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TITLE:

A Chromatin Immunoprecipitation Assay to Identify Novel NFAT2 Target Genes in Chronic Lymphocytic Leukemia

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KEYWORDS:

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SUMMARY:

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the western world. NFAT transcription factors are important regulators of development and activation in numerous cell types. Here, we present a protocol for the use of chromatin immunoprecipitation (ChIP) in human CLL cells to identify novel target genes of NFAT2.

ABSTRACT:

Chronic lymphocytic leukemia (CLL) is characterized by the expansion of malignant B cell clones and represents the most common leukemia in western countries. The majority of CLL patients show an indolent course of the disease as well as an anergic phenotype of their leukemia cells, referring to a B cell receptor unresponsive to external stimulation. We have recently shown that the transcription factor NFAT2 is a crucial regulator of anergy in CLL. A major challenge in

the analysis of the role of a transcription factor in different diseases is the identification of its specific target genes. This is of the great significance for the elucidation of pathogenetic mechanisms and potential therapeutic interventions. Chromatin immunoprecipitation (ChIP) is a classic technique to demonstrate protein-DNA interactions and can, therefore, be used to identify direct target genes of transcription factors in mammalian cells. Here, ChIP was used to identify *LCK* as a direct target gene of NFAT2 in human CLL cells. DNA and associated proteins are crosslinked using formaldehyde and subsequently sheared by sonication into DNA fragments of approximately 200-500 base pairs (bp). Cross-linked DNA fragments associated with NFAT2 are then selectively immunoprecipitated from cell debris using a αNFAT2 antibody. After purification, associated DNA fragments are detected via quantitative real-time PCR (qRT-PCR). DNA sequences with evident enrichment represent regions of the genome which are targeted by NFAT2 *in vivo*. Appropriate shearing of the DNA and the selection of the required antibody are particularly crucial for the successful application of this method. This protocol is ideal for the demonstration of direct interactions of NFAT2 with target genes. Its major limitation is the difficulty to employ ChIP in large-scale assays analyzing the target genes of multiple transcription factors in intact organisms.

INTRODUCTION:

Chronic lymphocytic leukemia (CLL) represents the most common leukemia in adults in western countries, exhibiting distinct accumulation of CD19, CD23, and CD5 expressing mature B cells[1]. Most patients exhibit an indolent disease course, which does not necessitate the specific treatment for many years. In contrast, some patients show rapid progression requiring immediate therapeutic interventions with immune-chemotherapy or other targeted therapies[2,3]. Nuclear factor of activated T cells (NFAT) is a family of transcription factors controlling various developmental and activation processes in numerous cell types[4-6]. We recently demonstrated overexpression and constitutional activation of NFAT2 in CLL cells from patients with indolent disease[7]. Here, it regulates an unresponsive state to B cell receptor stimulation called anergy[7]. To demonstrate that NFAT2 binds to the lymphocyte-specific protein tyrosine kinase (*LCK*) promoter and regulates *LCK* expression in human CLL cells, a specific chromatin immunoprecipitation assay (ChIP) was developed and employed.

ChIP is one of the several techniques to investigate the role of transcription factors in gene expression[8]. Gene expression is tightly orchestrated in a very complex manner by several regulators with transcription factors taking an irreplaceable part in this process[9-12]. Transcription factors regulating the gene expression in a spatial and temporal context have been identified in numerous species (*e.g.,* for development and differentiation)[13-18]. Errors in the intricate control mechanisms involving transcription factors can lead to a variety of pathologic processes including cancer[19,20]. Hence, identification of transcription factors and their respective targets might offer novel therapeutic avenues[21,22]. To investigate this intriguing field several methods are available like ChIP, electrophoretic mobility shift assay (EMSA), various DNA pull-down assays and reporter-assays[8,11,12,23,24].

To demonstrate that a certain transcription factor interacts with specific regions of the genome *in vivo,* ChIP is an ideal technique[25]. For this purpose, DNA and associated proteins in living cells

are cross-linked using UV irradiation or formaldehyde (cross-linked ChIP, XChIP). This step is omitted to obtain better DNA and protein recovery in the so-called native ChIP (NChIP)[26]. The DNA-protein complexes are subsequently sheared by sonication into fragments of approximately 200-500 base pairs (bp) and immunoprecipitated from the cell debris using a specific antibody against the transcription factor of interest. The associated DNA fragments are then purified and characterized by PCR, molecular cloning, and sequencing. Alternative techniques use microarrays (ChIP-on-Chip) or the next-generation sequencing (ChIP-Seq) to analyze the immunoprecipitated DNA.

