-depleted cells (B), relative to mock-transfected cells, in two independent experiments. Each mRNA is colored according to its average expression level and the color bar indicates fold changes. The number of mRNAs displayed is indicated below the panel. Genes and knockdown experiments were clustered applying Cluster (Eisen *et al.*, 1998). (C) Quantitative real-time PCR (qRT-PCR) validation of microarray data. Total RNA was extracted from HeLa cells transfected for 48 h with control (GL2) or U2AF³⁵ siRNAs, and RT-PCR amplification was carried out with specific primers (see Supplementary Table 2). The relative abundance of each mRNA in U2AF³⁵a- (1), U2AF³⁵b- (2), and U2AF³⁵a&b-depleted cells (3) was determined using U6 small nuclear RNA as an endogenous reference and the GL2-treated sample as the calibrator. For comparison between qRT-PCR and microarray data, values are presented as log₂.

the over- nor the underrepresented transcripts displayed any striking functional clustering (see Supplementary Table 1).

To validate the microarray data, we selected representative mRNAs and determined their relative levels by quantitative real-time PCR (qRT-PCR; Figure 4C). Although qRT-PCR analysis shows greater sensitivity and dynamic range than the microarray experiments, the data obtained with the two methods showed the same positive or negative trend (Figure 4C).

In summary, microarray analysis revealed that knockdown of each U2AF³⁵ isoform affects expression of distinct mRNAs.

U2AF³⁵a Is Required for Efficient Splicing of a Subset of Endogenous Transcripts

Previous evidence indicated that U2AF³⁵ is required for splicing of pre-mRNA substrates that contain AG-depen-

dent introns (Guth *et al.*, 1999, 2001; Merendino *et al.*, 1999; Wu *et al.*, 1999; Zorio and Blumenthal, 1999a). We therefore reasoned that mRNAs underrepresented in the microarray analysis of U2AF³⁵-depleted cells might correspond to these pre-mRNA substrates that fail to be efficiently spliced and consequently are retained and degraded in the nucleus. To test this hypothesis, we searched among the transcripts that were specifically and consistently underrepresented in U2AF³⁵-depleted cells for pre-mRNAs that contain introns with non-consensus 3' splice sites and that also encode proteins functionally relevant for cell proliferation and cell cycle control. Using these criteria, we selected 11 introns from five different genes (Figure 5A). For all these genes, the level of spliced mRNA was found underrepresented in cells depleted of U2AF³⁵a (Figure 5B). Splicing of each intron was analyzed

A

Gene	Microarray Result		Intron	3' Splice site sequence (5' - 3')		Intron Retention
	Fold change (Exp I/Exp II)					
Cdc16	1.8/2.4	In10-11	ttgtttatggtaaaattttgaacttttttag	GTGTCTTGGTTTGCAGTGGGATGTTAC	—	
		In18-19	gtgactccaaactgcatctctacatttag	GAACAGAGACCCGCCTTAAGAGACTGG	—	
Cdc23	1.4/1.7	In1-2	agctgatctagtttgtgtgggtgttccag	GTCGGCGGAGTTGGCTTTCTCTCTCCC	—	
		In15-16	tttgaacattttccctgactttgttgcag	ACCGGGGAAGAAGGTAAGGCCTTACTC	—	
Cdc27	1.6/1.8	In2-3	tagccttttttaaaaaatgtctgttttttag	TACACTCAGAAGAAGCCTTGTTTTTAC	—	
		In6-7	aatgacttggtagaaaatttctgttttcag	GAATTAACAGATTGAATTTAGAATCT	—	
		In11-12	cctttgtttactatgtgtgttttctcctag	AAGGTTTGATGAGCCTTCTTCGTGAAA	+	
POLA	2.2/3.5	In3-4	ttattgtttttctttaaccagtgttcttag	AATTGAAGTGAAGAAGAAGAGGAAAT	—	
		In6-7	aatacttttttccacttctgtggattgtag	TTCTGACCTGTAAGATAGAAGAACTTG	—	
PNKP	1.7/2.2	In3-4	tgctgttttgtctttccttggtgacaccag	GTGGCTGGCTTTGATCTGGACGGGACG	—	
		In12-13	gatctgtttgtgacctctcgtgtccccag	GACACGCTAGGCTCCTGGCAGCGCTGT	+	
Consensus ^a			yyyyyyyynyag GU			

