Nuclear Factor κB–dependent Gene Expression Profiling of Hodgkin's Disease Tumor Cells, Pathogenetic Significance, and Link to Constitutive Signal Transducer and Activator of Transcription 5a Activity

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Abstract

Constitutive nuclear nuclear factor (NF)-κB activity is observed in a variety of hematopoietic and solid tumors. Given the distinctive role of constitutive NF-κB for Hodgkin and Reed-Sternberg (HRS) cell viability, we performed molecular profiling in two Hodgkin's disease (HD) cell lines to identify NF-κB target genes. We recognized 45 genes whose expression in both cell lines was regulated by NF-κB. The NF-κB–dependent gene profile comprises chemokines, cytokines, receptors, apoptotic regulators, intracellular signaling molecules, and transcription factors, the majority of which maintain a marker-like expression in HRS cells. Remarkably, we found 17 novel NF-κB target genes. Using chromatin immunoprecipitation we demonstrate that NF-κB is recruited directly to the promoters of several target genes, including signal transducer and activator of transcription (STAT)5a, interleukin-13, and CC chemokine receptor 7. Intriguingly, NF-κB positively regulates STAT5a expression and signaling pathways in HRS cells, and promotes its persistent activation. In fact, STAT5a overexpression was found in most tumor cells of tested patients with classical HD, indicating a critical role for HD. The gene profile underscores a central role of NF-κB in the pathogenesis of HD and potentially of other tumors with constitutive NF-κB activation.

Key words: oncogene • tumor suppressor • survival • chromatin • microarray

Introduction

Members of the nuclear factor (NF)*-κB transcription factor family regulate immune, inflammatory, and acute phase responses, and their homozygous inactivation in mice results in severe immune system dysfunction (1–3). One of its most important functions is the activation of an antiapop- totic gene expression program (4–6). More recently, NF-κB activation has been connected to cell growth control (7–9). Because signaling pathways that govern proliferation and survival are important for tumor development, NF-κB has an intrinsic oncogenic potential.

Indeed, ample evidence linking Rel/NF-κB activity to oncogenesis has been accumulated in the past years (7–10). Transforming capacity has first been demonstrated for the viral oncoprotein v-Rel in vitro and in vivo (10). Moreover, oncogenic viruses, such as human T cell leukemia virus I or Epstein-Barr virus, activate NF-κB as part of the transformation process (11, 12). Similarly, cellular oncoproteins like Her-2/Neu and BCR-ABL induce NF-κB to achieve resistance to apoptosis or enhance transformation capacity (13, 14). Finally, chromosomal rearrangements of genes coding for Rel/NF-κB factors have been observed in

many human hematopoietic and solid tumors, and several human cancer cell types display persistent nuclear NF-κB activity as a result of constitutive activation of IκB kinases (IKKs) or mutations inactivating IκB subunits (7).

Most evidence for the role of Rel/NF-κB in human malignancies came from an analysis of Hodgkin's disease (HD). HD is the first hematopoietic tumor from which an aberrant constitutive NF-κB activation has been described (15). The malignant mononuclear Hodgkin and multinucleated Reed-Sternberg cells in HD only represent a fraction of the neoplastic lesions that are populated by eosinophils, neutrophils, T cells, B cells, plasma cells, histiocytes, and others. These reactive cells are attracted by cytokines and chemokines abundantly produced by Hodgkin and Reed-Sternberg (HRS) cells (16–18). Molecular single cell analysis has suggested that HRS cells are derived from germinal center B cells or B cells at later differentiation stages (19). Due to the scarcity of malignant cells it has been difficult to define the transforming molecular lesions that lead to the development of HD. Several HRS cell lines have been established and clonal identity with primary HRS cells has been demonstrated for L1236 cells, indicating that HRS cell lines can serve as suitable model systems (20). Constitutively activated NF-κB is a characteristic feature of HRS cell lines and primary cells (15, 21). Interestingly, mutations in the *ikbα* gene, producing nonfunctional or unstable IκBα proteins, are recurrent molecular abnormalities of HRS cell lines (22–24). However, most primary HRS cells lack mutations in the *ikbα* gene. Recent data indicate persistently activated IKK complex as the major cause of constitutive NF-κB activity (25). The inhibition of NF-κB by the expression of the superrepressor IκBΔN in various HRS cell lines led to decreased proliferation, enhanced apoptotic response, and strongly impaired tumor growth in immune-deficient mice (21, 26). Constitutive NF-κB activity regulates the expression of genes typically overexpressed by HRS cells, including the cell cycle regulatory protein cyclin D2, antiapoptotic proteins Bfl-1/A1, c-IAP2, TNFR–associated factor 1, and Bcl-x$_L$, and the cell surface receptors CD40 and CD86 (26). Thus, an important function of aberrant NF-κB activity in cell growth of malignant cells is well established. However, a determination of the full group of genes controlled by constitutive NF-κB will be important to understand its pathogenetic role in HD and other types of tumors.

For this purpose, we performed large-scale gene expression profiling in L428 and HDLM2 cells. In both cell lines, NF-κB activity could be efficiently blocked by adenoviral expression of the superrepressor IκBΔN, leading to massive spontaneous apoptosis within 48 h after infection. We identified 45 genes, which are affected upon NF-κB inhibition, encoding chemokines, cytokines, receptors, apoptotic regulators, intracellular signaling molecules, and transcription factors. Several known NF-κB target genes were found, which are overexpressed in primary or cultured HRS cells. Besides this, 17 novel genes could be verified as NF-κB targets by Northern blot or RT-PCR analysis. Importantly, most of these genes displayed elevated expression levels in HRS cells. Stimulus- and IKK-dependent induction in non-Hodgkin cells, as well

as direct recruitment of NF-κB to promoter regions, confirmed that many of these genes, including signal transducer and activator of transcription (STAT)5a, CCR7, or IL-13 are direct targets of NF-κB. We observed that NF-κB interferes with the Janus kinase/STAT signaling pathway and causes a high level of constitutive STAT5a activity in cultured and primary HRS cells. Because NF-κB controls a complex network of genes with central pathogenic importance in HD, we suggest that NF-κB is a key regulator in this malignancy.

Materials and Methods

Viral Infection. Ad5-IκBΔN and Ad5 control viruses were previously described (26). 10^6–10^7 pelleted cells were resuspended in RPMI 1640 containing 10% FCS at 10^7 cells/ml. Viruses were added (at a multiplicity of infection [m.o.i.] of 300 for L428; m.o.i. of 100 for HDLM2) and cells were incubated for 2 h at 37°C in 5% CO_2. After infection, cells were pelleted and resuspended at 3×10^5 cells/ml.

Cell Culture. Reh, Nam, L428, L1236, KMH-2, and HDLM2 cells were grown in RPMI 1640 (GIBCO BRL) supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, as well as 50 μM β-mercaptoethanol for 70Z/3 or 1.3E2 cells. Cells were treated with 200 ng/ml PMA (Calbiochem-Novabiochem), 10 μg/ml LPS (Sigma-Aldrich), or 25 μg/ml cycloheximide (CHX; Calbiochem-Novabiochem).

Extracts, Electrophoretic Mobility Shift Assay (EMSA), Western Blotting, and Immunoprecipitation. Preparation of whole cell extracts, Western blotting, and EMSA was performed as previously described (26). STAT5a gel shift oligonucleotides (sc-2565) were purchased from Santa Cruz Biotechnology, Inc. For immunoprecipitation, 400 μg protein whole cell extract was mixed with immunoprecipitation buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1mM dithiothreitol, protease and phosphatase inhibitors). Extracts were precleared with protein A–Sepharose for 1 h at 4°C. Immunoprecipitation using 2 μg STAT5a antibody (sc-1656; Santa Cruz Biotechnology, Inc.) was performed for 2 h at 4°C. Samples were washed four times with immunoprecipitation buffer and analyzed by SDS-PAGE and Western blotting. Mouse monoclonal antibodies against phosphotyrosine (05-321; Upstate Biotechnology), rabbit polyclonal antibodies against IκBα (C-21; Santa Cruz Biotechnology, Inc.), STAT5a (66621N; BD Biosciences), or CDK4 (H-22; Santa Cruz Biotechnology, Inc.), as well as horseradish peroxidase–conjugated anti–rabbit or anti–mouse antibodies (New England Biolabs, Inc.) were used for detection.

