

RESEARCH ARTICLE

Identification of novel Stat6 regulated proteins in IL-4-treated mouse lymphocytes

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Interleukin 4 (IL-4) has an indispensable role in the differentiation of naive T helper (Th) cells toward the Th2 phenotype and induction of B cells to produce the IgE class of Igs. By regulating these two cell types, IL-4 has a pre-eminent role in regulation of allergic inflammation. IL-4-mediated regulation of T and B cell functions is largely transmitted through signal transducer and activator of transcription 6 (Stat6). In this study, we have used metabolic labeling and 2-D electrophoresis to detect differences in the proteomes of IL-4 stimulated spleen mononuclear cells of Stat6^{−/−} and wild type mice and MS/MS for protein identification. With this methodology, we identified 49 unique proteins from 21 protein spots to be differentially expressed. Interestingly, in Stat6^{−/−} CD4⁺ cells the expression of isoform 2 of core binding factor b (CBFb2) was enhanced. CBFb is a non-DNA binding cofactor for the Runx family of transcription factors, which have been implicated in regulation of Th cell differentiation. We also found cellular nucleic acid binding protein (CNBP) to be downregulated in Stat6^{−/−} cells. None of the proteins identified in this study have previously been reported to be regulated *via* Stat6. The results highlight the importance of exploiting proteomics tools to complement the studies on Stat6 target genes identified through transcriptional profiling.

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1 Introduction

T helper cells (Th cells) are a subpopulation of T cells that express CD4 molecule on their cell membrane and are involved in activating and directing immune responses. Th cells can differentiate into functionally distinct subtypes, of which the most studied are Th1 and Th2 cells. The balance

between these cell types needs to be tightly controlled in order to acquire the adequate immune responses, and to avoid over-reactivity against both internal and external antigens. Sustained predominance of Th1 or Th2 cells is linked to a number of autoimmune and allergic diseases, respectively [1]. Th cell subtypes are defined by their cytokine expression profiles; Th1 cells produce mainly interferon (IFN) γ and the characteristic cytokines for Th2 cells are interleukin-4 (IL-4), IL-5, and IL-13 [2, 3].

IL-4 has a variety of functions in immune system. In naive Th cells, IL-4 drives the differentiation toward Th2 phenotype after the encounter of the specific antigen [4]. IL-4 also induces B cells to produce the IgE class of Igs. By regulating these two cell types, IL-4 has an important role in the regulation of allergic inflammation reactions [5]. IL-4 binding to its recep-

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Abbreviations: CBFb2, core-binding factor, beta subunit isoform 2; CNBP, cellular nucleic acid binding protein; MNC, mononuclear cell; IFN, interferon; IL, interleukin; RT, room temperature; Stat6, signal transducer and activator of transcription 6; Th, T helper

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tor leads to activation of intracellular phosphorylation cascade mediated by Jak1 and Jak3 kinases during which intracellular parts of IL-4 receptor become phosphorylated and provide docking sites for signal transducer and activator of transcription 6 (Stat6) transcription factor. Phosphorylation of Stat6 by receptor associated kinases releases the Stat6 molecule from the receptor and enables homodimerization. Dimerized Stat6 complexes translocate into the nucleus, bind to the regulatory regions of responsive genes in a sequence specific manner, and with the help of cofactors regulate target gene transcription [6, 7]. In addition to IL-4, IL-13 can also activate Stat6-mediated transcription [7–9], but it cannot replace IL-4 as a Th2 differentiation factor [8, 10].

Experiments with Stat6 deficient (Stat6^{−/−}) mice have shown that this molecule is indispensable for optimal Th2 cell differentiation, production of IgE antibodies, and most importantly for the development of antigen-induced airway inflammation and hyper-responsiveness [11–14]. By analyzing the transcriptomes of Stat6^{−/−} Th cells and B lymphocytes, a group of STAT6 regulated genes have been identified [15, 16]. The findings with different cell types overlap to some extent, but also show that Stat6 has cell type specific target genes. Despite studies that have been carried out to identify Stat6 targets at the mRNA level [15, 16], so far no studies have been published on the protein complement of the transcriptome. Moreover, proteomics provides unique information of the complexity of gene regulation by enabling unbiased detection of different splice variants and PTMs. In this study, we have explored the role of Stat6 transcription factor in mouse mononuclear cells (MNC) by using 2-DE-based proteomics. We found 21 protein spots to be differentially expressed between IL-4 stimulated Stat6 deficient cells compared to wild type control cells, and identified 49 unique proteins from these spots. Interestingly, the proteins differentially expressed include core binding factor b (CBFb), which is a non-DNA binding cofactor for Runx family of transcription factors [17] and cellular nucleic acid binding protein (CNBP), which regulates gene expression both at the level of transcription and translation [18, 19].

2 Materials and methods

2.1 Cell culture

Stat6 deficient mice and control wild type Balb/cJ mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Mice were bred and maintained in the Central Animal Laboratory of the University of Turku and experiments conducted with the permission from the Lab-Animal Care and Use Committee of Turku University. MNCs were isolated from homogenized spleens with Mouse Lympholyte (Cedarlane, Hornby, Canada). Before culturing the cells for 2-DE separation the remaining erythrocytes were lysed hypotonically with 0.83% NH₄Cl. Cells were activated with plate-bound anti-mouse CD3 (clone 500A2, Pharmingen, San Diego, CA, USA) as follows: anti-CD3

(325 ng/250 µL/well) was incubated for 2 h at 37°C and washed three times with 0.5 mL D-PBS before the cells were added to the wells. Cells were cultured in RPMI (Sigma, St. Louis, MO, USA) containing 5% heat inactivated FCS, 1 mM Na-pyruvate (Sigma), 2 mM L-glutamine (Sigma), 50 µM β-mercaptoethanol (Invitrogen, Paisley, Scotland, UK), 1 × non-essential amino acids (Invitrogen), and penicillin + streptomycin (Sigma). Cells were stimulated with recombinant murine IL-4 (10 ng/mL, Pharmingen, San Diego, CA, USA). For metabolic labeling, Redivue Pro-Mix L ³⁵S-methionine and ³⁵S-cysteine (50 µCi/mL, Amersham Pharmacia Biotech) was added to the culture medium consisting methionine-free RPMI (Sigma) and all the supplements described above. The culturing density was 5 × 10⁶ cells/500 µL on 24-well plates. Cells were cultured for 24 h in 37°C, 5% CO₂. Based on flow cytometry analysis, the proportions of CD4⁺ T, CD8⁺ T, CD19⁺ B, Mac-1⁺, and NK cell populations were similar between wt and Stat6^{−/−} cells after activation and IL-4 stimulation, CD4⁺ T (41 ± 3%), CD8⁺ T (22 ± 3%), CD19⁺ B (44 ± 8%) cells being the most predominant cell populations.

CD4⁺ T cells were isolated from the MNC population with positive selection by using MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany). Isolated CD4⁺ T cells were activated with plate-bound anti-CD3 similarly to the MNCs, and cultured in the same culture medium in the presence of anti-CD28 (500 ng/mL, Pharmingen, San Diego, CA, USA). For neutralization, 10 µg/mL of anti-IL-12 and 10 µg/mL of anti-IFNγ were added to the culturing medium of IL-4 stimulated cells. In addition, 10 µg/mL of anti-IL-4 was added to samples not treated with IL-4 (all neutralizing antibodies from Pharmingen, San Diego, CA, USA).

2.2 2-DE and protein detection

Cells were lysed and proteins were separated with 2-DE as previously described [20]. Briefly, soluble proteins were absorbed into the 18 cm 3–10 NL IPG-strips (Amersham Pharmacia Biotech) for 24 h at room temperature (RT). IEF to a total of 40 kVh was carried out at 20°C, and the focused strips were equilibrated for 25 min at RT. The second dimension was vertical 12% SDS-PAGE with gel thickness of 1 mm. Proteins were detected with silver staining [21] and autoradiography (24 h) [22]. Autoradiography images from three independent experiments were used for the comparison of protein expression levels. Gel image normalization and comparison was made with the PDQuest program (version 6.2, BioRad, Hercules, CA, USA). Proteins that were regulated on average by at least two-fold were selected for identification. The reproducibility of differential expression was confirmed manually and only consistently regulated protein spots were selected to be identified with MS.

2.3 Protein identification

The proteins differentially expressed between IL-4-treated Stat6^{−/−} and wt cells, were in-gel digested, and the resulting

peptides were analyzed by nano-LC-MS/MS as previously described [23–26]. Briefly, the excised gel spot was cut into pieces, washed twice, and dehydrated with ACN. Proteins were reduced with 20 mM DTT (Sigma), alkylated with 55 mM iodoacetamide (Sigma), and in-gel digested with trypsin (Sequencing Grade Modified Trypsin, Promega Corporation, Madison, WI, USA) at +37°C overnight. Peptides were extracted with 5% formic acid, 50% ACN, and dried with a vacuum centrifuge. Peptides were dissolved to 2% HCOOH prior to MS analysis. Analyses were made with a QStar Pulsar™ ESI-hybrid quadrupole-TOF instrument (Applied Biosystems/MDS Sciex, Toronto, Canada) coupled on-line with nanoHPLC (Famos, SwitchosII and Ultimate, LC Packings, Amsterdam, Netherlands) as previously described [26].

Peak lists from the MS/MS spectra were created from the Analyst QS program (version 1.1, Applied Biosystems) using the MASCOT.dll script with the following parameters: centroided mass, double and triple charged peptides as precursors only, peak threshold 0.01% of maximum, precursor mass tolerance for grouping 0.2, minimum 1 and a maximum ten cycles between groups. These spectra were analyzed using MASCOT software (www.matrixscience.com, version 02.01.02) via MASCOT Daemon 2.1.6 software (Matrix Science, London, UK) and searched against Swiss-Prot (version 51.5) and TrEMBL database (version 34.5) using the following parameters: mammalia specific taxonomy, trypsin digestion with maximum one missed cleavage, carbamidomethyl modification of cysteine as a fixed modification and oxidation of methionine as a variable modification, peptide tolerance maximum ± 0.3 Da, MS/MS tolerance maximum ± 0.3 Da, peptide charge +2 or +3, monoisotopic mass. All keratin, trypsin, and albumin related identifications were excluded from the list. If mouse specific protein identification was acquired, identifications from all other taxonomy groups were excluded. Only proteins with *p*-value below 0.05 and protein score above 50 in the MASCOT peptide summary report were included in the protein identification list.