B

Figure 5. Several mRNAs underrepresented in U2AF³⁵-depleted cells contain introns with nonconsensus 3' splice sites. (A) Sequences of the 3' splice sites of introns that were selected for RT-PCR analysis. Intron sequences are in lower case letters; exons are in upper case letters. The numbers that identify each intron refer to the flanking exons (for instance, In10–11 indicates the intron between exons 10 and 11). The 3' splice site consensus sequence according to Burge *et al.* (1999) is indicated (Consensus^a); y, pyrimidine nucleotide; n, any nucleotide. All mRNAs indicated were found underrepresented in U2AF³⁵-depleted cells in the microarray analysis. The fold difference, given by the ratio between mRNA levels in control and U2AF³⁵-depleted cells, is indicated for each independent experiment (Exp I and Exp II). (B) qRT-PCR analysis of mRNA levels of the selected genes. Results shown were normalized to the U6 small nuclear mRNA. Data are presented as means ± SD for at least three independent experiments; * p < 0.05 relative to control (Student's *t* test).

by semiquantitative RT-PCR of total RNA with specific primers targeting the flanking exons (primer sequences are described in Supplementary Table 2). On the basis of early studies, we searched for an increase in ratio of unspliced to spliced RNA (Pikielny and Rosbash, 1985). The results are shown in Figure 6 and summarized in Figure 5A. From all

introns tested, we only detected changes in intron 11–12 (i.e., the intron between exons 11 and 12) from Cdc27 and intron 12–13 from PNKP. Cdc27 is an essential subunit of the anaphase-promoting complex (APC), which is an ubiquitin-protein ligase responsible for the metaphase to anaphase transition and thus for exit from mitosis (Harper *et al.*, 2002),

Figure 6. Reduced splicing efficiency of endogenous transcripts in U2AF^{35a}-depleted cells. RT-PCR analysis of (A) Cdc27 and (B) PNKP expression using primers flanking Cdc27 intron 11–12 and PNKP intron 12–13, respectively (C27.E11F/C27.E12R and PNKP.E12F/PNKP.E13R; see Supplementary Table 2). RNA samples were extracted from HeLa cells transfected for 48 h with control (GL2, lane 1, top panels) or U2AF³⁵ siRNAs, as indicated (lanes 2 and 3, top panels). Mock RT-PCRs lacking reverse transcriptase indicated no contamination with genomic DNA. The relative positions of unspliced and spliced products are identified on the right, and molecular weight markers are indicated on the left. Bottom panels depict the ratios between unspliced and spliced RNA products as determined by qRT-PCR analysis. Separate primer pairs for spliced and unspliced RNAs were used (C27.E3F/C27.E4R and PNKP.EJ12–13/PNKP.E13F for Cdc27 and PNKP mRNA, respectively, and C27.I11/C27.E12R2 and PNKP.I12F/PNKP.E13R for unspliced Cdc27 and PNKP transcripts, respectively; Supplementary Table 2). Results are expressed relative to the ratio between unspliced and spliced RNA products in mock-transfected cells and are presented as means $\pm$ SD for $n = 3$. (C) Western blots of HeLa cells treated for 60 h with control (GL2) or U2AF³⁵ siRNAs, as indicated. Total cell lysates were probed with antibodies against Cdc27, U2AF³⁵, and α -actin. Note that after treatment with U2AF³⁵ siRNAs, two closely migrating bands corresponding to U2AF³⁵ isoforms are detected; the lower band corresponds to the most abundant protein isoform a and is not altered by U2AF^{35b}-directed siRNA, whereas the upper band is no longer visible after treatment with U2AF^{35b} siRNA (lanes 3 and 4). (D) Immunofluorescence analysis of HeLa cells treated for 60 h with control (GL2) or U2AF³⁵ siRNAs, as indicated. Mitotic cells (b, c, e, and f) and interphase cells (a and d) were immunolabeled with monoclonal anti-Cdc27. Identical exposure times were used for imaging both control and U2AF^{35a}-depleted cells. DNA was visualized with TOPRO iodide staining. Bar, 10 μ m.