DNA Microarray Analysis. Total RNA was prepared from Ad5 control– or Ad5-IκBΔN–infected L428 or HDLM2 cells 24 h after infection (RNeasy Kit; QIAGEN). Samples for Affymetrix microarray analysis were prepared according to the manufacturer's instructions. The HuGeneFL GeneChip microarray was hybridized with RNA at 45°C for 16 h, washed, and stained using the GeneChip Fluidies station according to the manufacturer's instructions. DNA chips were scanned with a GeneChip scanner and signals were processed by the GeneChip expression analysis algorithm (version 3.2; Affymetrix). The quantification of each gene expression was obtained from the hybridization intensities of 20 perfectly matched and mismatched control probe pairs. All chip files were scaled to a uniform intensity value of 1,000. For a comparative chip file, the experimental file (Ad5-IκBΔN–infected cells) was compared with the baseline file (Ad5 control–infected cells).

Genes that fit the following criteria were considered decreased/increased genes upon NF-κB inhibition: the change call was either decreased or induced, the change in the average difference was greater than twofold, the sort score value was >1 or <1, and an absolute call of "presence" was associated with the baseline file.

Northern Blotting. Total RNA preparation and Northern blotting was performed as previously described (26). For the generation of probes, I.M.A.G.E. cDNA clones (Resource Center of the German Human Genome Project, Max-Planck Institute for Molecular Genetics, Berlin, Germany) were either digested or amplified by PCR. Isolated fragments were labeled with Megaprime DNA Labeling System (Amersham Biosciences). Detailed information is available upon request.

RT-PCR. 5 μg total RNA (see above) were reverse transcribed using oligo dT primers and the Superscript™ first strand synthesis RT-PCR system (Life Technologies) according to the manufacturer's instructions. cDNA was amplified by CombiPol DNA polymerase (Invitek). To establish relative quantities, serial cDNA dilutions were amplified with β-actin–specific primers (22 cycles) for standardization. To semi-quantify expression levels of potential target genes, cDNAs were amplified with specific PCR primer pairs (30–40 cycles) in a volume of 50 μl. 10 μl of the reactions were analyzed on 1% agarose gels. Nucleotide sequences and detailed PCR conditions are available upon request.

Chromatin Immunoprecipitation (ChIP) Assay. ChIP was performed as previously described (27). The following are sequences of promoter-specific primers: IκBα: 5′ GACGACCCCAAT-TCAAATCG 3′ (s), 5′ TCAGGCTCGGGGAATTTCC 3′ (as); c-jun: 5′ CGACTGTAGGAGGGCAGCGG 3′ (s), 5′ AGCC-CTTATCCAGCCCGAGC 3′ (as); IL-13: 5′ GGAAGAGAGG-GTGGGCAAGC 3′ (s), 5′ GTTCCTAGTGCCACTGGGGC 3′ (as); CCR7: 5′ CCAGAAGCCAGAGGGAAAGC 3′ (s), 5′ TGACAGTCGCTGGTCATAGG 3′ (as); CD44: 5′ GAGGG-GCCCGCCCGGGAGGG 3′ (s), 5′ GCGAACGGAGGGGCG-CGGGCG 3′ (as); STAT5a: 5′ GCAGCTGGGCTGGC-CCCTCC 3′ (s), 5′ CCCCCTCTCCAAGAGGCCCC 3′ (as); thiopurine methyltransferase (TPMT): 5′ CCCGGGGCTCAC-CTTTGCGC 3′ (s), 5′ GAGGGTGCGGGGTGGGTATC 3′

(as); tissue factor pathway inhibitor (TFPI)-2: 5′ CACAA-ACGCTCCCTCAGGGC 3′ (s), 5′ TCACCCCGCCGCCC-CCGCGC 3′ (as); and GLUT5: 5′ CCAGTCATTCACCAT-GAGCC 3′ (s), 5′ GTAAGTGGGTGCCCCCTGGG 3′ (as).

Immunohistochemistry. Paraffin blocks from lymph node biopsies of patients with classical HD were from files of the Consultation and Reference Center for Hematopathology, Free University, Berlin, Germany. The diagnosis of classical HD was according to the criteria of the World Health Organization's classification (28). Four micrometer sections were deparaffinized and subjected to an antigen retrieval protocol to optimally visualize antigens in paraffin-embedded tissue. For that, tissue sections were immersed in 10 mmol citrate, pH 6.0, and cooked under high pressure in a pressure cooker for 2 min. Sections were incubated with anti-STAT5a antibody (sc-1656; Santa Cruz Biotechnology, Inc.) at a dilution of 1:200. Bound antibody was visualized using the streptavidin–biotin-alkaline phosphatase method and Fast Red as chromogen (kit 5005; Dako). The specificity of STAT5a immunodetection in HRS cells was confirmed by competition with a specific peptide (sc-1656p; Santa Cruz Biotechnology, Inc.).

Results

NF-κB–dependent Gene Profiling in HRS Cells. The identification of target genes is an important step to understand the oncogenic potential of NF-κB and its function in HRS cells. To provide a representative gene profile, we performed a parallel microarray analysis of two different HRS cell lines. We previously established adenovirus-mediated expression of the superrepressor IκBΔN to down-modulate constitutive NF-κB activity in the HRS cell line L428 (26). As a second HRS cell line, HDLM2 cells were used. As observed for L428 cells, the expression of IκBΔN nearly abolished NF-κB DNA binding activity in HDLM2 cells and caused a dramatic growth defect pri-
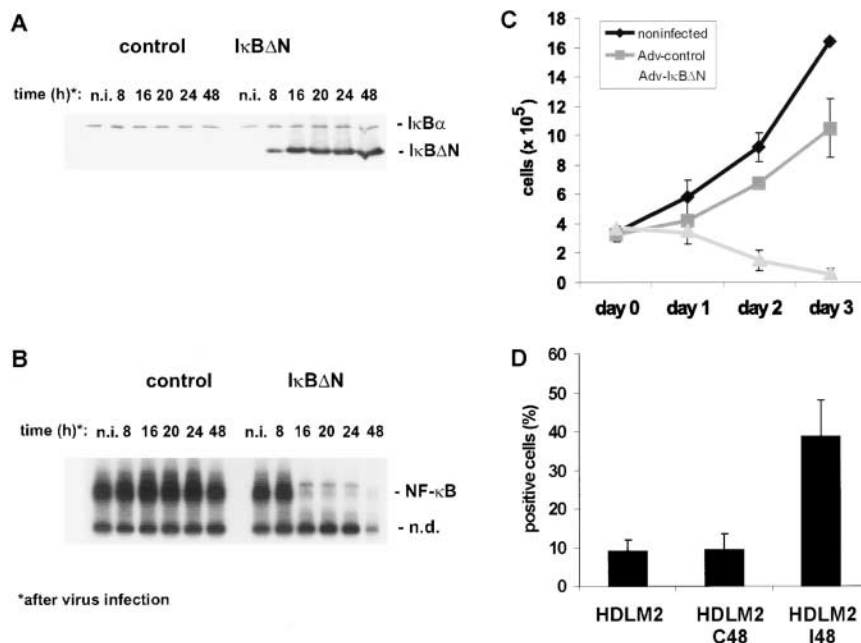
Figure 1. Adenovirus-mediated IκBΔN expression abrogates NF-κB activity and induces massive spontaneous apoptosis in HDLM2 cells. (A) HDLM2 cells were infected with Ad5-IκBΔN or Ad5 control (m.o.i. of 100). Control infection with a β-galactosidase–expressing adenovirus indicated 80% infection efficiency (m.o.i. of 100; not depicted). Whole cell extracts prepared at the indicated time points were analyzed by Western blotting for IκBα. (B) Whole cell extracts of HDLM2 cells infected with Ad5 control or Ad5-IκBΔN were analyzed by EMSA using an H2K binding site probe. (C) Growth rates of noninfected or infected HDLM2 cells, as indicated, were determined in five independent experiments. (D) Apoptotic cells were determined by annexin V staining in noninfected or infected cells.

marily due to massive spontaneous apoptosis (Fig. 1). NF-κB–mediated protection against apoptosis is due to its transcriptional regulation of a distinct set of antiapoptotic genes (26). RNA prepared 24 h after the infection of L428 and HDLM2 cells with either Ad5 control or Ad5-IκBΔN viruses was used for hybridization to high density DNA microarrays. The DNA arrays contained 7,133 gene sequences and expressed sequence tags. Before hybridization, RNA from each of the samples was converted to target according to standard procedures. The hybridized chips were then processed and analyzed as described in Materials and Methods.