ChIP was first introduced by Gilmour and Lis in 1984 when they used UV light to covalently cross-link DNA and bound proteins in living bacteria[27]. Upon cell lysis and immunoprecipitation of bacterial RNA polymerase, specific probes of known genes were used to map the *in vivo* distribution and density of RNA polymerase. The method was subsequently used by the same investigators to analyze the distribution of eukaryotic RNA polymerase II on heat shock protein genes in *Drosophila*[28]. The XChIP assay was further refined by Varshavsky and coworkers who first used formaldehyde cross-linking to study the association of histone H4 with heat shock protein genes[29,30]. The NChIP approach, which carries the advantage of a better DNA and protein recovery due to naturally intact epitopes and, therefore, greater antibody specificity, was first described by Hebbes and colleagues in 1988[31].

The advantage of ChIP in comparison to other techniques to analyze DNA-protein interactions is in fact, that the actual interaction of a transcription factor can be investigated *in vivo* and no probes or artificial conditions created by buffers or gels are employed[8,11,12]. By combining ChIP with the next-generation sequencing, multiple targets can be identified simultaneously.

Major limitations of this technique are its limited applicability to large-scale assays in intact organisms[25]. The analysis of the differential gene expression patterns can also be challenging using ChIP techniques if the respective proteins are expressed only at low levels or during narrow time windows. Another potentially limiting factor is the availability of an appropriate antibody suited for ChIP[11].

The ChIP protocol presented here can be employed for the *in vivo* identification of target genes of a transcription factor by quantitative real-time PCR (qRT-PCR). Specifically, the goal was to identify novel target genes of NFAT2 in CLL. ChIP was chosen because of its potential to directly demonstrate the binding of NFAT2 to the promoter regions of different target genes under natural conditions in human CLL patient cells.

PROTOCOL:

All experiments conducted with the human material were approved by the Ethics Committee of the University of Tübingen and written informed consent was obtained from all patients who contributed samples to this study.

1. Isolation and Stimulation of Jurkat cells

Note: To optimize the protocol, use the Jurkat cell line which is known to express the high levels of NFAT2. All steps are performed under a laminar flow hood.

1.1. Prepare 50 mL of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin by warming it to 37 °C in a water bath.

1.2. Culture Jurkat cells for 1-2 d in a cell culture flask at 37 °C and 5% CO_2 in 20 mL of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin to a density of approximately 2 x 10^6 cells/mL (cells are counted with Trypan blue staining and a Neubauer chamber under the microscope) and harvest them by centrifuging for 5 min at 200 x g.

1.3. Remove the supernatant with a pipette and resuspend the cells in 10 mL of RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin. Subsequently, count the cells with a conventional trypan blue staining and a Neubauer chamber under the microscope.

1.4. Centrifuge the cells from step 1.3 for 5 min at 200 x g and remove the supernatant by aspiration. Then, resuspend the cells at a density of 2 x 10^7 cells/mL in RPMI 1640 supplemented with 10% FCS and 1% penicillin/streptomycin and transfer 1 mL in a new conventional 5 mL tube by pipetting.

1.5. Stimulate the cells by adding 32.4 nM phorbol 12-myristate 13-acetate (PMA) and 1 µM ionomycin. Then incubate the cells for 16 h at 37 °C and 5% CO_2 with the lid of the tube opened (to avoid contamination, a sealing film permeable to air can be used).

2. Isolation and Stimulation of Primary CLL Cells from Human Patients

Note: Patient samples were acquired and stimulated as previously described[7]. All steps are performed under a laminar flow hood.

2.1. Prepare the equipment necessary to draw blood from patients and to perform a density gradient separation: 21-G needles, tourniquet, NH_4-heparin-blood collection syringes (e.g., S-monovettes), 1x PBS, and density gradient medium (density 1.077 g/mL).

2.2. Upon venipuncture, draw the blood from CLL patients (ca. 40 mL blood per patient) into the syringe. Then transfer the blood in 50 mL conical tubes. wash the syringe with 10 mL PBS and pool the blood-PBS-suspension in the 50 mL tubes (adjust the number of tubes to the volume of blood).