and the PNKP gene encodes a polynucleotide kinase/phosphatase implicated in DNA repair after ionizing radiation or oxidative damage (Jilani *et al.*, 1999).

RT-PCR analysis of intron 11–12 from Cdc27 and intron 12–13 from PNKP revealed approximately a twofold increased ratio between unspliced and spliced RNA in U2AF^{35a}-depleted cells when compared with control cells or U2AF^{35b}-depleted cells (Figure 6, A and B). No significant change in the amount of the corresponding unspliced pre-mRNAs was detected by qRT-PCR (Supplementary Figure S1), consistent with the view that unspliced mRNAs in the nucleus are readily targeted for degradation by the exosome (Mitchell and Tollervay, 2001).

Western blotting and immunofluorescence microscopy further revealed a specific reduction of Cdc27 protein levels in U2AF^{35a}-depleted cells (Figure 6, C and D). Taken together, these results suggest that knockdown of U2AF^{35a} impairs splicing of a subset of pre-mRNAs, leading to decreased levels of the corresponding mRNAs and proteins.

Depletion of Either U2AF^{35a} or U2AF^{35b} Alters Alternative Splicing of Cdc25 Transcripts

Recently, an RNAi study in cultured *Drosophila* cells revealed that reducing the levels of both U2AF subunits modulated alternative splicing (Park *et al.*, 2004). This prompted us to investigate a potential role of U2AF³⁵ as regulator of alternative splicing in HeLa cells. To monitor alternative splicing, we chose to analyze the Cdc25B and Cdc25C genes, which play a key role in mitosis (Donzelli and Draetta, 2003)

and encode well-described alternatively spliced isoforms (Baldin *et al.*, 1997; Forrest *et al.*, 1999; Wegener *et al.*, 2000). Moreover, both genes contain nonconsensus 3' splice sites, and Cdc25B was found underrepresented in the microarray analysis of U2AF^{35a}-depleted cells.

To study the effect of U2AF³⁵ on alternative splicing of Cdc25B and Cdc25C transcripts, we performed semiquantitative RT-PCR and quantitative real time PCR. Primers for RT-PCR were designed based on exons 5 and 7 of Cdc25B to detect isoforms B2, B3, and B4 as products of 126, 249, and 354 bp, respectively (Figure 7A). To analyze Cdc25C expression, primers were designed based on exons 2 and 7 to yield three different products of about 522, 400, and 303 bp (Figure 7B). The major 522-bp band corresponds to the Cdc25C1 variant, and the 303-bp product represents the Cdc25C5 isoform. The 400-bp product corresponds to both Cdc25C3 and Cdc25C4 variants, because these cannot be distinguished based on their size; we therefore refer to this band as C3/C4.

Depletion of U2AF^{35a}, but not U2AF^{35b}, altered the ratios of alternatively spliced Cdc25B mRNAs (Figure 7, A and C), whereas depletion of both U2AF^{35a} and U2AF^{35b} affected alternative splicing of Cdc25C mRNAs (Figure 7, B and D). For Cdc25B (Figure 7C), there was a significant increase in the B2 isoform relative to B3, the most abundant variant in control HeLa cells (B3:B2 = 5.0 $\pm$ 1.1 in U2AF^{35a}-depleted cells and B3:B2 = 12.4 $\pm$ 0.8 in control cells). For Cdc25C (Figure 7D), depletion of U2AF^{35a} (either alone or in conjunction U2AF^{35b}) induced a significant decrease in the C1