A total of 45 genes met the criteria that expression was considered decreased or increased upon NF-κB inhibition in both L428 and HDLM2 cell lines (Fig. 2). Other genes fulfilled the criteria for NF-κB–dependent regulation in only one cell line. In L428 cells, the expression of 15 additional genes was decreased and 6 were increased upon NF-κB inhibition. In HDLM2 cells, 43 additional genes had decreased and 23 had increased expression (Fig. 3). Among the candidates identified as common for both cell lines, the expression of only one gene, L-fucosidase, was increased, indicating a potential repressor function of NF-κB. Expression of all others was decreased greater than twofold, in turn suggesting that these genes are induced by constitutive NF-κB activity in HRS cells.

The target genes could be classified into these groups: cytokines and chemokines, cell surface receptors, cell adhesion molecules, regulators of apoptosis, signaling molecules, transcription factors, and a group of genes with miscellaneous functions (Fig. 2). The presence of several genes known to be NF-κB target in other cell types like lympho-
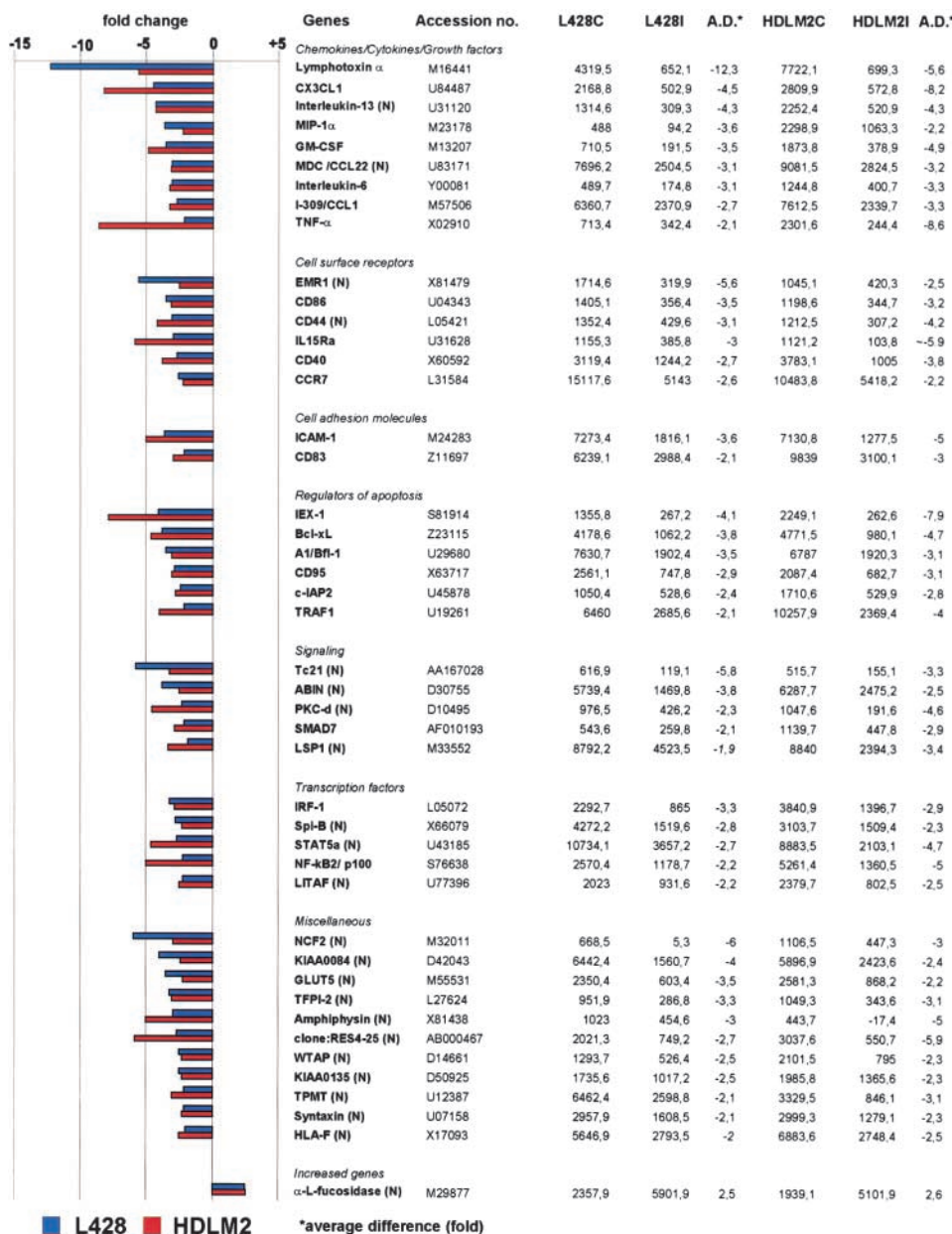
fold change (−15 to +5)	Genes	Accession no.	L428C	L428I	A.D.*	HDLM2C	HDLM2I	A.D.*
	Chemokines/Cytokines/Growth factors							
	Lymphotoxin α	M16441	4319,5	652,1	-12,3	7722,1	699,3	-5,6
	CX3CL1	U84487	2168,8	502,9	-4,5	2809,9	572,8	-8,2
	Interleukin-13 (N)	U31120	1314,6	309,3	-4,3	2252,4	520,9	-4,3
	MIP-1α	M23178	488	94,2	-3,6	2298,9	1063,3	-2,2
	GM-CSF	M13207	710,5	191,5	-3,5	1873,8	378,9	-4,9
	MDC /CCL22 (N)	U83171	7696,2	2504,5	-3,1	9081,5	2824,5	-3,2
	Interleukin-6	Y00081	489,7	174,8	-3,1	1244,8	400,7	-3,3
	I-309/CCL1	M57506	6360,7	2370,9	-2,7	7612,5	2339,7	-3,3
	TNF-α	X02910	713,4	342,4	-2,1	2301,6	244,4	-8,6
	Cell surface receptors							
	EMR1 (N)	X81479	1714,6	319,9	-5,6	1045,1	420,3	-2,5
	CD86	U04343	1405,1	356,4	-3,5	1198,6	344,7	-3,2
	CD44 (N)	L05421	1352,4	429,6	-3,1	1212,5	307,2	-4,2
	IL15Ra	U31628	1155,3	385,8	-3	1121,2	103,8	~-5,9
	CD40	X60592	3119,4	1244,2	-2,7	3783,1	1005	-3,8
	CCR7	L31584	15117,6	5143	-2,6	10483,8	5418,2	-2,2
	Cell adhesion molecules							
	ICAM-1	M24283	7273,4	1816,1	-3,6	7130,8	1277,5	-5
	CD83	Z11697	6239,1	2988,4	-2,1	9839	3100,1	-3
	Regulators of apoptosis							
	IEX-1	S81914	1355,8	267,2	-4,1	2249,1	262,6	-7,9
	Bcl-xL	Z23115	4178,6	1062,2	-3,8	4771,5	980,1	-4,7
	A1/Bfl-1	U29680	7630,7	1902,4	-3,5	6787	1920,3	-3,1
	CD95	X63717	2561,1	747,8	-2,9	2087,4	682,7	-3,1
	c-IAP2	U45878	1050,4	528,6	-2,4	1710,6	529,9	-2,8
	TRAF1	U19261	6460	2685,6	-2,1	10257,9	2369,4	-4
	Signaling							
	Tc21 (N)	AA167028	616,9	119,1	-5,8	515,7	155,1	-3,3
	ABIN (N)	D30755	5739,4	1469,8	-3,8	6287,7	2475,2	-2,5
	PKC-d (N)	D10495	976,5	426,2	-2,3	1047,6	191,6	-4,6
	SMAD7	AF010193	543,6	259,8	-2,1	1139,7	447,8	-2,9
	LSP1 (N)	M33552	8792,2	4523,5	-1,9	8840	2394,3	-3,4
	Transcription factors							
	IRF-1	L05072	2292,7	865	-3,3	3840,9	1396,7	-2,9
	Spi-B (N)	X66079	4272,2	1519,6	-2,8	3103,7	1509,4	-2,3
	STAT5a (N)	U43185	10734,1	3657,2	-2,7	8883,5	2103,1	-4,7
	NF-kB2/ p100	S76638	2570,4	1178,7	-2,2	5261,4	1360,5	-5
	LITAF (N)	U77396	2023	931,6	-2,2	2379,7	802,5	-2,5
	Miscellaneous							
	NCF2 (N)	M32011	668,5	5,3	-6	1106,5	447,3	-3
	KIAA0084 (N)	D42043	6442,4	1560,7	-4	5896,9	2423,6	-2,4
	GLUT5 (N)	M55531	2350,4	603,4	-3,5	2581,3	868,2	-2,2
	TFPI-2 (N)	L27624	951,9	286,8	-3,3	1049,3	343,6	-3,1
	Amphiphysin (N)	X81438	1023	454,6	-3	443,7	-17,4	-5
	clone:RES4-25 (N)	AB000467	2021,3	749,2	-2,7	3037,6	550,7	-5,9
	WTAP (N)	D14661	1293,7	526,4	-2,5	2101,5	795	-2,3
	KIAA0135 (N)	D50925	1735,6	1017,2	-2,5	1985,8	1365,6	-2,3
	TPMT (N)	U12387	6462,4	2598,8	-2,1	3329,5	846,1	-3,1
	Syntaxin (N)	U07158	2957,9	1608,5	-2,1	2999,3	1279,1	-2,3
	HLA-F (N)	X17093	5646,9	2793,5	-2	6883,6	2748,4	-2,5
	Increased genes							
	α-L-fucosidase (N)	M29877	2357,9	5901,9	2,5	1939,1	5101,9	2,6