2.4 Western blotting

Whole cells and insoluble protein pellet fractions were boiled with 62.5 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.1% w/v bromophenol blue. Cytosolic and nuclear protein fractions were boiled with 5 × SDS sample buffer. SDS-PAGE and protein transfer to NC membranes were carried out according to standard protocols [27]. When proteins, that were first separated with 2-DE using Protean II (BioRad), were transferred to NC membrane, the 2-DE gels were cut into convenient parts to be transferred using either Mini Trans-Blot cell or Criterion blotter (BioRad). The following antibody dilutions were used for Western blots: mouse anti-CBFB 1:250 (BD Transduction Laboratories no. 610514), rabbit CNBP antiserum 1:500, and b-actin 1:10 000 (Sigma A-5441). CNBP antiserum was a generous gift from Dr. Paola Pierandrei-Amaldi. Secondary antibodies used in this study: 1:10 000 HRP goat anti-mouse (sc-2005, Santa Cruz Bio-

technology), 1:5000 HRP goat anti-rabbit (no. 554021, BD Pharmingen). All results are representative from at least three independent experiments unless otherwise stated.

2.5 Nuclear fractionation and Runx1 DNA binding activity

Harvested cells were washed with PBS and cell membrane lysed with a hypotonic lysis buffer (0.2% Tween, 1 mM NaF, 1 mM Na₃VO₃, 1 mM DTT and complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) in 22 mM Hepes, pH 7.9) for 2 min on ice, nuclear fraction was spun down, and cytosolic fraction collected. Nuclei were incubated in the extraction buffer (1.0% glycerol, 430 mM NaCl, 1 mM NaF, 1 mM Na₃VO₃, 1 mM DTT and complete protease inhibitor cocktail (Roche Diagnostics) in 22 mM HEPES, pH 7.9) for 1 h on ice. Extracted nuclear proteins were collected after the cell debris was spun down. Alternatively, after removal of cytosolic fraction and incubation in the nuclear extraction buffer, the nuclear protein pool was sonicated with the cell debris, samples were centrifuged (12 000 rpm for 4 min, +4°C), and supernatants collected. Cytosolic and nuclear fractions were stored at –70°C. Protein concentration in each fraction was quantified with Bradford method or DC protein assay reagents package (BioRad 500-0116). Runx1 DNA binding activity was measured with TransAM AML-1/Runx1 kit (Active Motif no. 47396). Nuclear extracts from HL-60 cell line were used for the standard curve against which the A values were normalized. Equal amount of protein in each nuclear sample was used for the measurement.

2.6 Confocal microscopy

0.1–0.5 × 10⁶ cells/sample were fixed with 3.65% formaldehyde for 15 min at RT and permeabilized with 0.2% saponin or 0.1% Triton X-100. Unspecific binding was blocked with 10% BSA. Primary antibody incubations were done in 1.0% BSA/permeabilization buffer overnight at 4°C and secondary antibody incubations in the same buffer 45 min at RT. Antibodies used were: CBFB (BD no. 610514, 1:25) and Runx1 (Active motif no. 39000, 1:100), goatF(ab')₂ anti-mouse IgG (H + L), FITC conjugate (Caltag no. M35001, 1:200), and goat anti-rabbit Alexa 568 nm (Molecular Probes, 1:400). Nuclei were stained separately with dapi or by using dapi-containing mounting medium (Molecular probes Slow-Fade, no. S36938). Zeiss LSM510 META laser scanning confocal microscope was used in image acquisition.

3 Results and discussion

3.1 Differentially expressed proteins in IL-4 stimulated Stat6–/– and wt mononuclear cells

To identify Stat6 target proteins, MNCs were isolated from the spleens of wt and Stat6–/– mice, activated through CD3

and cultured for 24 h in the presence of IL-4 and radioactive ^{35}S -Met/Cys. Proteins were subsequently separated with 2-DE and newly expressed proteins were detected with autoradiography. Protein spots that were differentially expressed by at least two-fold between wt and Stat6 $^{-/-}$ samples were analyzed with MS/MS. With this methodology, we found 21 protein spots to be differentially expressed between wt and Stat6 $^{-/-}$ cells, 16 of which (spots number 1–16 in Fig. 1) were more highly expressed in wt and 5 (spots number 17–21 in Fig. 1) in Stat6 $^{-/-}$ samples. Altogether 49 unique proteins were identified from these protein spots (Table 1) indicating that separation power of 2-DE was exceeded. This is a common phenomenon reflecting the complexity of the proteome of whole cell lysate [28] and has been also reported previously in the experimental setups similar to the one used in this study [29, 30]. To our knowledge, none of the proteins identified in this study have previously been reported to be Stat6 regulated in transcriptomics studies conducted with either mouse B cells or CD4 $^{+}$ T cells [15, 16]. There are several factors which can explain the differences between the results acquired with these two methodologies. In fact, this is in concordance with most of the studies showing weak or absent correlation between mRNA and protein expression levels [31, 32]. Many genes which were found to be differentially expressed in the transcriptomics studies in question represent genes which code low-abundant, secreted or transmembrane proteins making them difficult to be detected with 2-DE. Due to the sample preparation and separation methods used, the analyzed proteins in 2-DE studies are from soluble cellular fraction and exclude the proteins with

extreme pI and molecular mass. On the other hand, transcriptomics cannot be utilized to detect post-translational regulation, which is an important and common part of protein regulation. In addition to the technical aspects, the cell types and designs of these experiments differ from each other, thus preventing true comparison. The present study complements the current knowledge of the Stat6-mediated signaling and opens interesting views on the post-transcriptional target protein expression.

The proteins identified have a wide range of cellular functions, varying from growth regulation to transcriptional regulation, which is an important and common part of protein regulation. For example, GST P1 (identified from spot number 7 with a higher expression in wt cells) has been reported to suppress the activity of JNK and Stat (especially Stat1, Stat3, and Stat5) proteins, and its removal leads to a selective expansion of B lymphocytes [33]. Stat6-dependent regulation of this protein has not been reported before. FK506-binding protein 4 (identified from spot number 1 having higher expression in wt cells) has been reported to inhibit the transcriptional activity of interferon regulatory factor 4 protein [34], which is expressed in B and mature T cells regulating their homeostasis as well as IL-4 signaling and Th2 type immune responses [35–40].

Two of the identified proteins that were found to be reproducibly more expressed in wt than Stat6 $^{-/-}$ cells (Table 1, protein spot 8 and 14, respectively), namely core-binding factor β subunit isoform 2 (CBFb2) and CNBP, were selected on the basis of their reported functions for validation and further studies. CBFb2 and CNBP are both known to be

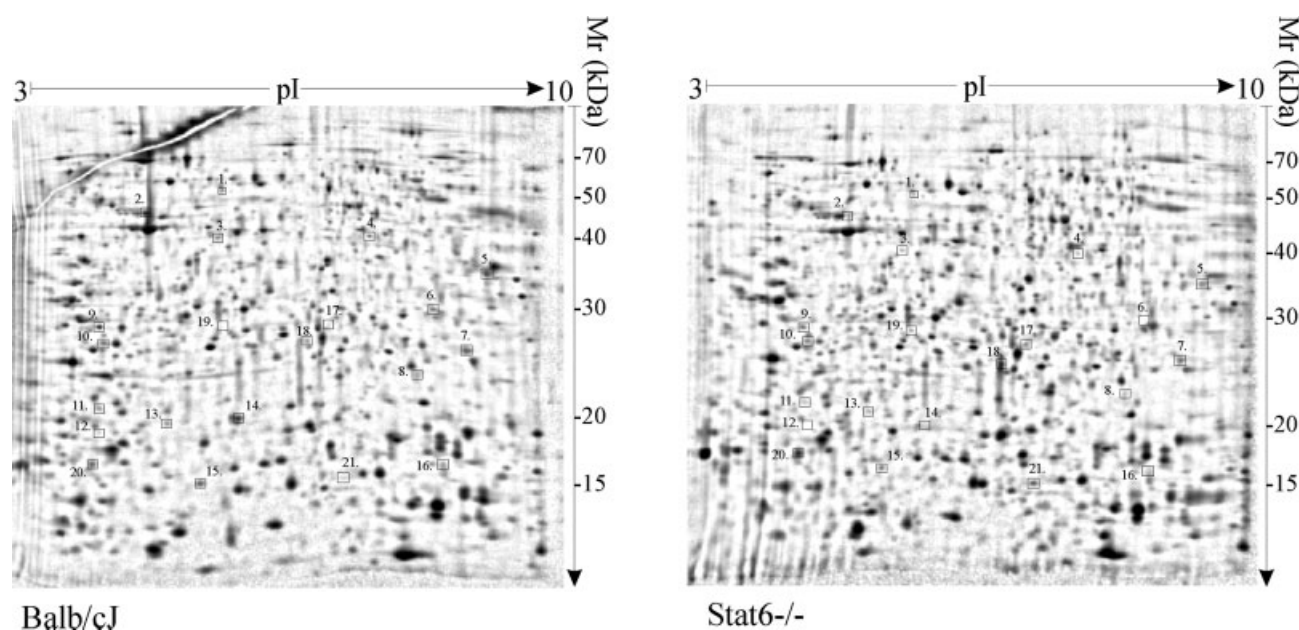


Figure 1. Stat6 regulated protein spots in mouse MNCs. Isolated wt or Stat6 $^{-/-}$ MNCs were activated through CD3 and metabolically labeled for 24 h in the presence of IL-4. Cells were lysed and soluble proteins were separated with 2-DE, and metabolically labeled proteins were detected with autoradiography. Spots 1–16 showed at least two-fold higher expression in wt and spots number 17–21 in Stat6 $^{-/-}$ samples.