Figure 7. Depletion of U2AF³⁵ affects alternative splicing of Cdc25B and Cdc25C transcripts. RT-PCR analysis of (A) Cdc25B and (B) Cdc25C splicing variants. The top panels show a schematic representation of Cdc25B and Cdc25C pre-mRNA sequences and their alternatively spliced products. PCR was carried out with primer pairs C25B.E5F/C25B.E7R and C25C.E2F/C25C.E7R (see Supplementary Table 2; arrows). The bottom panels show the RT-PCR products obtained from RNA samples extracted from HeLa cells treated for 48 h with control (GL2) or U2AF³⁵ siRNAs, as indicated. PCR products are identified on the right and molecular weight markers are indicated on the left. (C and D) Quantitative real time PCR analysis of the relative expression of Cdc25B3 and B2 isoforms (C) and Cdc25C1 and C5 isoforms (D). A schematic representation of the gene-specific primer pairs (arrows) used to amplify and detect each isoform is depicted below (Cdc25B3 isoform was amplified with primers C25B.EJ5-6F/C25B.E6R and B2 isoform with primers C25B.EJ5-7F/C25B.E7R; Cdc25C1 isoform was amplified with primers C25C.E3F/C25C.EJ5-6R and C5 isoform was amplified with primers C25C.EJ2-4F/C25C.EJ4-7R; see Supplementary Table 2). The ratios between isoform levels were calculated from the formula $2^{-\Delta\Delta Ct}$ and results are presented as means $\pm$ SD for at least three independent experiments; * $p < 0.05$ relative to control (Student's *t* test). (E and F) RT-PCR analysis of Cdc25B (E) and Cdc25C (F) splice variants in HeLa cells treated with 60 ng/ml nocodazole for 6 h (lane 2) and 16 h (lane 3). PCR products are identified on the right, and molecular weight markers are indicated on the left.

isoform relative to C5 (C1:C5 = 1.1 ± 0.2 in U2AF^{35a}-depleted cells and C1:C5 = 2.9 ± 0.9 in control cells), whereas depletion of U2AF^{35b} increased C1 relative to C5 (C1:C5 = 5.4 ± 0.6). Noteworthy, all introns whose splicing was found to be U2AF³⁵-dependent contain nonconsensus 3' splice sites.

To determine whether the observed effects on alternative splicing are specific for U2AF^{35a}, we performed RNAi knockdown of the SKIP protein. SKIP was chosen because it was recently identified as a splicing factor required for cell division in HeLa cells (Kittler *et al.*, 2004). The efficiency of the SKIP knockdown experiments was confirmed by West-

ern blotting. As shown in Figure 7, A and B, depletion of SKIP did not affect the ratios of the alternatively spliced isoforms of both Cdc25B and Cdc25C relative to control cells. To further exclude the possibility that the observed changes in alternative splicing of Cdc25B and Cdc25C transcripts were a nonspecific response to impaired cell cycle progression, we analyzed RNA samples isolated from HeLa cells treated with nocodazole, a microtubule-destabilizing drug that arrests mitosis at prometaphase (Jordan *et al.*, 1992). RNA was extracted from cells incubated in the presence of 60 ng/ml nocodazole for 6 and 16 h and analyzed by semiquantitative RT-PCR (Figure 7, E and F). In clear contrast to the effect of knockdown of U2AF³⁵, nocodazole treatment had no significant effect on the ratio of alternatively spliced variants of Cdc25B (Figure 7E) and Cdc25C (Figure 7F) transcripts.

In conclusion, these data argue that both U2AF³⁵ isoforms can function as regulators of alternative splicing in human cells.