■ L428 ■ HDLM2   *average difference (fold)

Figure 2. NF-κB–dependent gene profiling in HRS cells. L428 and HDLM2 cells were infected with Ad5 control (L428C, HDLM2C) or Ad5-IκBΔN (L428I, HDLM2I). Total RNA was prepared 24 h after infection, which is the shortest time point of complete NF-κB inhibition in both cell lines, and subjected to DNA microarray analysis. Potential target genes, which fit the criteria for decreased/increased genes upon NF-κB inhibition in both cell lines, are grouped based on their molecular functions. Hybridization intensities for the potential target genes of each sample and average difference values (right) as well as a graphical representation (left) are shown. N, novel NF-κB target genes.
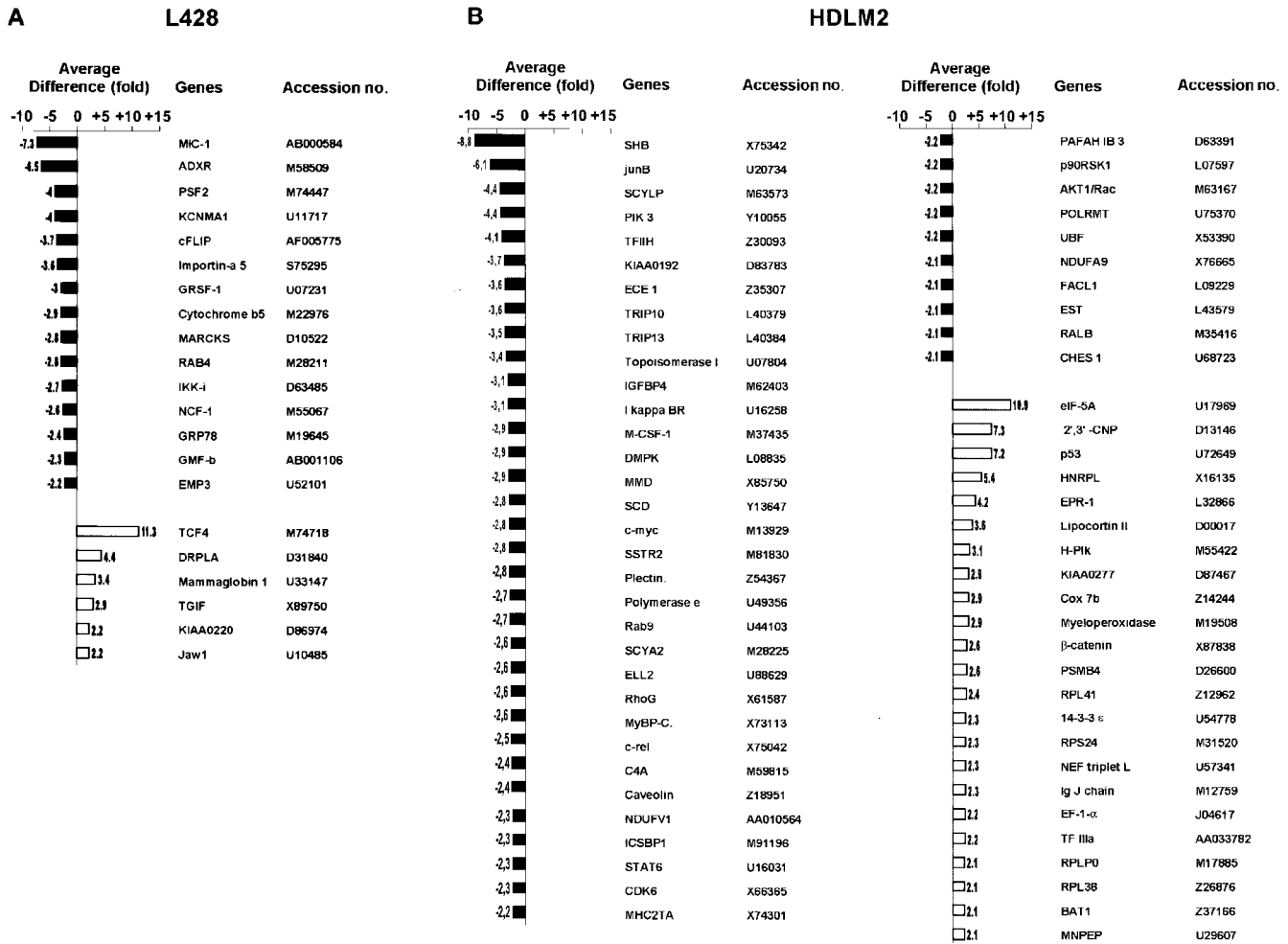
A L428

Average Difference (fold)	Genes	Accession no.
-7.3	MIC-1	AB000584
-4.5	ADXR	M58509
-4	PSF2	M74447
-4	KCNMA1	U11717
-3.7	cFLIP	AF005775
-3.6	Importin-a 5	S75295
-3	GRSF-1	U07231
-2.9	Cytochrome b5	M22976
-2.8	MARCKS	D10522
-2.8	RAB4	M28211
-2.7	IKK-i	D63485
-2.6	NCF-1	M55067
-2.4	GRP78	M19645
-2.3	GMF-b	AB001106
-2.2	EMP3	U52101
11.3	TCF4	M74718
4.4	DRPLA	D31840
3.4	Mammaglobin 1	U33147
2.9	TGIF	X89750
2.2	KIAA0220	D86974
2.2	Jaw1	U10485

B HDLM2

Average Difference (fold)	Genes	Accession no.
-8.8	SHB	X75342
-6.1	junB	U20734
-4.4	SCYLP	M63573
-4.4	PIK 3	Y10055
-4.1	TFIIH	Z30093
-3.7	KIAA0192	D83783
-3.6	ECE 1	Z35307
-3.6	TRIP10	L40379
-3.5	TRIP13	L40384
-3.4	Topoisomerase I	U07804
-3.1	IGFBP4	M62403
-3.1	I kappa BR	U16258
-2.9	M-CSF-1	M37435
-2.9	DMPK	L08835
-2.9	MMD	X85750
-2.8	SCD	Y13647
-2.8	c-myc	M13929
-2.8	SSTR2	M81830
-2.8	Plectin	Z54367
-2.7	Polymerase e	U49356
-2.7	Rab9	U44103
-2.6	SCYA2	M28225
-2.6	ELL2	U88629
-2.6	RhoG	X61587
-2.6	MyBP-C.	X73113
-2.5	c-rel	X75042
-2.4	C4A	M59815
-2.4	Caveolin	Z18951
-2.3	NDUFV1	AA010564
-2.3	ICSBP1	M91196
-2.3	STAT6	U16031
-2.3	CDK6	X66365
-2.2	MHC2TA	X74301