Table 1. The proteins differentially expressed between wt and Stat6^{−/−} MNCs after IL-4 stimulation

Spot no.	Fold change	Repro-ducibility	Entry name ^{a)}	The identified protein	MW (kDa)	pI	Protein score	No. of peptides	Sequence coverage (%)
Proteins expressed more in wt than Stat6^{−/−} cells									
1	2.2	3/3	COR1A_MOUSE	Coronin-1A (Coronin-like protein p57, Coronin-like protein A, Clipin-A)	51 641	6.05	143	4	7.4
			FKBP4_MOUSE	FK506-binding protein 4	51 939	5.54	131	4	9.2
			CH60_MOUSE	60 kDa heat shock protein, mitochondrial precursor (Hsp60)	61 088	5.91	57	2	3.1
2	2.2	3/3	ACTB_MOUSE	Actin	42 052	5.29	167	5	15.2
			ACTC_MOUSE						
			ACTG_MOUSE						
			ACTS_MOUSE						
			HNRPF_MOUSE	Heterogeneous nuclear ribonucleoprotein F (hnRNP F)	46 043	5.31	164	8	22.9
3	2.0	2/3	PSD13_MOUSE	26S proteasome non-ATPase regulatory subunit 13	43 124	5.46	426	16	44.1
			Q9CQT1_MOUSE	RIKEN clone:3010001P22 product: hypothetical Initiation factor 2B-containing protein	39 671	5.6	199	5	13.8
			SHLB1_MOUSE	SH3 domain GRB2-like protein B1 (Endophilin-B1)	41 115	5.78	52	3	7.4
4	2.4	3/3	IF33_MOUSE	Eukaryotic translation initiation factor 3 subunit 3 (eIF-3 γ)	39 978	6.2	513	14	31.5
			TWF2_MOUSE	Twinfilin-2 (Twinfilin-1-like protein, A6-related protein, mA6RP)	39 674	6.33	216	7	24.1
			COR1A_MOUSE	Coronin-1A (Coronin-like protein p57, Coronin-like protein A, Clipin-A)	51 641	6.05	63	2	3.5
			CTBP1_MOUSE	C-terminal binding protein 1 (EC 1.1.1.1-, CtBP1)	48 084	6.28	52	1	2.5
5	2.3	2/2	G3P_MOUSE	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12, GAPDH)	36 072	8.44	484	9	26.7
			MDHM_MOUSE	Malate dehydrogenase	36 030	8.83	187	3	9.2
6	3.6	3/3	GBLP_MOUSE	Guanine nucleotide binding protein subunit β 2-like 1	35 511	7.6	347	7	25.6
			CH1B2_MOUSE	Charged multivesicular body protein 1b-2 (Chromatin-modifying protein 1b-2, CHMP1b-2)	22 204	7.82	106	3	12.6
			CH1B1_MOUSE	Charged multivesicular body protein 1b-1 (Chromatin-modifying protein 1b-1, CHMP1b-1)	22 167	7.82	91	3	12.6
			RSU1_MOUSE	Ras suppressor protein 1 (Rsu-1, RSP-1)	31 531	8.86	84	2	5.8
			PSA4_MOUSE	Proteasome subunit α type	29 737	7.59	53	1	3.8
7	2.4	3/3	PSB1_MOUSE	Proteasome subunit β type	26 583	7.67	386	12	50.4
			GSTP1_MOUSE	Glutathione S-transferase P 1	23 765	7.68	247	7	35.2
8	2.0	3/3	Q3UYR3_MOUSE ^{b)}	Core binding factor subunit β (CBF- β), isoform 2	16 120	5.52	105	3	23.9
			PEBB_MOUSE		22 245	5.59	86	3	17.6
9	2.4	3/3	Q3UIQ7_MOUSE	Heat shock protein HSP 90- β (HSP 84)	83 513	4.98	229	10	12.3
			Q71LX8_MOUSE						
			LMNB1_MOUSE	Lamin-B1	66 973	5.11	141	6	9.9
			PGK1_MOUSE	Phosphoglycerate kinase 1	44 907	7.53	57	2	8.4
			ACTB_MOUSE	Actin	42 052	5.29	55	3	9.6
			ACTG_MOUSE						
10	2.5	2/3	LMNB1_MOUSE	Lamin-B1	66 973	5.11	355	10	15.6
			GDIR_MOUSE	Rho GDP-dissociation inhibitor 1 (Rho GDI 1, Rho-GDI α , GDI-1)	23 450	5.12	250	6	33.8
			ACTB_MOUSE	Actin	42 052	5.29	93	3	9.9

Table 1. Continued

Spot no.	Fold change	Repro-ducibility	Entry name ^{a)}	The identified protein	MW (kDa)	pI	Protein score	No. of peptides	Sequence coverage (%)			
11	2.8	2/2	ACTG_MOUSE	Heat shock protein HSP 90	85 134	4.93	64	1	1.6			
			HS90A_MOUSE									
			HS90B_MOUSE									
			LASP1_MOUSE	LIM and SH3 domain protein 1 (LASP-1, MLN 50)	30 374	6.61	64	1	4.9			
			Q9QY93_MOUSE									
			PSB9_MOUSE									
			VIME_MOUSE	Vimentin	53 712	5.11	148	2	42.4			
			Q6PFA2_MOUSE	Clathrin, light polypeptide	23 496	5.06	120	4	13.7			
			Q3THU7_MOUSE	Chromobox protein homolog 3	23 606	4.39	108	2	11.6			
			CBX3_MOUSE									
			DBLOH_MOUSE									
12	3.5	2/2	ACTB_MOUSE	Actin	21 013	5.13	92	2	14.8			
			ACTG_MOUSE									
			No identification									
			MTND_MOUSE	1,2-Dihydroxy-3-keto-5-methylthiopentene dioxxygenase (Aci-reductone dioxxygenase, ARD)	26 984	6.15	92	3	13.1			
			PEBP1_MOUSE									
			Q3U935_MOUSE									
			Q3ULK8_MOUSE	Phosphatidylethanolamine binding protein 1 (PEBP-1, HCNPpp)	21 567	5.31	222	8	53.1			
			H2A1_MOUSE ^{c)}									
			Not identified									
			UB2D3_MOUSE	Cellular nucleic acid binding protein (CNBP)	20 988	5.19	111	5	31.6			
			H2A1_MOUSE ^{c)}									
Ubiquitin-conjugating enzyme E2 D3												
13	2.4	3/3	MTND_MOUSE	Histone H2A	19 984	8	165	4	32.9			
			PEBP1_MOUSE									
			Q3U935_MOUSE									
			Q3ULK8_MOUSE	Histone H2A	14 127	11.05	68	2	12.3			
			H2A1_MOUSE ^{c)}									
			Not identified									
			UB2D3_MOUSE	Ubiquitin-conjugating enzyme E2 D3	16 904	7.67	154	4	42.9			
			H2A1_MOUSE ^{c)}									
			Histone H2A									
			14	10.6	2/2	Q3U935_MOUSE	Histone H2A	14 153	11.05	104	4	35.4
						Q3ULK8_MOUSE						
H2A1_MOUSE ^{c)}												
H2A1_MOUSE ^{c)}	Histone H2A	14 127				11.05	108	2	21.5			
Not identified												
UB2D3_MOUSE												
UB2D3_MOUSE	Ubiquitin-conjugating enzyme E2 D3	16 904				7.67	154	4	42.9			
H2A1_MOUSE ^{c)}												
Histone H2A												
15	4.8	2/2				UB2D3_MOUSE	Histone H2A	14 153	11.05	104	4	35.4
						H2A1_MOUSE ^{c)}						
			Histone H2A									
			Proteins expressed more in Stat6 ^{−/−} than wt cells			DOCK2_MOUSE	Dedicator of cytokinesis protein 2	212 966	6.5	373	7	4
						TPIS_MOUSE						
						THY1_MOUSE						
						PSB3_MOUSE	Triosephosphate isomerase	27 038	6.9	174	4	20.1
						RB11A_MOUSE						
						RB11B_MOUSE						
						RHOA_MOUSE	Thy-1 membrane glycoprotein precursor	18 297	9.16	138	3	22.2
						GRP78_MOUSE						
GRP78_MOUSE												
19	2.1	3/3				GRP78_MOUSE	Proteasome subunit β type 3	23 235	6.15	51	1	3.4
						RB11A_MOUSE						
			RB11B_MOUSE									
			RHOA_MOUSE	Ras-related protein Rab-11	24 492	6.12	296	8	33.8			
			GRP78_MOUSE									
			GRP78_MOUSE									
			20	2.5	3/3	H2A1_MOUSE ^{c)}	Transforming protein RhoA	22 110	5.83	75	5	18.1
						ACTC_MOUSE						
						ACTS_MOUSE						
						COR1A_MOUSE	78 kDa glucose-regulated protein precursor (GRP 78, Heat shock 70 kDa protein 5)	72 492	5.07	387	12	10.8
						UBE2A_MOUSE						
NUCL_MOUSE												
21	2.2	3/3				NUCL_MOUSE	Histone H2A	14 127	11.05	108	2	21.5
						PSA1_MOUSE						
						Proteasome subunit α type 1						
						PSA1_MOUSE	Actin	42 334	5.23	108	3	10.1
						COR1A_MOUSE						
			UBE2A_MOUSE									
			UBE2A_MOUSE	Coronin-1A	51 641	6.05	61	1	2.2			
			NUCL_MOUSE									
			PSA1_MOUSE									
			PSA1_MOUSE	Ubiquitin-conjugating enzyme E2 A	17 419	5.06	54	1	6.6			
			NUCL_MOUSE									
PSA1_MOUSE												
PSA1_MOUSE	Nucleolin (Protein C23)	76 734	4.69	309	7	8.2						
PSA1_MOUSE												
Proteasome subunit α type 1												
PSA1_MOUSE	Proteasome subunit α type 1	29 813	6	193	6	26.2						
PSA1_MOUSE												
Proteasome subunit α type 1												

Isolated wt or Stat6^{−/−} MNCs were activated through CD3 and cultured for 24 h in the presence of IL-4. Proteins in the 2-DE spots having at least two-fold increase or decrease in the protein expression between the mouse strains were identified with MS/MS. The fold change and reproducibility of differential expression as well as the full name, entry name, theoretical molecular weight and theoretical pI are indicated in the columns. Confidence of the protein identification is presented as protein score in MASCOT peptide summary report, number of the identified peptides and sequence coverage of the protein.

a) Entry names of all identifications can be found from the Supporting Information.

b) Entry Q3UYR3_MOUSE contains isoform 2 specific peptide. See Supporting Information for further information.

c) Several members of Histone H2A protein family were identified from the same spot with same peptide identification. See Supporting Information for further information.

involved in the regulation of transcription and were therefore seen as interesting candidates in relation to the downstream effects of Stat6. Previous studies have also linked Runx family of transcription factors in the regulation of Th cell differentiation and deletion of CBFb has been shown lead to asthma-like symptoms, making CBFb a very interesting protein in the context of these studies [41].