DISCUSSION

Orthologues of the human U2AF³⁵ were known to be essential in *D. melanogaster* (Rudner *et al.*, 1996), *S. pombe* (Wentz-Hunter and Potashkin, 1996), *C. elegans* (Zorio and Blumenthal, 1999b), and zebrafish (Golling *et al.*, 2002). However, before this study it was unclear whether U2AF³⁵ plays any vital role in mammalian cells.

Previously, we have shown that primary transcripts encoding splicing factor U2AF³⁵ in higher vertebrates are alternatively spliced (Pacheco *et al.*, 2004). Here, we used RNAi to specifically ablate the expression of U2AF^{35a} and b isoforms in human HeLa cells. RNAi is currently used extensively to inhibit expression of specific genes in the cells of several organisms, including mammals (Huppi *et al.*, 2005). By exploiting this evolutionarily conserved process, a specific gene can be silenced by a siRNA complementary to the mRNA product of the gene, which anneals to the mRNA and triggers its degradation. The assumption in any RNAi experiment is that the siRNA will selectively target its complementary mRNA for degradation; however, recent reports suggest that siRNAs can also nonspecifically alter the expression of a significant number of genes involved in diverse cellular functions (Jackson *et al.*, 2003; Semizarov *et al.*, 2003; Persengiev *et al.*, 2004). To minimize nonspecific effects, we used two different oligonucleotides to target each U2AF³⁵ isoform, and similar phenotypic effects were observed with both.

Our results show that knockdown of U2AF^{35a} abrogated cell proliferation (Figure 2A), decreased cell viability to ~64% (Figure 2B), increased about threefold the fraction of apoptotic cells (Figure 2C), and caused a massive delay in mitotic progression (Figure 2F). Importantly, more than 80% of depleted mitotic cells failed to progress through cytokinesis during a 45-min observation period, whereas the steady state proportion of G2/M phase cells increased only from 14 to 22% in U2AF^{35a}-depleted cells. This is probably because the mitotically arrested cells die by apoptosis and become therefore excluded from the population. On the basis of these results, we conclude that U2AF^{35a} plays an essential role in cell division.

Strikingly, an siRNA screen for genes required for HeLa cell division identified several components of the splicing machinery, namely *SNRPA1*, *SNRPB*, *SNW1/SKIP*, *DHX8*, *DDX5*, *LSM6*, and *SART1* (Kittler *et al.*, 2004). The phenotypes observed after knockdown of these splicing proteins included mitotic arrest and spindle abnormalities (Kittler *et al.*, 2004). Moreover, conditional mutations in several splic-

ing factors in yeast induce cell cycle arrest at the restrictive temperature (Dahan and Kupiec, 2004 and references therein) and genetic inactivation of splicing factor ASF/SF2 induced cell cycle arrest and apoptosis in chicken B-cells (Li *et al.*, 2005). Thus, cell cycle arrest appears to be a common phenotype induced by depletion of splicing proteins. An involvement of RNA and RNA-binding proteins in mitotic spindle assembly was recently discovered (Blower *et al.*, 2005), raising the possibility that some splicing factors may have an as yet unidentified direct role in cell division. Alternatively (or additionally), depletion of splicing proteins may indirectly lead to abnormal pre-mRNA processing of transcripts encoding proteins essential for cell division. Consistent with the latter hypothesis, in U2AF^{35a}-depleted cells we detected an almost twofold increase in the ratio between unspliced and spliced transcripts encoding Cdc27, a twofold decrease of Cdc27 mRNA and correspondingly reduced Cdc27 protein levels (Figures 5B and 6, A, C, and D). Cdc27 is an essential subunit of the APC responsible for the metaphase-to-anaphase transition (Harper *et al.*, 2002), and therefore reduced levels of Cdc27 protein are expected to prevent metaphase cells from progressing to anaphase. However, we found that U2AF^{35a}-depleted cells accumulated predominantly in prometaphase, arguing that U2AF^{35a} is probably required for efficient splicing of several pre-mRNAs, and the observed phenotype results from the cumulative effect of many reduced proteins.