Average Difference (fold)	Genes	Accession no.
-2.2	PAFAH IB 3	D63391
-2.2	p90RSK1	L07597
-2.2	AKT1/Rac	M63167
-2.2	POLRMT	U75370
-2.2	UBF	X53390
-2.1	NDUFA9	X76665
-2.1	FACL1	L09229
-2.1	EST	L43579
-2.1	RALB	M35416
-2.1	CHES 1	U68723
10.9	eIF-5A	U17969
7.3	2',3'-CNP	D13146
7.2	p53	U72649
5.4	HNRPL	X16135
4.2	EPR-1	L32866
3.6	Lipocortin II	D00017
3.1	H-PIk	M55422
2.9	KIAA0277	D87467
2.9	Cox 7b	Z14244
2.9	Myeloperoxidase	M19508
2.6	β-catenin	X87838
2.6	PSMB4	D26600
2.4	RPL41	Z12962
2.3	14-3-3 ε	U54778
2.3	RPS24	M31520
2.3	NEF triplet L	U57341
2.3	Ig J chain	M12759
2.2	EF-1-α	J04617
2.2	TF IIIa	AA033782
2.1	RPLP0	M17885
2.1	RPL38	Z26876
2.1	BAT1	Z37166
2.1	MNPEP	U29607

Figure 3. NF-κB target genes in individual HRS cell lines (A) L428 and (B) HDLM2. Analysis was performed as described in Fig. 2. Genes with decreased expression genes upon NF-κB inhibition are listed first followed by genes with increased expression. Graphical representations of average difference values are shown.

toxin-α, IL-6, GM-CSF, TNF-α, intercellular adhesion molecule (ICAM)-1, or CD95 verified the efficacy of the screen (29). Because NF-κB activity protects HRS cells from cell death (Fig. 1; reference 26), it was expected to detect antiapoptotic genes (Fig. 2). All of them are known NF-κB target genes and with the exception of IEX-1, the overexpression in HRS cells has been documented (26, 29, 30). In agreement with our prior observations (26), the cell surface receptors CD40 and CD86 were found to be activated by NF-κB. We discovered 23 novel NF-κB target genes activated in both cell lines including IL-13, macrophage-derived chemokine (MDC), CD44, and STAT5a. Importantly, several of these including IL-13, CD44, leukocyte-specific protein (LSP)-1, and STAT5a (see below) are overexpressed in HRS cells (31–34).

High Level Expression of NF-κB Target Genes in HRS Cells. All novel candidates identified for both L428 and HDLM2 cells and a group of known NF-κB target genes were selected for Northern analysis to validate NF-κB–dependent expression in HRS cells. Because the microchip data were obtained from two independent experiments and

two different cell lines, the rate of false positives should be minimal. For some candidates, semi-quantitative RT-PCR was performed. We analyzed mRNA expression in Ad5-IκBΔN– or Ad5 control–infected L428 and HDLM2 cells, and in uninfected HRS and control cell lines. For all samples we confirmed that NF-κB DNA binding activity was constitutive only in HRS cells and was repressed by IκBΔN (Fig. 4 A). Pronounced NF-κB–dependent regulation could be verified for the vast majority of genes (Fig. 4, B and C). Moreover, the observed change of mRNA expression levels upon NF-κB inhibition correlated well with the DNA microarray data (Fig. 2). CX3CL, MIP1-α, CCR7, IL-15Rα, CD83, IEX-1, SMAD7, interferon regulatory factor 1, and NF-κB p100, previously proposed to be regulated by NF-κB in other cell types (29, 35–39), could be confirmed as cellular target genes in HRS cells. Abundant CCR7 and CD83 expression was detected exclusively in HRS cells (Fig. 3 B), supporting recent observations (38, 40). Likewise, the mRNA patterns of CX3CL, IEX-1, IL-15Rα, and p100 indicate strongly elevated expression in HRS cells compared with non-HRS cells (Fig. 4 B).
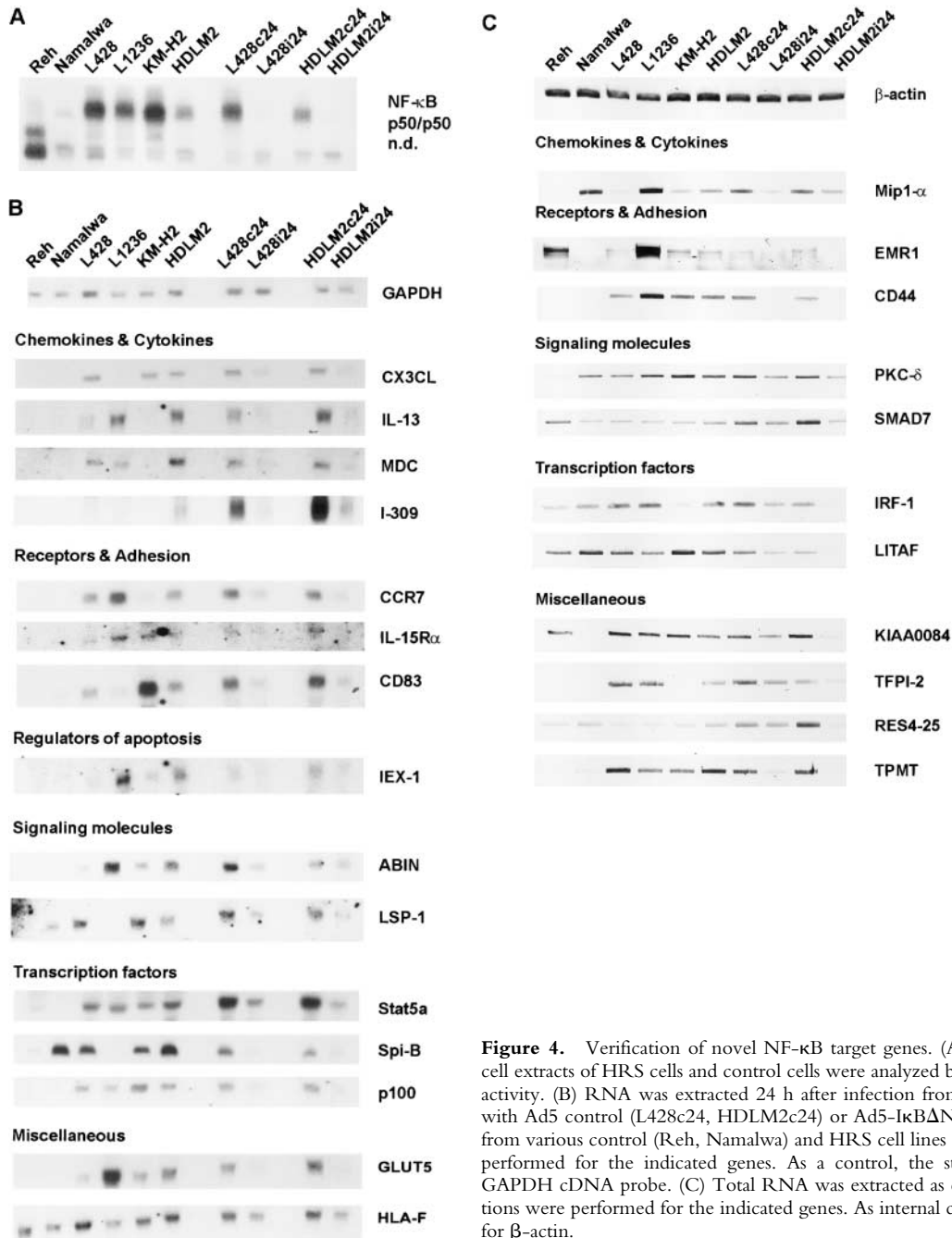
Figure 4. Verification of novel NF-κB target genes. (A) NF-κB binding activity. Whole cell extracts of HRS cells and control cells were analyzed by EMSA for NF-κB DNA binding activity. (B) RNA was extracted 24 h after infection from L428 and HDLM2 cells infected with Ad5 control (L428c24, HDLM2c24) or Ad5-IκBΔN (L428i24, HDLM2i24), as well as from various control (Reh, Namalwa) and HRS cell lines as indicated. Northern blotting was performed for the indicated genes. As a control, the stripped blot was reprobed with a GAPDH cDNA probe. (C) Total RNA was extracted as described in B and RT-PCR reactions were performed for the indicated genes. As internal control, RT-PCRs were performed for β-actin.

Among the novel target genes, 17 could be validated by Northern blotting or RT-PCR, namely IL-13, MDC, I-309, EMR1, CD44, ABIN, LSP-1, protein kinase C (PKC)-δ, STAT5a, Spi-B, LPS-induced TNF-α factor (LITAF), HLA-F, glucose transporter protein GLUT5, TFPI-2, TPMT, KIAA0084, and RES4–25 (Fig. 4, B and C). The chemokine I-309 was strongly and NF-κB dependently expressed in virally infected cells (Fig. 4 B). In contrast, only weak amounts of mRNA could be detected in noninfected HRS cells, indicating a stimulating event caused by adenoviral infection. The remaining six candidates, like TC21 or NCF2 (Fig. 2), could not be confirmed

(unpublished data). This might be caused by the fact that these candidates display very low average values or that the observed change in the average difference was near the cut-off criteria (Fig. 2). In general, the microarray analysis was confirmed by Northern and RT-PCR analysis.