3.2 Expression of CNBP protein is decreased in Stat6^{-/-} cells

CNBP, a highly conserved protein sharing more than 95% sequence identity between human, chicken, rat, and mouse [42–45] has a number of cellular functions. CNBP binds both to ssDNA and RNA containing unpaired guanosine-rich sequences [46]. CNBP is not a classical transcription factor because it binds preferentially to single-stranded nucleic acids. However, it has been reported to function either as a repressor [47, 48] or an activator [19] of gene transcription. CNBP can also modulate its target gene expression by binding to 5' untranslated regions of terminal oligo pyrimidine-containing mRNA molecules [18]. CNBP regulates transcription of c-Myc [19, 45], Six3, Bf-1, and Hesx1 [49, 50], which are all involved in the regulation of cell cycle progression and development. It has also been shown that overexpression of CNBP increases cell proliferation [45]. Likewise, Stat6 has been implicated in cell cycle regulation, since Stat6^{-/-} B and T cells have impaired proliferation rate compared to their wild type littermates [11–13, 51]. The importance of CNBP is underlined by the fact that Cnbp^{-/-} mice are embryonically lethal and show severe defects in organogenesis. Multiple defects in growth and organogenesis have been described also in CNBP heterozygous mutant mice [50].

CNBP is a zinc-finger protein with three isoforms which are produced by alternative splicing. CNBP isoforms have the same *in vitro* DNA binding specificity, but interestingly, they can have different roles in their target gene regulation. This indicates a putative extra level of regulation of CNBP functions by competition of binding sites between the isoforms [48]. Based on the MS/MS data, we could not conclude which isoform was differentially expressed in our dataset (Fig. 2a). In addition to the Stat6 regulated protein form, CNBP was also identified from two adjacent protein spots that were not regulated by Stat6 (Fig. 2a and Supporting Information). The MS/MS data acquired from the original 2-DE gels or larger starting material did not provide sufficient information on the modificational or structural differences between these different CNBP forms. Most probably this was limited by the quantity of this protein present in 2-DE gels. We further studied the expression of CNBP in wt and Stat6^{-/-} MNCs with Western blotting, using both 2-D electrophoresis and SDS-PAGE for protein separation. 2-DE Western blotting confirmed that one spot detected for CNBP was expressed at a higher level in wt than in Stat6^{-/-} cells (Fig. 2b and Fig. 2 of Supporting Information). The total

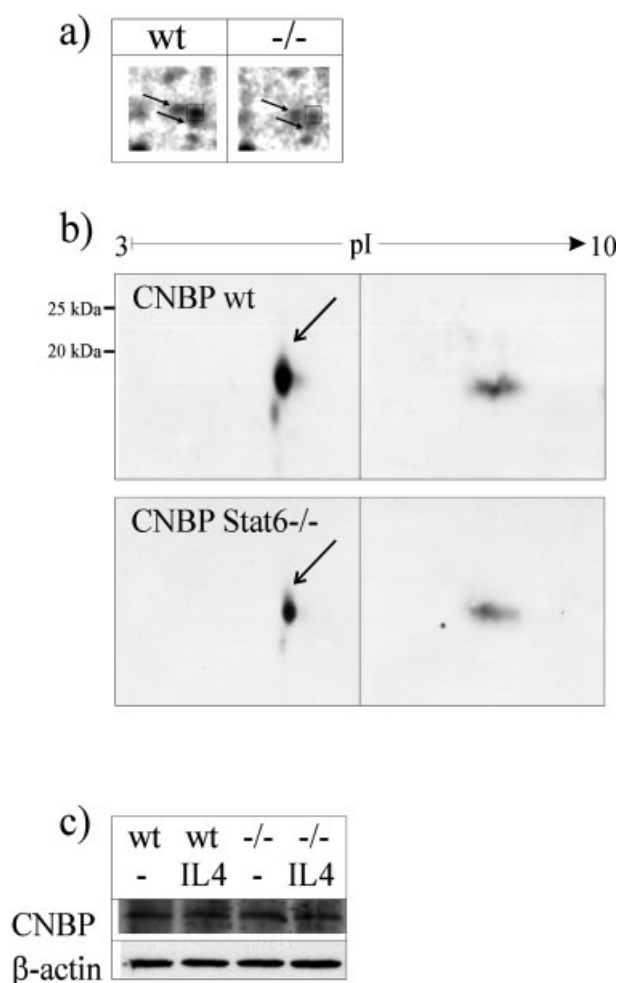


Figure 2. The expression of one form of CNBP is regulated by Stat6. (a) The differentially expressed CNBP form is marked with a rectangle. CNBP was also identified from the neighboring 2-DE protein spots marked with an arrow. Shown here is the surroundings of the spot 14 from Fig. 1 enlarged. The expression of CNBP in MNC cells was studied further with 2-DE and 1-DE Western blotting after activation through CD3 and culturing for 24 h with or without IL-4. (b) 2-DE Western blot of CNBP shows that the expression of one CNBP form (marked with an arrow) is higher in wt than Stat6^{-/-} cells after IL-4 stimulation. 2-DE gels were cut into smaller pieces before the transfer, the site of the cut is indicated by vertical line in the middle of the images. (c) 1-DE Western blot analysis of CNBP shows that the total protein level of CNBP does not differ between wt and Stat6^{-/-} cells.

expression of CNBP did not differ between wt and Stat6^{-/-} cells (Fig. 2c), again suggesting that the difference identified is due to some modification. Interestingly in this context, the function of CNBP has been reported to be regulated by cAMP-dependent protein kinase (PKA)-mediated phosphorylation. It has been suggested that CNBP phosphorylation might be involved in the determination of mRNA conformation or chromatin remodeling and consequently regulate CNBP target mRNA translation or recruitment of other

trans-acting factors [52]. Based on our observations there may also be other modifications of CNBP yet to be characterized.

3.3 Isoform 2-specific upregulation of CBFb protein in Stat6^{-/-} cells

CBFb protein was also identified from a 2-DE protein spot that was expressed more in wt MNCs compared to Stat6^{-/-} cells. Altogether, four peptides belonging to CBFb2 protein were identified in the MS/MS analysis including MS/MS data for the isoform 2-specific peptide VSQLLAVTGK (Table 1 and Supporting Information). CBFb2 is the major isoform of four CBFb proteins reported in the Swiss-Prot database. CBFb is a non-DNA binding cofactor for Runx family of transcription factors [53–55]. The Runx family consists of three members, Runx1, Runx2, and Runx3, which share a conserved DNA binding Runt domain [56]. Interaction between a common cofactor CBFb and Runx proteins confers

high affinity DNA binding [17] and the stability of at least Runx1 protein [57]. All members of the Runx family are implicated in the regulation of development: Runx1 is needed for haematopoiesis, Runx2 for osteogenesis, and Runx3 regulates CD8⁺ T cell maturation and neuronal development [58–62]. The importance of these factors in various stages of normal development and regulation of growth is well documented by studies indicating severe and fatal defects in Runx knock out animals, and by a direct link between Runx proteins and cancer [63].

In addition to its role in early haematopoiesis, Runx1 is also important in the regulation of Th cell differentiation. Runx1 represses the expression of Gata3, which is one of the master regulators of Th2 cell development [64]. Runx1 can also activate transcription of IFN γ [65], and is suggested to play a role in repression of IL-4 gene in Th1 cells [41]. Interestingly, inactivation of CBFb in CD4⁺ cells leads to the development of asthma-like symptoms including increased production of IgE and infiltration of lymphoid cells into the lungs. Also, the expression of IL-4 is significantly increased

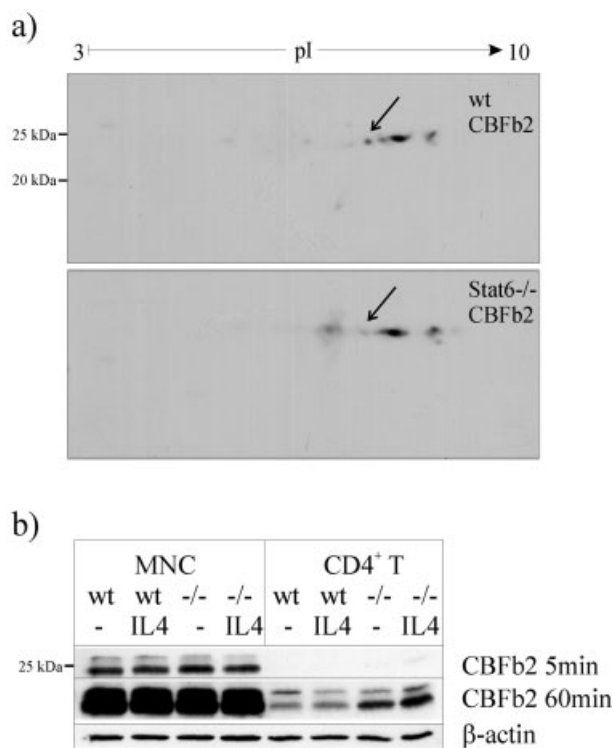


Figure 3. The smaller form of CBFb is expressed at a higher level in Stat6^{-/-} than in wt CD4⁺ T cells. Isolated MNCs were activated through CD3 and CD4⁺ T cells through CD3 and CD28, and the cells were cultured for 24 h in the presence or absence of IL-4. The expression of CBFb was detected with immunoblotting. (a) 2-DE Western blot showing that one CBFb form (marked with an arrow) is expressed more in IL-4-treated wt than in Stat6^{-/-} MNC cells as detected in two independent experiments. (b) 1-DE Western blot analysis shows that the total protein expression level of CBFb does not differ between wt and Stat6^{-/-} MNCs, but the smaller form of the protein is clearly expressed more in Stat6^{-/-} CD4⁺ T cells.