2.3. Prepare 15 mL of the density gradient medium in a fresh 50 mL conical tube. Subsequently, layer 35 mL of the blood-PBS-suspension carefully on the density gradient medium. For this purpose, hold the tube at a flat angle and slowly pipette the blood-PBS-suspension against the bottom wall of the 50 mL conical tube. As more of the blood-PBS-suspension is accumulating in

the 50 mL conical tube, increase the angle of the tube up to a right angle. Repeat this step according to the volume of the blood-PBS-suspension.

2.4. Perform centrifugation at 560 x g for 20 min (without brake), then extract the mononuclear cell phase which contains the peripheral blood mononuclear cells (PBMCs). To do so, collect the white interphase of the density gradient separation with a 5 mL serological pipette and transfer it to a new 50 mL conical tube.

2.5. Fill the cell-suspension to 50 mL with PBS and centrifuge for 5 min at 360 x g. Remove the supernatant by aspiration. Repeat this step with centrifugation for 5 min at 275 x g. Then, pool the cells of one patient in 5-10 mL of PBS in a 50 mL conical tube.

2.6. Count the cells as described in 1.3.

Note: It depends on the proportion of CLL cells in the isolated PBMCs, if the cells can be used directly for this procedure or a B cell isolation has to be conducted, *e.g.,* via magnetic cell sorting (MACS). The B cell isolation is recommended but can be omitted if the proportion of CLL cells is above 95% of total lymphocytes (determined via flow cytometry). Blood of CLL patients is fixed and lysed according to the manufacturer's instructions. Then a staining with CD19-FITC and CD5-PE antibodies is performed according to the manufacturer's instructions and the cells are analyzed via flow cytometry. One exemplary patient is shown in **Figure 1**, in which the proportion of CLL cells is 89.03%. Thus, a B cell isolation has to be performed in this patient. The proportion of physiological B cells is negligible (3.72% in the example).

2.7. Wash the cells with 10 mL of pre-warmed (37 °C) RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin, 100 mM non-essential amino acids, 1 mM sodium pyruvate and 50 mM β-mercaptoethanol for 5 min at 200 x g and remove the supernatant by aspiration.

2.8. Adjust the cells to 2 x 10^7 cells/mL in RPMI 1640 supplemented with 10% FCS, 1% penicillin/streptomycin, 100 mM non-essential amino acids, 1 mM sodium pyruvate and 50 mM β-mercaptoethanol (37 °C) and fill 1 mL of the cell suspension into a 5 mL tube.

Note: The ß-mercaptoethanol is employed to counteract the oxidative stress.

2.9. Stimulate the cells by adding 32.4 nM PMA and 1 µM ionomycin. Then incubate the cells for 16 h at 37 °C and 5% CO_2 with the lid of the tube opened (to avoid contamination, a sealing film permeable to air can be used).

Note: To reduce the level of stress the cells can be rested for 1 h at 37 °C and 5% CO_2 in the incubator prior to the 16 h of stimulation.

3. Fixation, Cell Lysis, and Chromatin Shearing

Note: Patient samples and Jurkat cells are fixed and lysed with a commercially available ChIP kit according to the manufacturer's instructions with modifications as described previously[7]. The fixation is performed under a laminar flow hood.

3.1. Prepare a 1.5 mL tube with 1 mL of 37% formaldehyde, a 1.5 mL tube with 1 mL of 1.25 M glycine, a 5 mL tube with 4 mL of 1x PBS and a box with ice for the fixation of the cells.

3.2. After stimulation of the cells for the respective incubation time in step 1.5 or 2.9, spin the 5 mL tubes at 200 x g for 5 min and resuspend the cells in 500 µL of PBS in the 5 mL tubes.

3.3. Add 340 µM formaldehyde (13.5 µL of 37% formaldehyde) to the cells. After brief mixing by carefully pipetting up and down incubate the suspension for 2.5 min (Jurkat cells) or 5 min (patient sample) at room temperature.

Note: Prolonged fixation time is needed for primary cells to assure optimal shearing.

3.4. Add 125 mM glycine (57 µL of 1.25 M glycine) to stop the fixation and incubate for another 5 min. Put the cells on ice immediately thereafter and centrifuge at 500 x g for 5 min at 4 °C. Remove the supernatant by aspiration.

3.5. Wash the cells twice with 1 mL of ice-cold PBS at 200 x g for 5 min at 4 °C and remove the supernatant every time by aspiration.