As an important observation, many of the novel target genes, including MDC, IL-13, CD44, ABIN, LSP-1, STAT5a, GLUT5, TPMT, and TFPI-2 display high level expression in HRS compared with non-HRS cells, thus correlating with constitutive NF-κB activity. CD44 and IL-13 have been suggested as crucial factors in the pathogenesis of HD (31, 33).

Novel Target Genes Are Inducible by IKK-dependent Signaling. For additional verification, a subgroup of novel target genes was analyzed in 70Z/3 pre-B lymphoma cells and their IKKγ-deficient variant 1.3E2, which is defective in IKK signaling (41). Cells were stimulated with PMA, LPS, or LPS in combination with CHX, and mRNA expression of CCR7, Spi-B, LITAF, PKC-δ, and ABIN was analyzed (Fig. 5). CCR7 and Spi-B mRNA expression was significantly induced by LPS, whereas no or only weak induction was observed with PMA or LPS in the presence of CHX. Because CHX blocks protein synthesis, these observations suggest an additional protein requirement for NF-κB–dependent activation of CCR7 and Spi-B. In contrast, LITAF, PKC-δ, and ABIN mRNAs were induced by all stimuli in 70Z/3 cells. The lack of induction of all five genes in 1.3E2 cells and the results from previous experiments (Fig. 2 and Fig. 4, B and C) reveals that all are regulated through the IKK–NF-κB pathway.

NF-κB Recruitment to Target Promoters. To analyze if NF-κB is recruited to target promoters, ChIP assays (27) were performed with L428 cells. The p65 antibody precipitated an IκBα gene promoter fragment that could be blocked with an antibody-specific peptide, although it did not precipitate the *c-jun* promoter. However, both promoter fragments could be precipitated with an anti–c-Jun antibody (Fig. 6 A). These data demonstrate the specificity of the procedure and are in agreement with the conservation of binding sites in the two genes. Next, ChIP assays were performed with L428 cells that were uninfected or infected with Ad5-IκBΔN or Ad5 control. p65 recruitment to the IκBα promoter was strongly diminished in IκBΔN-expressing cells compared with infected and uninfected controls (Fig. 6 B). We also investigated the association of NF-κB with IL-13, CCR7, CD44, STAT5a, TPMT, TFPI-2, or Glut5 promoter regions (Fig. 6 B). In all cases, we observed NF-κB binding in noninfected and Ad5 control–infected cells. Again, no or only weak binding was observed in the presence of peptide or in cells infected with Adv-IκBΔN. Promoter sequences in the databases (GenBank and euGenes) showed that all analyzed genes contain NF-κB binding site motifs according to the consensus sequence GGGRNNYYCC (Fig. 6 B; reference 42). The data provide strong evidence that these genes are under direct transcriptional control of NF-κB.

NF-κB Induces STAT5a Overexpression and Activation in HRS Cells. The transcription factor STAT5a is an interesting novel target gene, because STAT5a activity is linked to cell growth control. Moreover, constitutively activated STAT5a has been observed in a variety of tumors (43, 44). Similar to mRNA expression (Fig. 4 B), we observed high level protein expression of STAT5a in all HRS cell lines but not in non-HRS cells (Fig. 7 A). STAT5a protein expression was dependent on NF-κB because it was reduced in both L428 and HDLM2 cells 48 h after infection with Adv-IκBΔN. Furthermore, a constitutive STAT5a DNA binding activity was observed in the majority of HRS cell lines, most strongly in L1236 and L540 cells (Fig. 7 B). In line with this, constitutive tyrosine phosphorylation, a prerequisite for STAT5a activation, was observed in HRS cells (Fig. 7 C). Intriguingly, NF-κB inhibition led to a rapid loss of STAT5a phosphorylation and DNA binding activity 24 h after infection with Ad5-IκBΔN (Fig. 7, B and C). Thus, NF-κB controls both expression and activation of STAT5a.

Patients with Classical HD Reveal a High Level of STAT5a Expression in Malignant Cells. As predicted from the data obtained with cell lines, all patients tested with classical HD (24 cases) revealed high level cytoplasmic and nuclear staining for STAT5a in >80% of the HRS cells in the lymph node sections (Fig. 7 D and unpublished data). Tonsil sections, as benign tissue, revealed elevated STAT5a expression in germinal center cells, albeit not at the same level as in HRS cells (unpublished data). Nuclear staining of HRS cells implies that STAT5a is constitutively active in primary HRS cells. In contrast to classical HD, only in a subset (4 out of 14 cases) of lymphocyte predominance HD were all malignant lymphocytic and histiocytic cells significantly stained for STAT5a (unpublished data). Overall, STAT5a staining was weaker in lymphocyte predominance HD compared with classical HD.
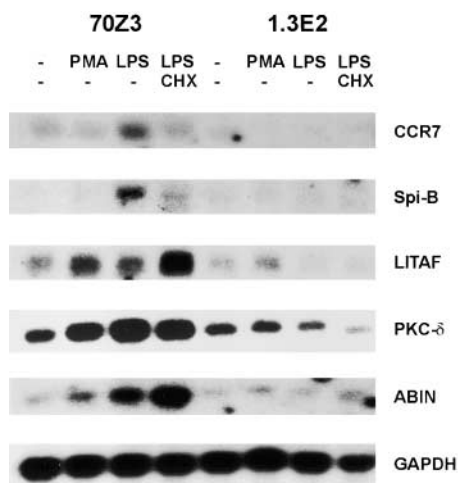
Discussion

A considerable body of work has linked deregulated NF-κB activity to oncogenesis (7). High level constitutive nuclear NF-κB is a characteristic and important property of the malignant cells of HD (15, 18, 21, 26). There is ample evidence that cell death protection is a key function of constitutive NF-κB activity in HRS cells (Figs. 1 and 2; references 21 and 26). However, additional contributions to the pathogenesis of HD are poorly understood. Our large-scale gene profiling revealed that NF-κB regulates a
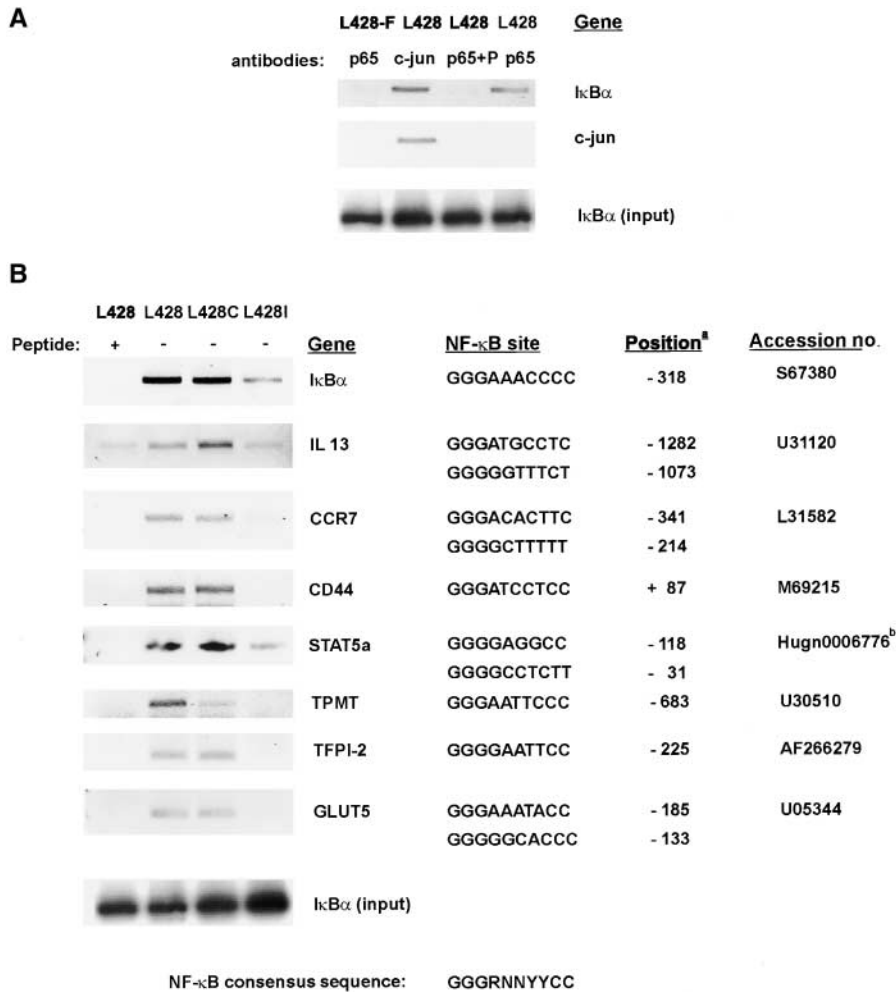

Figure 5. Novel NF-κB target genes are induced by IKK-dependent signaling in pre-B cells. 70Z/3 and 1.3E2 cells were stimulated with PMA, LPS, or LPS in combination with CHX for 2 h. RNA was extracted and Northern blotting was performed for the indicated genes. The stripped blot was reprobed with a GAPDH probe.