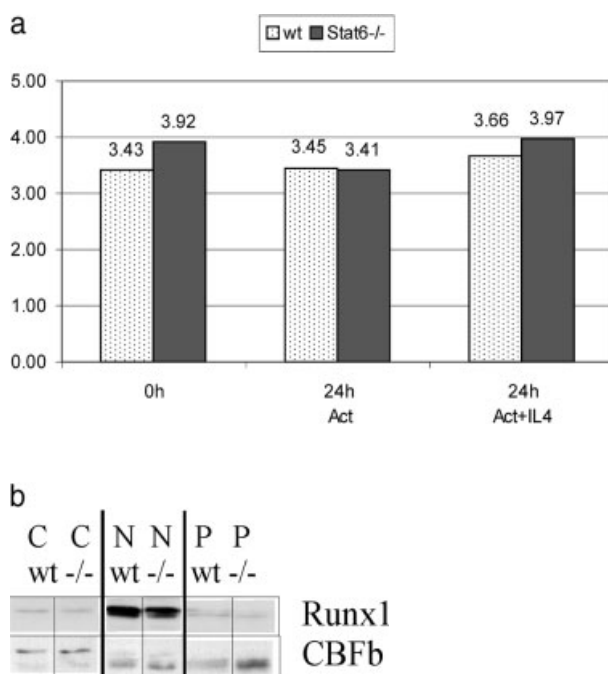


Figure 4. Runx1 DNA binding activity is similar in wt and Stat6^{-/-} CD4⁺ T cells. (a) Runx1 DNA binding activity was measured from nuclear preparations of CD4⁺ T lymphocytes prior to activation (0 h samples) and after activation through CD3 and CD28 for 24 h with and without IL-4. No significant differences were detected in the Runx1 DNA binding activity. (b) Western blot analysis of activated wt and Stat6^{-/-} CD4⁺ cells showing that most of the differentially expressed CBFb is localized to the pellet fraction (P), remaining after extraction of cytosolic (C) and nuclear (N) proteins. Protein loading was equalized according to protein concentration and corresponding fractions from wt and Stat6^{-/-} cells were analyzed in parallel. Similar results were obtained from two independent cultures. Vertical lines separate the data from different gels.

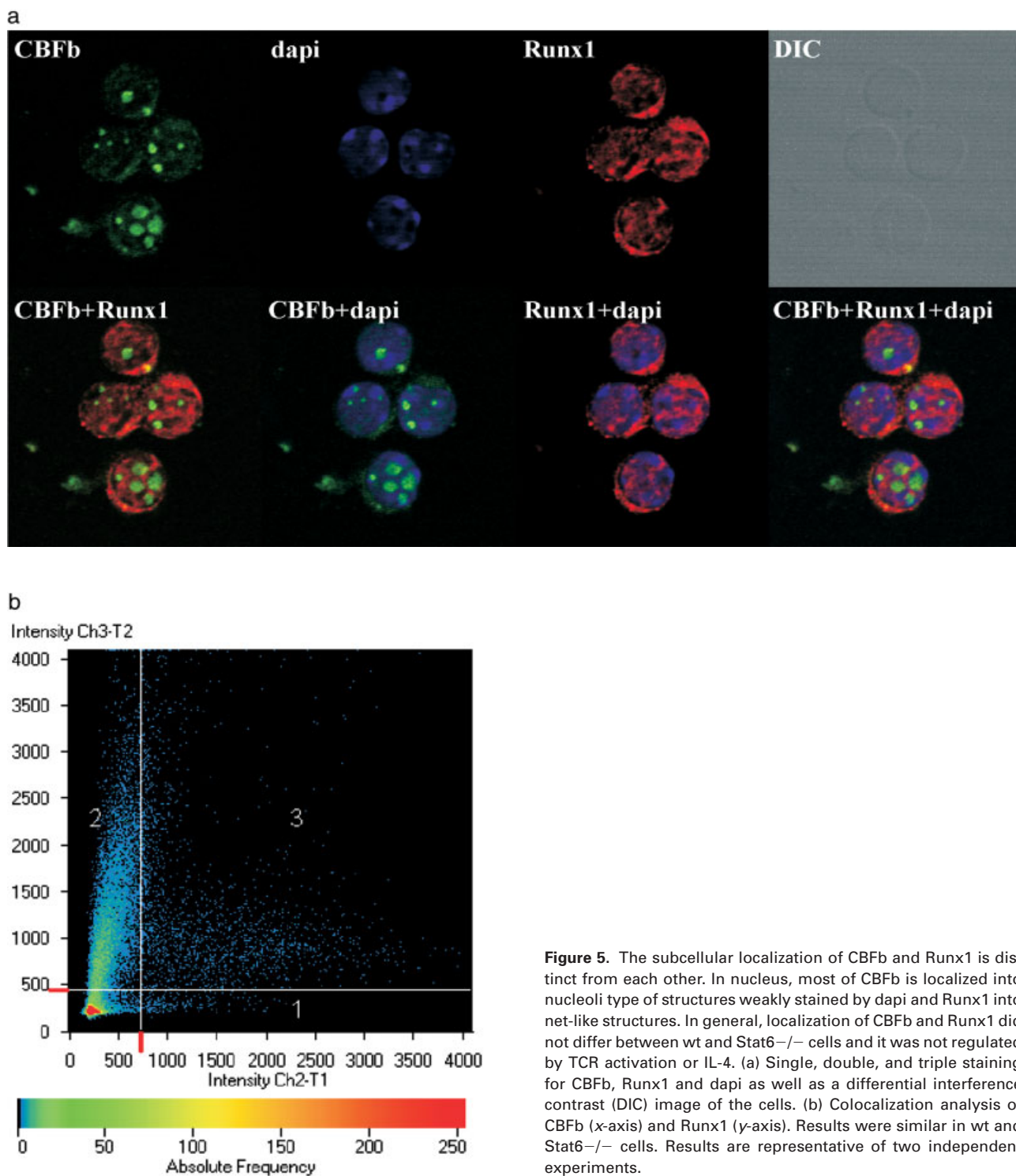


Figure 5. The subcellular localization of CBFb and Runx1 is distinct from each other. In nucleus, most of CBFb is localized into nucleoli type of structures weakly stained by dapi and Runx1 into net-like structures. In general, localization of CBFb and Runx1 did not differ between wt and Stat6^{−/−} cells and it was not regulated by TCR activation or IL-4. (a) Single, double, and triple staining for CBFb, Runx1 and dapi as well as a differential interference contrast (DIC) image of the cells. (b) Colocalization analysis of CBFb (x-axis) and Runx1 (y-axis). Results were similar in wt and Stat6^{−/−} cells. Results are representative of two independent experiments.

in CBFb^{flox/flox}/CD4 Cre cells indicating that CBFb represses IL-4 production in CD4⁺ cells [41].

We studied the expression of CBFb in wt and Stat6^{−/−} mononuclear and CD4⁺ T cells with Western blotting, using both 2-DE and SDS-PAGE for protein separation (Fig. 3).

2-DE Western blot analysis showed that one form of CBFb, localizing to the same area in the gel as differentially expressed CBFb2 in the initial 2-DE gels, was expressed more in wt cells (Fig. 3a and Fig. 3 of Supporting Information). Interestingly, we found that although there were no differences in

the total expression of CBFb in MNCs, the smaller form of CBFb was expressed more in Stat6^{−/−} compared to wt CD4⁺ T cells (Fig. 3b). The differential expression of CBFb was not dependent on TCR activation or IL-4 stimulation (Fig. 3b and data not shown), suggesting that Stat6 also has phosphorylation independent activity in CD4⁺ cells as previously reported in non-small cell lung cancer cells [66]. As the total expression of CBFb is markedly higher in MNC population than in CD4⁺ T cells (Fig. 3b), the difference in CD4⁺ T cells is likely to be masked by equally expressed CBFb in other MNCs when analyzed with 1-DE Western blotting.

Since, CBFb has been reported to stabilize Runx1 protein [57], we next studied Runx1 protein levels from Stat6^{−/−} and wt cells to see whether they are regulated in a Stat6 dependent manner. The total protein expression of Runx1 was similar between wt and Stat6^{−/−} MNCs and CD4⁺ T cells (data not shown). We also studied, whether the upregulation of CBFb2 in Stat6^{−/−} CD4⁺ T cells could lead to increased Runx1 DNA binding activity. However, no significant differences in Runx1 DNA binding activity were detected between wt and Stat6^{−/−} cells (Fig. 4a). Runx1 activity was neither regulated by activation or IL-4 stimulation. We therefore studied, the localization of CBFb and compared it to the localization of Runx1, since these proteins have been reported to have distinct cellular distribution in NIH 3T3 cell line [67]. The cytoplasmic and nuclear proteins were extracted with hypotonic buffers and insoluble pellet fraction was lysed with SDS buffer. Subsequently, the corresponding fractions from wt and Stat6^{−/−} cells were analyzed in parallel in order to identify the localization of differentially expressed CBFb. The smaller CBFb form was found predominantly in the nuclear and pellet fractions, whereas the larger form was mainly in the cytoplasm (Fig. 4b). In contrast to the CBFb, Runx1 is in turn enriched in the extracted nuclear protein pool. The Stat6-regulated CBFb is found in the pellet fraction (Fig. 4b), suggesting that this differentially expressed CBFb pool might not be freely available for interaction with transcriptionally active Runx1 proteins in the soluble nuclear pool. The cellular localization of Runx1 and CBFb proteins was also studied with confocal microscopy. Using this method, we could not detect prevailing differences in CBFb or Runx1 cellular localization between wt and Stat6^{−/−} cells. In general, TCR activation or IL-4 stimulation did not affect the localization these proteins. In the nucleus, Runx1 and CBFb were largely localized into distinct compartments (Fig. 5). CBFb was mainly localized into the nuclear regions that were poorly stained with dapi, thus having a nucleolus-like localization pattern [68, 69]. Runx1 was in turn localized into net-type structures in the nuclei. Runx1 has been reported to associate with the nuclear matrix [70], and our staining pattern is consistent with this finding. Although the general *in vitro* DNA binding activity of Runx1 was not increased in Stat6^{−/−} cells, and Runx1 and CBFb were mainly localized into different cellular compartments, this does not rule out the possibility that Runx1 activity is

regulated in a Stat6 dependent manner in distinct promoter regions of Runx1 target genes. Future studies might reveal, if such promoter regions can be identified from developing Th2 cells.