Note: After the fixation, the protocol can be paused. Fixed cells should be stored at -80 °C. To test, if the epitope of the antibody intended to use in the following protocol is not masked via fixation, additional samples can be used for analysis via SDS-PAGE gel electrophoresis and Western blot. These steps are performed with 100 µg of protein according to manufacturer's instructions. All αNFAT2 antibodies are used at a dilution of 1:1000, the GAPDH antibody at a dilution of 1:10000. An exemplary image of fixed samples from Jurkat cells with appropriate and inappropriate antibodies is shown in **Figure 2**.

3.6. Resuspend the cell pellet in 5 mL of commercially available lysis buffer 1 by pipetting up and down. Then, place the samples on the ice on a shaker and incubate them for 10 min while shaking.

Note: Prior to lysis, the cells can be disintegrated for 10 s in liquid nitrogen. This is useful for Jurkat cells but should be avoided in primary patient samples. For the disintegration, place the 5 mL tubes for 5 s in 5-10 mL of liquid nitrogen in a specific container. Wear safety glasses and appropriate safety gloves.

3.7. Subsequently, centrifuge the tubes at 500 x g for 5 min at 4 °C and remove the supernatant carefully by pipetting.

3.8. Homogenize the cells in 5 mL of commercially available lysis buffer 2 by pipetting up and down and incubate for 10 min on ice while shaking. Then centrifuge the tubes at 500 x g for 5 min at 4 °C and remove the supernatant carefully by pipetting.

3.9. Resuspend the cell pellet in 500 µL (Jurkat cells) or 140 µL (patient samples) of shearing buffer 1 containing 1x protease inhibitor (5 µL or 1.4 µL, respectively) and incubate the mixture for 10 min on ice.

3.10. For chromatin shearing, transfer 140 µL of the cell-suspension from step 3.9 into sonicator tubes (avoid producing air bubbles and repeat this step if necessary due to sample volume). Place the tubes in the focused ultra-sonicator at a temperature of 7 °C and shear for 10 min (cell line) or 7.5 min (patient samples) with an average incident power of 9.375 W to obtain 200-500 bp DNA fragments.

3.11. Finally, centrifuge at 15700 x g for 10 min at 4 °C in the small centrifuge and collect the supernatant in a new 1.5 mL tube.

3.12. To test for the appropriate fragment sizes, analyze 20 µL of the sheared chromatin via gel-electrophoresis in 1.5% TBE agarose gel. An exemplary image of sheared chromatin from Jurkat cells of good and bad quality is shown in **Figure 3**. At this point, the protocol can be potentially paused. Sheared chromatin should be stored at -80 °C.

4. Chromatin Immunoprecipitation

Note: The chromatin immunoprecipitation was performed with a commercially available ChIP kit according to the manufacturer's instructions with modifications[7].

4.1. Calculate the number of immunoprecipitations (15 µL of sheared chromatin plus one antibody) and prepare 20 µL of protein A coated beads per precipitation in a 1.5 mL tube. Wash the beads in 40 µL of 1x ChIP buffer 1 per precipitation by pipetting up and down, let the beads rest in a magnetic rack for 1 min and remove the supernatant by pipetting. Perform this step four times in total. Eventually, resuspend the beads in the original volume with 1x ChIP buffer 1.

4.2. For the immunoprecipitation itself, use 10 µg of the αNFAT2 antibody (7A6) to capture NFAT2 with its bound target sequences. Use 2.5 µg of a rabbit IgG antibody (DA1E) as an IgG-control. Prepare the reactions as indicated in fresh 1.5 mL tubes as indicated in **Table 1**.

Note: It is important to use a monoclonal antibody with high affinity whose epitope is not masked during fixation for this protocol. Polyclonal antibodies introduce an additional level of variation making it significantly more difficult to appropriately interpret the received results.

4.3. Incubate the mixture from step 4.2 overnight at 4 °C on a rotating wheel at 6 rpm.

4.4. On the next day spin the tubes for 5 s at 7,000 x g in the small centrifuge, incubate them in a magnetic rack for 1 min and remove the supernatant by aspiration. Wash the beads once with each of the wash buffers 1, 2, 3 and 4 consecutively.

4.5. To do so, resuspend the beads in 350 µL of wash buffer and incubate them for 5 min at 4 °C on a rotating wheel at 6 rpm.