Because we did not use splicing-specific microarrays, we could not identify genome-wide effects on splicing caused by loss U2AF^{35a}. However, we detected reduced Cdc27 and PNKP mRNA levels both by microarray analysis and qRT-PCR, and we showed an increased ratio between the corresponding unspliced and spliced transcripts induced by U2AF^{35a} depletion. These observations support the view that several underrepresented mRNAs in the microarray analysis may result from impaired splicing.

In a recent study, RNAi-mediated depletion of the essential splicing factor SF3a was shown to produce ~90% cell death and cause a general block in splicing (Tanackovic and Kramer, 2005). On the basis of our results showing that depletion of the major U2AF^{35a} isoform caused ~40% cell death and affected splicing of only a subset of the transcripts analyzed, we conclude that human U2AF³⁵ is not a constitutive splicing factor in vivo.

Although alternative splicing is well known to be regulated by RNA-binding proteins that modulate the association of core components of the spliceosome with the pre-mRNA, a recent study using RNAi in *Drosophila* cells has unexpectedly shown that reducing the levels of core components of the spliceosome can affect alternative splicing (Park *et al.*, 2004). These core splicing factors included snRNP proteins, DExH/D-box proteins and both subunits of U2AF (Park *et al.*, 2004). Consistent with that finding, we show here that depletion of U2AF^{35a} in HeLa cells specifically altered the ratios of alternatively spliced isoforms of transcripts encoding the cell cycle phosphatases Cdc25B and Cdc25C. Importantly, the Cdc25B phosphatase is essential for mitotic entry (Lindqvist *et al.*, 2005) and is thought to play an oncogenic role (Galaktionov *et al.*, 1995). In particular, the Cdc25B2 splice variant (which increased after depletion of U2AF^{35a}) is not detected in nonneoplastic lymphoid tissues but is progressively up-regulated in indolent and aggressive human non-Hodgkin's lymphomas (Hernandez *et al.*, 2000) and has also been shown to be differentially regulated in colorectal carcinomas (Hernandez *et al.*, 2001). We therefore speculate that changing the levels of U2AF³⁵ in

a cell may promote the production of Cdc25B splice variants with different oncogenic potential.

The finding that knockdown of U2AF^{35b} causes minor effects when compared with knockdown of U2AF^{35a} is not surprising given that U2AF^{35b} is 9–18-fold less abundant than U2AF^{35a} in mammalian tissues (Pacheco *et al.*, 2004). However, U2AF^{35b} has been highly conserved throughout evolution, suggesting that this protein plays an important role in metazoans (Pacheco *et al.*, 2004). Accordingly, we observe that U2AF^{35b} depletion interferes with cell proliferation and augments the defects caused by knockdown of U2AF^{35a}. Furthermore, microarray analysis revealed that loss of U2AF^{35b} specifically altered the expression level of 138 mRNAs, and qRT-PCR experiments demonstrated that U2AF^{35b} can specifically influence alternative splicing of Cdc25C transcripts.

In conclusion, this study establishes a link between the most abundantly expressed U2AF^{35a} isoform and cell division and provides evidence suggesting that both the major and minor U2AF³⁵ isoforms act as regulators of alternative splicing in human cells.

ACKNOWLEDGMENTS

We thank Juan Valcárcel for stimulating discussions and for reagents. We are also grateful to Carol Featherstone for editing the manuscript; to W. van Venrooij, T. Maniatis, and I. Herman for providing antibodies; and to Sandra Caldeira for help with FACS analysis. This work was supported by grants from Fundação para a Ciência e Tecnologia (FCT), Portugal (POCTI/MGI/36547/2000 and POCI/MMO/57700/2004), the Human Frontier Science Program Organization (RG0300/2000-M), and the European Commission (EU-RASNET). T.R.P. and A.Q.G. were supported by FCT fellowships (PRAXIS XXI/BD/18044/98 and POCTI SFRH/BPD/9388/2002, respectively).

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