4 Concluding remarks

Studies on Stat6^{−/−} mice have shown the pivotal role of Stat6 in the regulation of Th cell differentiation. The development of Th2 cells responsible for enhancement of antibody production by B cells and activation of mast cells and eosinophils is severely defective in Stat6^{−/−} mice. Moreover, these mice are resistant to experimentally induced airway hyper-responsiveness. In this study, we have identified by using 2-DE-based proteomics a group of 21 protein spots representing altogether 49 proteins to be regulated by Stat6 transcription factor in IL-4 stimulated mouse MNCs. The most important findings in this study, namely the differential expression of certain forms of CNBP and CBFb proteins in Stat6^{−/−} and wt cells, provide the basis for future studies on Stat6-mediated cellular responses.

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5 References

- [1] Romagnani, S., Th1 and Th2 in human diseases. *Clin. Immunol. Immunopathol.* 1996, **80**, 225–235.
- [2] Farrar, J. D., Asnagli, H., Murphy, K. M., T helper subset development: Roles of instruction, selection, and transcription. *J. Clin. Invest.* 2002, **109**, 431–435.
- [3] Glimcher, L. H., Murphy, K. M., Lineage commitment in the immune system: The T helper lymphocyte grows up. *Genes Dev.* 2000, **14**, 1693–1711.
- [4] Seder, R. A., Paul, W. E., Acquisition of lymphokine-producing phenotype by CD4⁺ T cells. *Annu. Rev. Immunol.* 1994, **12**, 635–673.
- [5] Renaud, J. C., New insights into the role of cytokines in asthma. *J. Clin. Pathol.* 2001, **54**, 577–589.
- [6] Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J., Paul, W. E., The IL-4 receptor: Signaling mechanisms and biologic functions. *Annu. Rev. Immunol.* 1999, **17**, 701–738.
- [7] Hebenstreit, D., Wirnsberger, G., Horejs-Hoeck, J., Duschl, A., Signaling mechanisms, interaction partners, and target

- genes of STAT6. *Cytokine Growth Factor Rev.* 2006, 17, 173–188.
- [8] Barner, M., Mohrs, M., Brombacher, F., Kopf, M., Differences between IL-4R alpha-deficient and IL-4-deficient mice reveal a role for IL-13 in the regulation of Th2 responses. *Curr. Biol.* 1998, 8, 669–672.
- [9] McKenzie, G. J., Emson, C. L., Bell, S. E., Anderson, S. *et al.*, Impaired development of Th2 cells in IL-13-deficient mice. *Immunity* 1998, 9, 423–432.
- [10] Sornasse, T., Larenas, P. V., Davis, K. A., de Vries, J. E., Yssel, H., Differentiation and stability of T helper 1 and 2 cells derived from naive human neonatal CD4⁺ T cells, analyzed at the single-cell level. *J. Exp. Med.* 1996, 184, 473–483.
- [11] Kaplan, M. H., Schindler, U., Smiley, S. T., Grusby, M. J., Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. *Immunity* 1996, 4, 313–319.
- [12] Shimoda, K., van Deursen, J., Sangster, M. Y., Sarawar, S. R. *et al.*, Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. *Nature* 1996, 380, 630–633.
- [13] Takeda, K., Tanaka, T., Shi, W., Matsumoto, M. *et al.*, Essential role of Stat6 in IL-4 signalling. *Nature* 1996, 380, 627–630.
- [14] Kuperman, D., Schofield, B., Wills-Karp, M., Grusby, M. J., Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J. Exp. Med.* 1998, 187, 939–948.
- [15] Chen, Z., Lund, R., Aittokallio, T., Kosonen, M. *et al.*, Identification of novel IL-4/Stat6-regulated genes in T lymphocytes. *J. Immunol.* 2003, 171, 3627–3635.
- [16] Schroder, A. J., Pavlidis, P., Arimura, A., Capece, D., Rothman, P. B., Cutting edge: STAT6 serves as a positive and negative regulator of gene expression in IL-4-stimulated B lymphocytes. *J. Immunol.* 2002, 168, 996–1000.
- [17] Bushweller, J. H., CBF—a biophysical perspective. *Semin. Cell Dev. Biol.* 2000, 11, 377–382.
- [18] Pellizzoni, L., Lotti, F., Rutjes, S. A., Pierandrei-Amaldi, P., Involvement of the *Xenopus laevis* Ro60 autoantigen in the alternative interaction of La and CNBP proteins with the 5'UTR of L4 ribosomal protein mRNA. *J. Mol. Biol.* 1998, 281, 593–608.
- [19] Michelotti, E. F., Tomonaga, T., Krutzsch, H., Levens, D., Cellular nucleic acid binding protein regulates the CT element of the human c-myc protooncogene. *J. Biol. Chem.* 1995, 270, 9494–9499.
- [20] Nyman, T. A., Rosengren, A., Syyrakki, S., Pellinen, T. P. *et al.*, A proteome database of human primary T helper cells. *Electrophoresis* 2001, 22, 4375–4482.
- [21] O'Connell, K. L., Stults, J. T., Identification of mouse liver proteins on two-dimensional electrophoresis gels by matrix-assisted laser desorption/ionization mass spectrometry of in situ enzymatic digests. *Electrophoresis* 1997, 18, 349–359.
- [22] Johnston, R. F., Pickett, S. C., Barker, D. L., Autoradiography using storage phosphor technology. *Electrophoresis* 1990, 11, 355–360.
- [23] Rosenfeld, J., Capdevielle, J., Guillemot, J. C., Ferrara, P., In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Anal. Biochem.* 1992, 203, 173–179.
- [24] Shevchenko, A., Wilm, M., Vorm, O., Mann, M., Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* 1996, 68, 850–858.
- [25] Nyman, T. A., Matikainen, S., Sareneva, T., Julkunen, I., Kalkkinen, N., Proteome analysis reveals ubiquitin-conjugating enzymes to be a new family of interferon-alpha-regulated genes. *Eur. J. Biochem.* 2000, 267, 4011–4019.
- [26] Rosengren, A. T., Nyman, T. A., Lahesmaa, R., Proteome profiling of interleukin-12 treated human T helper cells. *Proteomics* 2005, 5, 3137–3141.
- [27] Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D. *et al.*, Current protocols in Molecular Biology, John Wiley and Sons., New York 1995.
- [28] Gygi, S. P., Corthals, G. L., Zhang, Y., Rochon, Y., Aebersold, R., Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc. Natl. Acad. Sci. USA.* 2000, 97, 9390–9395.
- [29] Rautajoki, K. J., Marttila, E. M., Nyman, T. A., Lahesmaa, R., Interleukin-4 inhibits caspase-3 by regulating several proteins in the Fas pathway during initial stages of human T helper 2 cell differentiation. *Mol. Cell. Proteomics* 2007, 6, 238–251.
- [30] Rakkola, R., Matikainen, S., Nyman, T. A., Proteome analysis of human macrophages reveals the upregulation of manganese-containing superoxide dismutase after toll-like receptor activation. *Proteomics* 2007, 7, 378–384.
- [31] Yarmush, M. L., Jayaraman, A., Advances in proteomic technologies. *Annu. Rev. Biomed. Eng.* 2002, 4, 349–373.
- [32] Nie, L., Wu, G., Culley, D. E., Scholten, J. C., Zhang, W., Integrative analysis of transcriptomic and proteomic data: Challenges, solutions and applications. *Crit. Rev. Biotechnol.* 2007, 27, 63–75.
- [33] Gate, L., Majumdar, R. S., Lunk, A., Tew, K. D., Increased myeloproliferation in glutathione S-transferase pi-deficient mice is associated with a deregulation of JNK and Janus kinase/STAT pathways. *J. Biol. Chem.* 2004, 279, 8608–8616.
- [34] Mamane, Y., Sharma, S., Petropoulos, L., Lin, R., Hiscott, J., Posttranslational regulation of IRF-4 activity by the immunophilin FKBP52. *Immunity* 2000, 12, 129–140.
- [35] Matsuyama, T., Grossman, A., Mittrucker, H. W., Siderovski, D. P. *et al.*, Molecular cloning of LSIRF, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE). *Nucleic Acids Res.* 1995, 23, 2127–2136.
- [36] Grumont, R. J., Gerondakis, S., Rel induces interferon regulatory factor 4 (IRF-4) expression in lymphocytes: Modulation of interferon-regulated gene expression by rel/nuclear factor kappaB. *J. Exp. Med.* 2000, 191, 1281–1292.
- [37] Gupta, S., Jiang, M., Anthony, A., Pernis, A. B., Lineage-specific modulation of interleukin 4 signaling by interferon regulatory factor 4. *J. Exp. Med.* 1999, 190, 1837–1848.
- [38] Mittrucker, H. W., Matsuyama, T., Grossman, A., Kundig, T. M. *et al.*, Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. *Science* 1997, 275, 540–543.
- [39] Tominaga, N., Ohkusu-Tsukada, K., Udono, H., Abe, R. *et al.*, Development of Th1 and not Th2 immune responses in mice lacking IFN regulatory factor-4. *Int. Immunol.* 2003, 15, 1–10.