4.6. Thereafter, spin the tubes for 5 s at 7000 x g in the small centrifuge. Then, place them for 1 min in a magnetic rack, remove the supernatant and add the next wash buffer.

4.7. After the last washing step, remove the supernatant by pipetting, take up the beads in 100 µL of elution buffer 1 and incubate them for 30 min on a rotating wheel at 6 rpm.

4.8. Spin the tubes shortly (5 s at 7000 x g in the small centrifuge) and place them in a magnetic rack for 1 min.

4.9. Then transfer the supernatant by pipetting to a new 1.5 mL tube and add 4 µL of elution buffer 2.

4.10. To create the input control, mix 1 µL of the sheared chromatin with 99 µL of elution buffer 1 and 4 µL of elution buffer 2 (in this setup, there are three tubes per patient: one input control, one IgG-control and one αNFAT2 sample).

4.11. Incubate the samples for 4 h at 65 °C and 1300 rpm in a 1.5 mL tube shaker. Add 100 µL of 100% isopropanol, vortex and spin the samples for 5 s at 7000 x g in the small centrifuge. Resuspend the available magnetic beads by thoroughly vortexing, add 10 µL of the beads to every sample and incubate for 1 h at room temperature on a rotating wheel at 6 rpm.

4.12. After the incubation, spin the tubes for 5 s at 7000 x g in the small centrifuge and place them for 1 min in a magnetic rack. Remove the supernatant by aspiration.

4.13. Resuspend the beads in 100 µL of wash buffer 1. Invert the tubes to mix and incubate for 5 min at room temperature on a rotating wheel at 6 rpm.

4.14. Spin the tubes for 5 s at 7000 x g in the small centrifuge, place them for 1 min in a magnetic rack and remove the supernatant by pipetting. Repeat the steps 4.13-4.14 with wash buffer 2.

4.15. Subsequently, remove the wash buffer by aspiration, resuspend the beads in 55 µL of elution buffer and incubate for 15 min at room temperature on the rotating wheel at 6 rpm to elute the precipitated DNA. Finally, spin the tubes for 5 s at 7000 x g in the small centrifuge, place them for 1 min in a magnetic rack and transfer the supernatant into new 1.5 mL tubes.

Note: Here the protocol can be potentially paused. The eluted DNA should then be stored at -20 °C.

5. Detection of NFAT target genes in CLL cells.

5.1. Conduct the qRT-PCR reaction in duplicate for every sample as indicated in **Table 2**.

Note: For the detection of target genes a quantitative real-time PCR (qRT-PCR) is conducted using a commercially available qRT-PCR Mix with a Real-time PCR instrument.

5.2. Use white 96-well reaction plates for the reactions and the RT-PCR instrument. The cycling conditions are as follows: 95 °C for 30 s, [95 °C for 5 s, 60 °C for 30 s] x 40 cycles.

Note: A serial dilution of the input (undiluted or diluted 1:10, 1:100 and 1:1000 in nuclease-free water) is used to calculate the relative enrichment. In Jurkat cells, *IL-2* was used as a positive control[32]. *CD40L*, a well-known target of NFAT2 in B cells, was used as a positive control in CLL cells and *LCK* as an example for a novel target gene of NFAT2 in CLL[33,34]. The primers for the *CD40L*, the *IL-2* and *LCK* promoter region are described in the table of specific reagents and equipment.

6. Normalization and Data Analysis

Note: Relative enrichment for the promoter regions of interest (*LCK*) is calculated using the IgG-control for normalization.

6.1. First, determine Ct (threshold cycle) values for the input serial dilution, the *IL-2*, *CD40L* and the *LCK* promoter region via the evaluation software from qRT-PCR data.

6.2. Define a standard curve for the concentrations via the serial dilution of the input. With this standard curve calculate the concentration for the *IL-2, CD40L* and the *LCK* promoter region for each duplicate of every sample.

6.3. Next, calculate the mean of the duplicates. Then, set the IgG immunoprecipitation sample as a reference. Define the relative enrichment for this sample as 1.0 and compare the other sample (from the NFAT2 immunoprecipitation) to this reference.

6.4. Finally, calculate the relative enrichment by dividing the concentration of the samples by the concentration of the IgG reference.