Figure 6. Recruitment of p65 to target gene promoters. (A) For ChIP assays, L428 cells were treated with (L428) or without (L428-F) formaldehyde and sheared chromatin fragments were precipitated with antibodies against c-Jun or p65 in the absence or presence (+P) of antibody-specific peptide. *IκBα* and *c-jun* gene fragments were amplified by PCR as indicated. DNA from input chromatin (input) were analyzed for the presence of *IκBα* sequences by PCR reactions using ^{32}P-dCTP. (B) ChIP assay was performed with uninfected L428 cells or with cells infected with Ad5 control (L428C) or Ad5-IκBΔN (L428I) 24 h after infection. Immunoprecipitation with anti-p65 antibody was performed in the absence or presence of antibody-specific competitor peptide as indicated. p65 coprecipitated gene fragments were analyzed by semi-quantitative RT-PCR with promoter-specific primers for the individual genes as indicated. NF-κB binding sites in promoters under investigation are shown, [a], position relative to the predicted transcriptional start site; [b], euGenes identification. Control of input chromatin was performed as described in A. Accession numbers are indicated.

complex network of genes, which are overexpressed in primary and cultured HRS cells (Table I; references 18, 26, 31, 32, 38, 40, and 45–49). A significant fraction of these genes appears to determine important characteristic properties of malignant cells in HD.

In addition to the antiapoptotic function, NF-κB might render tumor cells resistant to chemotherapy, as thiopurine TPMT, which catalyses S-methylation of thiopurines such as 6-mercaptopurine and 6-thioguanine, was identified as a novel target with high level expression in HRS cells (Figs. 2, 4 C, and 6). Indeed, TPMT activity is relevant for chemotherapy treatment, as has been reported for childhood acute lymphoblastic leukemia (50).

The malignant HRS cells invoke the infiltration of reactive cells including granulocytes, plasma cells, and T cells. The expression of the chemokine MDC with the adhesion molecule ICAM-1 and CD86 by HRS cells has been proposed to account for a preferential influx of Th2-type T cells and the suppression of Th1-type immune response (45). Recent data suggested that TNF-α secretion by HRS cells induces eotaxin in fibroblasts of HD tissue, which subsequently recruits T cells and eosinophils (51). Likewise, CX3CL1 and CD83 have the potential to attract T cells

and might contribute to T cell influx into the affected lymph nodes (52, 53). Similarly, cytokines like IL-6 and GM-CSF stimulate plasma cells, Th2 cells, and eosinophils (45, 54). Taken together, these NF-κB target genes are strongly implicated to contribute to the architecture of affected lymph nodes in HD.

Tumor cells of classical HD are predominantly found in the interfollicular zone or less frequently in the follicular mantle zone of partially infiltrated nodes (55). Thus, confining tumor cells to distinct lymphoid compartments might be mediated by chemokine receptors like CCR7. In agreement with recent data, CCR7 was determined as a bona fide NF-κB target gene in HRS cells (38). CCR7 might not only contribute to distinct dissemination of neoplastic cells into lymphoid organs, but also seems to have a more general role in tumor cell migration because a critical role in breast cancer metastasis has been described (56). Likewise, CD44 is expressed at high levels in HRS cells and implicated in the dissemination of HRS cells (31). Moreover, the expression of CD44 splice variant v10 is associated with an unfavorable clinical prognosis (31). Finally, the serine protease inhibitor TFPI-2, which has a proinvasive effect in hepatocellular carcinoma cells, might
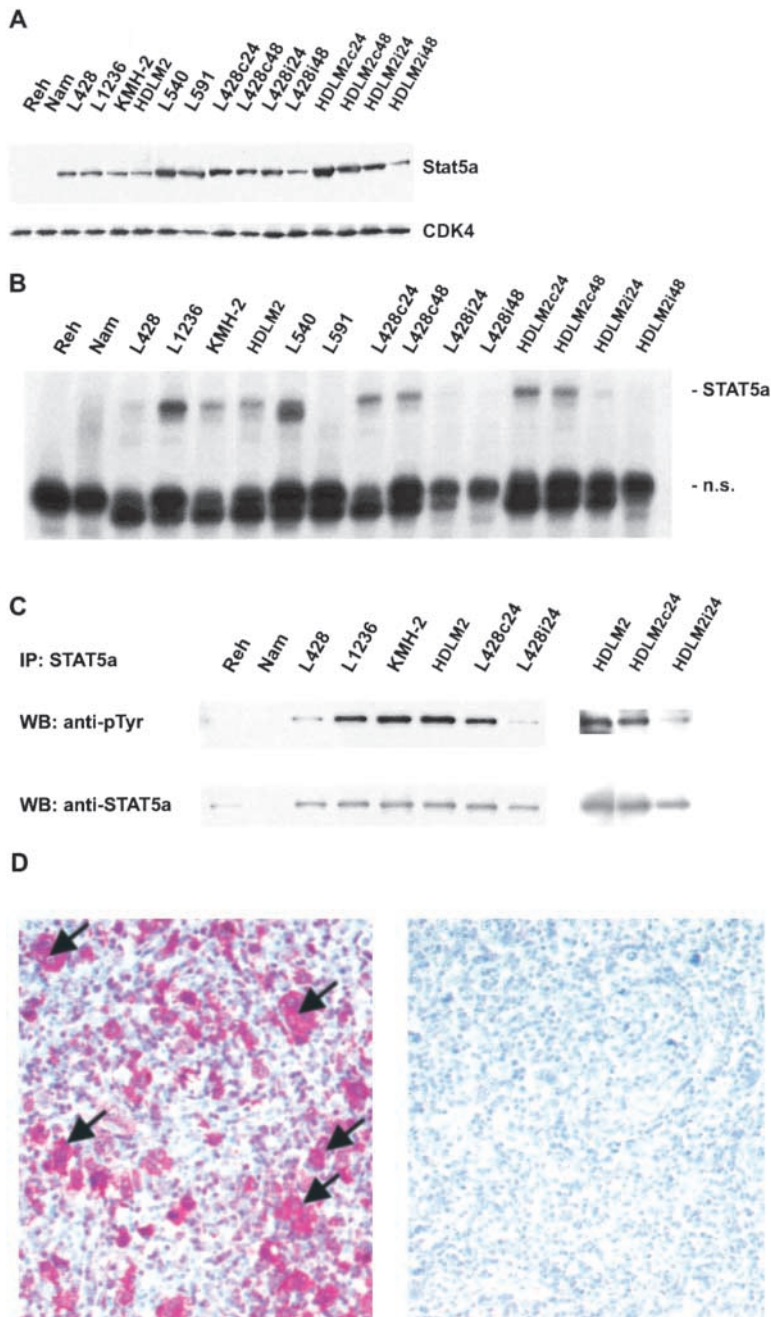
Figure 7. NF-κB induces STAT5a overexpression and activation in HRS cells. (A) Whole cell extracts of control and HRS cells, or infected L428 and HDLM2 cells as indicated, were analyzed by Western blotting with anti-STAT5a antibody (top). Loading control blots were probed with anti-CDK4 antibody (bottom). (B) STAT5a DNA binding activity in whole cell lysates of control and HRS or infected L428 and HDLM2 cells was analyzed by EMSA. Specificity of STAT5a–DNA complexes was confirmed in competition experiments (unpublished data). (C) Immunoprecipitations were performed with whole cell lysates of control and HRS or infected L428 and HDLM2 cells. STAT5a tyrosine phosphorylation was detected by Western blotting using antibodies against phosphotyrosine (top left). Analysis of HDLM2 cells is shown in a separate experiment (top right). Blots were reprobed with antibodies against STAT5a (bottom). (D) Patients with classical HD reveal high level of STAT5a expression in malignant cells. Immunohistochemistry of classical HD. All HRS cells (left; arrows indicate representative cells) reveal strong STAT5a staining compared with surrounding benign cells. Specificity of STAT5a detection was confirmed by competition with a specific peptide (right).

also be involved in the migration of HD tumor cells (57). Altogether, NF-κB controls a set of genes that likely regulates tumor cell localization.