- [40] Hu, C. M., Jang, S. Y., Fanzo, J. C., Pernis, A. B., Modulation of T cell cytokine production by interferon regulatory factor-4. *J. Biol. Chem.* 2002, **277**, 49238–49246.
- [41] Naoe, Y., Setoguchi, R., Akiyama, K., Muroi, S. *et al.*, Repression of interleukin-4 in T helper type 1 cells by Runx/Cbf beta binding to the Il4 silencer. *J. Exp. Med.* 2007, **204**, 1749–1755.
- [42] Warden, C. H., Krisans, S. K., Purcell-Huynh, D., Leete, L. M. *et al.*, Mouse cellular nucleic acid binding proteins: A highly conserved family identified by genetic mapping and sequencing. *Genomics* 1994, **24**, 14–19.
- [43] Yasuda, J., Mashiyama, S., Makino, R., Ohyama, S. *et al.*, Cloning and characterization of rat cellular nucleic acid binding protein (CNBP) cDNA. *DNA Res.* 1995, **2**, 45–49.
- [44] van Heumen, W. R., Claxton, C., Pickles, J. O., Sequence and tissue distribution of chicken cellular nucleic acid binding protein cDNA. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 1997, **118**, 659–665.
- [45] Shimizu, K., Chen, W., Ashique, A. M., Moroi, R., Li, Y. P., Molecular cloning, developmental expression, promoter analysis and functional characterization of the mouse CNBP gene. *Gene* 2003, **307**, 51–62.
- [46] Armas, P., Nasif, S., Calcaterra, N. B., Cellular nucleic acid binding protein binds G-rich single-stranded nucleic acids and may function as a nucleic acid chaperone. *J. Cell. Biochem.* 2007, **103**, 1013–1036.
- [47] Rajavashisth, T. B., Taylor, A. K., Andalibi, A., Svenson, K. L., Lusis, A. J., Identification of a zinc finger protein that binds to the sterol regulatory element. *Science* 1989, **245**, 640–643.
- [48] Flink, I. L., Morkin, E., Alternatively processed isoforms of cellular nucleic acid-binding protein interact with a suppressor region of the human beta-myosin heavy chain gene. *J. Biol. Chem.* 1995, **270**, 6959–6965.
- [49] Abe, Y., Chen, W., Huang, W., Nishino, M., Li, Y. P., CNBP regulates forebrain formation at organogenesis stage in chick embryos. *Dev. Biol.* 2006, **295**, 116–127.
- [50] Chen, W., Liang, Y., Deng, W., Shimizu, K. *et al.*, The zinc-finger protein CNBP is required for forebrain formation in the mouse. *Development* 2003, **130**, 1367–1379.
- [51] Mohrs, M., Lacy, D. A., Locksley, R. M., Stat signals release activated naive Th cells from an anergic checkpoint. *J. Immunol.* 2003, **170**, 1870–1876.
- [52] Lombardo, V. A., Armas, P., Weiner, A. M., Calcaterra, N. B., *In vitro* embryonic developmental phosphorylation of the cellular nucleic acid binding protein by cAMP-dependent protein kinase, and its relevance for biochemical activities. *FEBS J.* 2007, **274**, 485–497.
- [53] Wang, S., Wang, Q., Crute, B. E., Melnikova, I. N. *et al.*, Cloning and characterization of subunits of the T-cell receptor and murine leukemia virus enhancer core-binding factor. *Mol. Cell. Biol.* 1993, **13**, 3324–3339.
- [54] Ogawa, E., Inuzuka, M., Maruyama, M., Satake, M. *et al.*, Molecular cloning and characterization of PEBP2 beta, the heterodimeric partner of a novel Drosophila runt-related DNA binding protein PEBP2 alpha. *Virology* 1993, **194**, 314–331.
- [55] Ogawa, E., Maruyama, M., Kagoshima, H., Inuzuka, M. *et al.*, PEBP2/PEA2 represents a family of transcription factors homologous to the products of the Drosophila runt gene and the human AML1 gene. *Proc. Natl. Acad. Sci. USA* 1993, **90**, 6859–6863.
- [56] Levanon, D., Groner, Y., Structure and regulated expression of mammalian RUNX genes. *Oncogene* 2004, **23**, 4211–4219.
- [57] Huang, G., Shigesada, K., Ito, K., Wee, H. J. *et al.*, Dimerization with PEBP2beta protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation. *EMBO J.* 2001, **20**, 723–733.
- [58] Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G., Downing, J. R., AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* 1996, **84**, 321–330.
- [59] Egawa, T., Tillman, R. E., Naoe, Y., Taniuchi, I., Littman, D. R., The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. *J. Exp. Med.* 2007, **204**, 1945–1957.
- [60] Komori, T., Yagi, H., Nomura, S., Yamaguchi, A. *et al.*, Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 1997, **89**, 755–764.
- [61] Levanon, D., Bettoun, D., Harris-Cerruti, C., Woolf, E. *et al.*, The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons. *EMBO J.* 2002, **21**, 3454–3463.
- [62] Taniuchi, I., Osato, M., Egawa, T., Sunshine, M. J. *et al.*, Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development. *Cell* 2002, **111**, 621–633.
- [63] Blyth, K., Cameron, E. R., Neil, J. C., The RUNX genes: Gain or loss of function in cancer. *Nat. Rev. Cancer* 2005, **5**, 376–387.
- [64] Komine, O., Hayashi, K., Natsume, W., Watanabe, T. *et al.*, The Runx1 transcription factor inhibits the differentiation of naive CD4+ T cells into the Th2 lineage by repressing GATA3 expression. *J. Exp. Med.* 2003, **198**, 51–61.
- [65] Ono, M., Yaguchi, H., Ohkura, N., Kitabayashi, I. *et al.*, Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature* 2007, **446**, 685–689.
- [66] Cui, X., Zhang, L., Luo, J., Rajasekaran, A. *et al.*, Unphosphorylated STAT6 contributes to constitutive cyclooxygenase-2 expression in human non-small cell lung cancer. *Oncogene* 2007, **26**, 4253–4260.
- [67] Lu, J., Maruyama, M., Satake, M., Bae, S. C. *et al.*, Subcellular localization of the alpha and beta subunits of the acute myeloid leukemia-linked transcription factor PEBP2/CBF. *Mol. Cell. Biol.* 1995, **15**, 1651–1661.
- [68] Pontes, O., Lawrence, R. J., Neves, N., Silva, M. *et al.*, Natural variation in nucleolar dominance reveals the relationship between nucleolus organizer chromatin topology and rRNA gene transcription in Arabidopsis. *Proc. Natl. Acad. Sci. USA* 2003, **100**, 11418–11423.
- [69] Porter, S. E., Penheiter, K. L., Jaehning, J. A., Separation of the Saccharomyces cerevisiae Paf1 complex from RNA polymerase II results in changes in its subnuclear localization. *Eukaryot. Cell* 2005, **4**, 209–220.
- [70] Zeng, C., van Wijnen, A. J., Stein, J. L., Meyers, S. *et al.*, Identification of a nuclear matrix targeting signal in the leukemia and bone-related AML/CBF-alpha transcription factors. *Proc. Natl. Acad. Sci. USA* 1997, **94**, 6746–6751.

PROTEOMICS

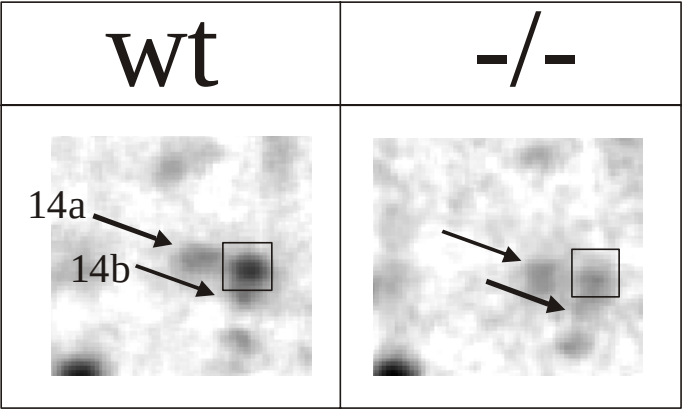
Supporting Information for Proteomics

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**Identification of novel Stat6 regulated proteins
in IL-4-treated mouse lymphocytes**

SUPPLEMENTARY DATA

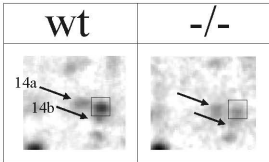


Supplementary Figure 1. Cellular nucleic acid binding protein (identification no 14, marked with a rectangle) was found to be more expressed in IL-4 treated wild type mononuclear cells than in Stat6^{-/-} cells. The same protein was also identified from neighboring spots 14a and 14b marked with arrows (supplementary table 1).