Deregulated proliferation is a typical event associated with malignant transformation. Therefore, the overexpression of factors involved in growth control is of great interest. Both IL-13 and CD40 play a critical role in B cell proliferation (18, 58). Remarkably, IL-13–neutralizing antibodies blocked the proliferation of HDLM-2 cells (33). Likewise, the IL-15–IL-15R signaling pathway was suggested as important for tumor propagation in multiple myeloma (59). Our data reveal IL-15R overexpression in HRS cells (Fig. 5). However, a potential role of the IL-15–

IL-15R signaling pathway in HD has to be established. Aside from deregulated proliferation, malignant cells display high rates of glucose uptake and glycolysis (60). The overexpression of GLUT5, as observed in this study, might permit the enhanced uptake of fructose and provide a metabolic advantage for HRS cells (61).

As a striking observation, constitutive NF-κB activates the STAT5a signaling pathway both by overexpression and by the induction of tyrosine phosphorylation of STAT5a in cultured HRS cells (Figs. 2, 4, and 7). Notably, all patients analyzed with classical HD express high level activated STAT5a in the entire tumor cell population (Fig. 7 D). These findings establish a new level of complexity in the

Table I. *Pathogenetic Relevance of NF-κB Target Genes for HD and Other Neoplastic Malignancies*

Gene	Known target	Verified target	Expression in HRS cells[a]	Expression in HRS cells[b]	Expression in other tumors	Potential involvement in pathogenesis
IL-6	+		+	+	Multiple myeloma, HNSCC	Induction of plasma cell infiltration
GM-CSF	+		+	+	HNSCC	Regulates eosinophil proliferation
CX3CL1	+		+	+	—	Recruits T cells to lymphoma
ICAM-1	+		+		Multiple myeloma	Recruits T cells to lymphoma
CD83	+	+	+	+	—	Recruits T cells to lymphoma
MDC		+		+	—	Recruits Th2-type T cells to lymphoma
CD86	+		+		B cell leukemia	Interacts with T cells, induces anergy?
Lymphotoxin-α	+		+		—	Inflammatory mediator
TNF-α	+		+		Multiple myeloma	Inflammatory mediator
IL-13		+	+	+	—	Proliferation
IL-15Rα	+	+		+	Multiple myeloma, ATL	Proliferation, survival
CD40	+		+		B cell lymphoma, carcinoma	Proliferation, survival
STAT5a		+		+	ATL, CML, ALL	Proliferation, survival
GLUT5		+		+	Breast carcinoma	Metabolic advantage
IEX-1	+	+		+	—	Antiapoptotic
Bcl-x$_L$	+		+		Breast carcinoma	Antiapoptotic
A1/Bfl-1	+		+		Gastric and colon carcinoma	Antiapoptotic
c-IAP2	+		+		—	Antiapoptotic
TRAF1	+		+		—	Antiapoptotic
TPMT		+		+	ALL	S-methylation of thiopurines
CCR7	+	+	+	+	ATL, breast carcinoma	Dissemination into lymphoid organs
TFPI-2		+		+	Ovarian carcinoma, HCC	Invasion
CD44		+	+	+	Colon and breast carcinoma, B-CLL, multiple myeloma	Dissemination in lymphoid organs associated with high risk of relapse
ABIN		+		+	—	?
LSP-1		+	+	+	B cell leukemia and lymphoma	?
NF-κB2/p100	+	+		+	Breast and colon carcinoma	?

Known target, NF-κB regulation was previously described (26, 29, 38, 77) for HRS or non-HRS cells; Verified target, NF-κB regulation in HRS cells was determined by Northern/RT-PCR; ATL, adult T cell leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; HCC, hepatocellular carcinoma cell; B-CLL, B cell chronic lymphocytic leukemia.
[a]Genes for which the high level expression in primary or cultured HRS cells was described in the literature (18, 26, 31, 32, 38, 40, 45–49).
[b]Genes for which the high level expression in primary or cultured HRS cells was demonstrated in this study.

oncogenic function of NF-κB. STAT5a has been implicated in hematopoietic cell growth and tumor development and may therefore present an important downstream effector of NF-κB (43, 44). Because STAT5a regulates cell cycle progression via activating D-type cyclins (62) and inhibits apoptosis by stimulating Bcl-x$_L$ expression (63), it might contribute to cyclin D2 and Bcl-x$_L$ induction in HRS cells in synergism with NF-κB (26). Because cyclin D2 could not be detected as a direct NF-κB target gene in the microarray analysis 24 h after Adv-IκBΔN infection but is affected at later time points (26), NF-κB could regulate cyclin D2 expression in part via STAT5a. In fact, both cyclin D2 and Bcl-x$_L$ contain functional STAT5 binding sites in their promoter regions (64, 65).

Aside from HD, the expression of aberrant NF-κB activity or mutant *rel/nfkb* genes has been noted in many human hematopoietic (e.g., multiple myeloma, adult T cell leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia, and B cell leukemia and lymphoma) and solid tumors (e.g., head and neck squamous cell [HNSCC], breast, colon, and ovarian carcinoma; reference 7). Correspondingly, many of the NF-κB target genes are overexpressed in these tumors and are implicated to contribute to their pathogenesis (Table I). In particular GLUT5, Bcl-x$_L$,

CCR7, CD44, and p100 are overexpressed in breast carcinoma (7, 56, 61, 66, 67). In colon carcinoma, high levels of CD44, Bfl-1/A1, and p100 have been observed (7, 67, 68). A high concentration of cytokines IL-6 and GM-CSF are produced by tumor cells of patients with HNSCC (69), whereas enriched levels of TFPI-2 were found in ovarian tumor samples (70). Different types of leukemia display aberrant expression of CD86, IL-15Rα, STAT5a, CCR-7, CD44, and LSP-1 (32, 44, 71–74). At last, the overexpression of the NF-κB target genes IL-6, TNF-α, ICAM-1, IL-15Rα, and CD44 seems to contribute to the pathogenesis of multiple myeloma (59, 75, 76). NF-κB–dependent gene profiling in the described tumors might extend these observations and further manifest the role of deregulated NF-κB activity in oncogenesis.

In addition to clinical implications, this study provides new insights into NF-κB–dependent gene regulation. Only a minority of previously described target genes (29, 77) could be identified as NF-κB regulated in HRS cells. Although several known target genes like RANTES or A20 are expressed in HRS cells, they either did not respond or only weakly responded upon NF-κB inhibition (unpublished data). Thus, constitutive NF-κB drives a specific network of genes in HRS cells that differs from signal-induced NF-κB–dependent regulation in other cell types. Although all HRS cell lines display constitutive NF-κB activity, there is some heterogeneity in the expression of several target genes (Fig. 4). Likewise, junB, which is regulated by NF-κB (29 and unpublished data), met the criteria for NF-κB target genes only in HDLM2 cells. These observations reflect variations in gene expression control among different HRS cells and suggest the requirement of additional factors for NF-κB–dependent gene activation. In fact, LPS stimulation of CCR7 and Spi-B expression in 70Z/3 cells was abrogated when protein synthesis was inhibited (Fig. 5). NF-κB might act in concert with other transcription factors such as AP-1, or certain coactivators like CBP/p300, to fully activate gene expression (78, 79). Furthermore, some promoters require modifications in chromatin structure to make NF-κB sites accessible (27). Taken together, NF-κB–dependent gene expression is a matter of both the NF-κB activation status and the molecular environment within a given cell type.

In summary, this study underlines a fundamental importance of NF-κB in HD. NF-κB controls a complex network of genes, which promotes the specific architecture of Hodgkin lymphoma, supports proliferation and migration, and confers resistance to apoptosis. Pharmacological manipulation of the NF-κB system or of selected target genes might have a therapeutic potential for HD and other neoplastic malignancies.

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