Supplementary Table 1

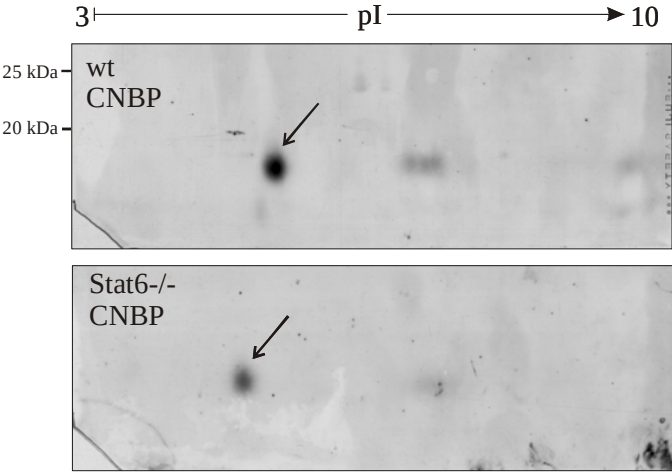
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14a	15	CNBP_MOUSE	Cellular nucleic acid-binding protein (CNBP) (Zinc finger protein 9) - Mus musculus (Mouse)	85	20833	3	23.6	178	7.76	128	495.93	1484.76	3	1484.82	0.15	120	131	0	26.06	15	37	0.75	1	K	CYSCGERGHQK	D	
	15									135	592.65	1774.94	3	1774.83	0.1	42	56	0	31.8	16	38	0.23	1	R	GFQFVSSSLPDCYR	C	
	15									138	635.62	1903.83	3	1903.68	0.15	66	80	0	27.61	21	37	0.44	1	K	DCDLQEDACVNCGR	G	
	15	Q3U5V2_MOUSE	Bone marrow macrophage cDNA, RIKEN full-length enriched library, clone:IB30157G09 product:cellular	85	20113	3	24.6	171	7.76	128	495.93	1484.76	3	1484.82	0.15	113	124	0	26.06	15	37	0.75	1	K	CYSCGERGHQK	D	
	15									135	592.65	1774.94	3	1774.83	0.1	35	49	0	31.8	16	38	0.23	1	R	GFQFVSSSLPDCYR	C	
	15									138	635.62	1903.83	3	1903.68	0.15	59	73	0	27.61	21	37	0.44	1	K	DCDLQEDACVNCGR	G	
	15	Q5QJQ9_MOUSE	Cellular nucleic acid binding protein (Osteocalcin-like cell cDNA, RIKEN full-length enriched librar	85	20833	3	23.6	178	7.76	128	495.93	1484.76	3	1484.82	0.15	120	131	0	26.06	15	37	0.75	1	K	CYSCGERGHQK	D	
	15									135	592.65	1774.94	3	1774.83	0.1	42	56	0	31.8	16	38	0.23	1	R	GFQFVSSSLPDCYR	C	
	15									138	635.62	1903.83	3	1903.68	0.15	66	80	0	27.61	21	37	0.44	1	K	DCDLQEDACVNCGR	G	
	18	ADAUV1_MOUSE	Hist1h2ah protein (Fragment) - Mus musculus (Mouse)	57	13811	2	12.6	127	11.03	24	425.8	849.58	2	849.52	0.07	82	88	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	21	29	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	18	Q0VE75_MOUSE	Histone cluster 1, H2ak - Mus musculus (Mouse)	57	14141	2	12.3	130	11.05	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	22	30	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	18	H2A1F_MOUSE	Histone H2A type 1-F - Mus musculus (Mouse)	57	14153	2	12.3	130	11.05	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
14b	18	H2A1H_MOUSE	Histone H2A type 1-H - Mus musculus (Mouse)	57	13942	2	12.5	128	11.03	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	22	30	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	18	H2A1K_MOUSE	Histone H2A type 1-K - Mus musculus (Mouse)	57	14141	2	12.3	130	11.05	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	22	30	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	18	H2A1_MOUSE	Histone H2A type 1 - Mus musculus (Mouse)	57	14127	2	12.3	130	11.05	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18	H2A2A_MOUSE	Histone H2A type 2-A (H2A.2) (H2a-614) (H2a-615) - Mus musculus (Mouse)	57	14087	2	12.3	130	10.9	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	22	30	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	18	H2A2C_MOUSE	Histone H2A type 2-C (H2a-613B) - Mus musculus (Mouse)	57	13980	2	12.4	129	10.9	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	22	30	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	18	H2A3_MOUSE	Histone H2A type 3 - Mus musculus (Mouse)	57	14113	2	12.3	130	11.05	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	22	30	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	18	H2AV_MOUSE	Histone H2AV (H2A.F1Z) - Mus musculus (Mouse)	57	13501	2	12.5	128	10.58	24	425.8	849.58	2	849.52	0.07	86	92	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	24	32	0	23.23	31	39	2	3	R	AGLQFPVGR	I	
	18	H2AX_MOUSE	Histone H2A.x (H2Ax) - Mus musculus (Mouse)	57	15133	2	11.2	143	10.74	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
18									55	472.81	943.6	2	943.52	0.07	22	30	0	23.23	31	39	2	3	R	AGLQFPVGR	V		
14c	18	H2AZ_MOUSE	Histone H2A.Z (H2Az) - Mus musculus (Mouse)	57	13545	2	12.5	128	10.58	24	425.8	849.58	2	849.52	0.07	86	92	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	24	32	0	23.23	31	39	2	3	R	AGLQFPVGR	I	
	18	Q0BAU5_MOUSE	Histone cluster 1, H2af - Mus musculus (Mouse)	57	14153	2	12.3	130	11.05	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	22	30	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	18	Q8CGF4_MOUSE	Histone protein Hist1h2aa (Histone cluster 1, H2aa) (OTTMUSP0000000555) - Mus musculus (Mouse)	57	14048	2	12.4	129	11.21	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	22	30	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	18	Q8R1M2_MOUSE	H2afj protein (H2A histone family, member J) (0 day neonate eyeball cDNA, RIKEN full-length enrich	57	14037	2	12.4	129	11.05	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	22	30	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	18	Q149V4_MOUSE	Hist1h2an protein (Hist1h2ac protein) - Mus musculus (Mouse)	57	14127	2	12.3	130	11.05	24	425.8	849.58	2	849.52	0.07	83	89	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	22	30	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	18	Q64426_MOUSE	Histone H2A (Fragment) - Mus musculus domesticus (western European house mouse)	57	14794	2	11.7	137	10.6	24	425.8	849.58	2	849.52	0.07	90	96	0	34.09	27	39	0.18	1	R	HLQLAIR	N	
	18									55	472.81	943.6	2	943.52	0.07	29	37	0	23.23	31	39	2	3	R	AGLQFPVGR	V	
	19	DCTN3_MOUSE	Dynactin subunit 3 (Dynactin light chain p24) - Mus musculus (Mouse)	53	21079	2	10.8	186	5.84	64	496.8	991.59	2	991.49	0.1	20	28	0	23.38	21	38	1.8	1	R	VYVVGPGGT	G	
	19									79	550.17	1098.73	2	1098.64	0.09	40	50	0	30.07	24	38	0.35	1	K	VQVALGNIAK	R	
14d	10	Q3U935_MOUSE	Bone marrow macrophage cDNA, RIKEN full-length enriched library, clone:IB30044A19 product:cellular	116	19984	3	17.1	170	8	122	888.37	1774.72	2	1774.63	0.09	59	72	0	53.43	26	38	0.0015	1	K	DCDLQEDACVNCGR	G	
	10									123	592.6	1774.78	3	1774.63	0.14	59	72	0	48.35	16	38	0.0051	1	K	DCDLQEDACVNCGR	G	
	10									124	888.5	1774.99	2	1774.83	0.15	35	49	0	64.07	21	38	0.00014	1	R	GFQFVSSSLPDCYR	C	
	10	Q3ULK8_MOUSE	4 cells embryo 4 cells cDNA, RIKEN full-length enriched library, clone:IOC00446C05 product:cellular	116	20704	3	16.4	177	8	122	888.37	1774.72	2	1774.63	0.09	66	79	0	53.43	26	38	0.0015	1	K	DCDLQEDACVNCGR	G	
	10									123	592.6	1774.78	3	1774.63	0.14	66	79	0	48.35	16	38	0.0051	1	K	DCDLQEDACVNCGR	G	
	10									124	888.5	1774.99	2	1774.83	0.15	42	56	0	64.07	21	38	0.00014	1	R	GFQFVSSSLPDCYR	C	
	14	ADAUV1_MOUSE	Hist1h2ah protein (Fragment) - Mus musculus (Mouse)	61	13811	2	12.6	127	11.03	21	425.8	849.58	2	849.52	0.07	82	88	0	22.8	24	39	2.4	1	R	HLQLAIR	N	
	14									51	472.81	943.6	2	943.52	0.07	21	29	0	38.56	24	39						

SUPPLEMENTARY FIGURE 1

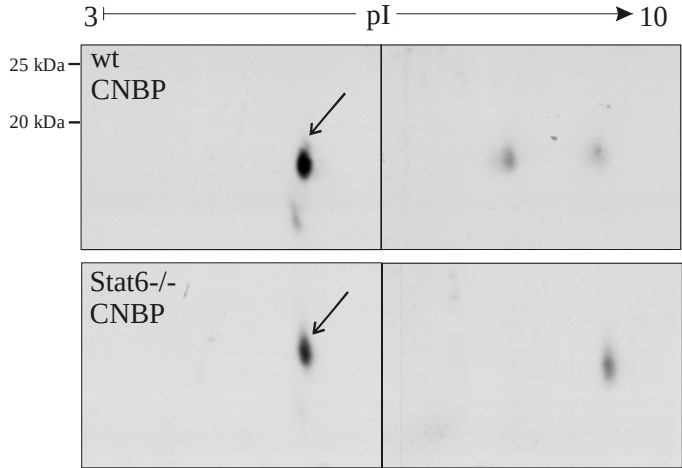


SUPPLEMENTARY FIGURE 2
CNBP 2-DE Western detections

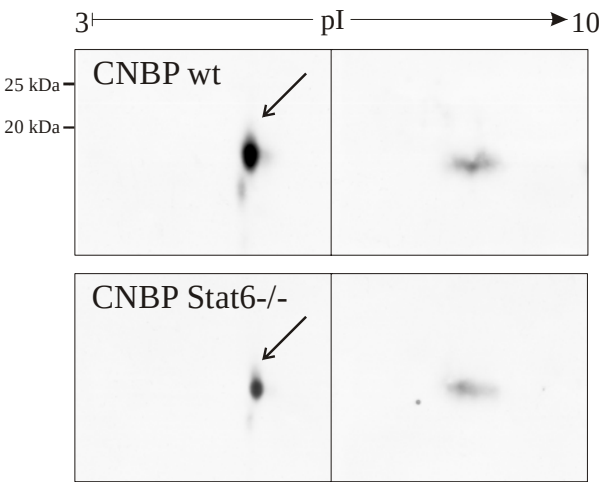
Experiment 1



Experiment 2

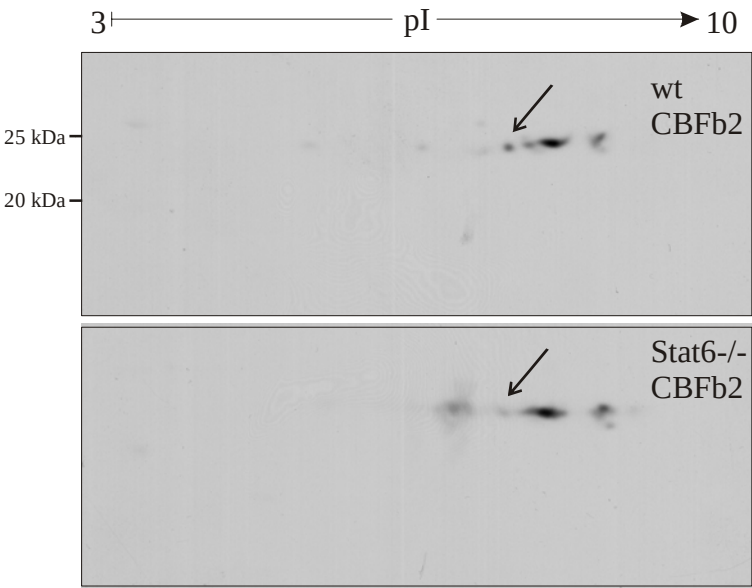


Experiment 3 (Figure 2b)



SUPPLEMENTARY FIGURE 3
CBFb2 2-DE Western detections

Experiment 1 (Figure 3a)



Experiment 2