REPRESENTATIVE RESULTS:

Figure 1 shows an exemplary flow cytometry analysis of a CLL patient performed after staining with CD19-FITC and CD5-PE antibodies. **Figure 1a** shows the gating of the lymphocytes, representing the majority of cells in the blood of CLL patients. **Figure 1b** shows the proportion

of $CD19^+/CD5^+$ CLL cells, which represent 89.03% of lymphocytes in this example. The proportion of $CD19^+/CD5^-$ B cells is 3.72% in the respective patient.

Figure 2 shows examples of antibody performance in Jurkat cells fixed for different time periods as assessed by SDS-PAGE and Western Blot. Different antibodies from different manufacturers were analyzed. **Figure 2a** shows the performance of the αNFAT2-antibody (clone 7A6) from different manufacturers detected with a green fluorescent anti-mouse antibody. The antibody of manufacturer 1 showed binding to its epitope without fixation (band A) and even when Jurkat cells were fixed for 2.5 min or 5 min (band B or C, respectively). The αNFAT2-antibody (clone 7A6) from manufacturer 2, on the other hand, showed a weaker performance even in Jurkat cells not fixed (band D). Upon fixation, the antibody of this manufacturer achieved even weaker results (bands E (2.5 min fixation) and F (5 min fixation)). **Figure 2b** shows the performance of the αNFAT2-antibody (clone D15F1) from a different manufacturer detected with a red fluorescent anti-rabbit antibody. This antibody also performed weakly in unfixed samples (band A) and the absence of a band indicates the masking of its epitope by the fixation (bands B (2.5 min fixation) and C (5 min fixation)).

Figure 3 shows examples of sheared chromatin from Jurkat cells obtained after different fixation and shearing times of good and poor quality as assessed by gel electrophoresis. Bands A and B (0 min fixation or 2.5 min fixation, respectively) show good quality sheared chromatin, which is characterized by a DNA fragment size of 200-500 bp which can be detected on an agarose gel as a typical smear. Sheared chromatin of poor quality, on the other hand, can be recognized by almost complete or complete absence of the expected DNA smear (band C) as well as a smear in a larger or smaller than expected fragment size range (bands D-F). The complete absence of the smear indicates the use of insufficient amounts of starting material. The detection of smaller DNA fragments hints to excessive DNA shearing (band E) while larger DNA fragments hint to insufficient shearing.

Figure 4 shows the results of a typical experiment with Jurkat cells. *LCK* was analyzed as a potential NFAT2 target. *IL-2* which is a well-defined target gene of NFAT2 in T cells was used as a positive control. An IgG-control antibody was utilized for normalization. **Figure 4a** shows an experiment with optimal results documented by significant enrichment of the *IL-2* positive control. From this experiment, it can be concluded that there is a significant enrichment of *LCK* sequences in the precipitated DNA demonstrating that *LCK* is a direct NFAT2 target in Jurkat cells. **Figure 4b** shows an experiment performed with poor quality DNA due to missing fixation or a suboptimal shearing procedure causing a low degree of enrichment in the *IL-2* positive control.

Figure 5 shows the results of an experiment performed with primary human CLL cells. *CD40L* which is a known NFAT2 target in B cells served as the positive control and *LCK* was analyzed as the experimental target. **Figure 5a** shows an experiment with a considerable level of enrichment of *CD40L* DNA in the positive control. From the even stronger enrichment of *LCK* DNA, it could be concluded that *LCK* is also a direct NFAT2 target in primary human CLL cells. **Figure 5b** shows an experiment performed with poor quality DNA most likely due to inadequate

shearing. No substantial enrichment of *CD40L* DNA could be detected in the positive control rendering the experimental data meaningless.

FIGURE AND TABLE LEGENDS:

Figure 1: Exemplary Flow cytometry analysis of CLL patients. (a) Samples of CLL patients were analyzed via flow cytometry after staining with CD19-FITC and CD5-PE antibodies and lymphocytes were gated. (b) Subsequently, the proportion of $CD19^+/CD5^+$ CLL cells and $CD19^+/CD5^-$ B cells were determined. Here, $CD19^+/CD5^+$ CLL cells account for 89.03% of lymphocytes, whereas $CD19^+/CD5^-$ B cells represent 3.72% of lymphocytes. One exemplary patient is shown.

Figure 2: Examples of antibody performance in fixed Jurkat cells assessed by SDS-PAGE and Western-Blot. (a) Jurkat cells were fixed for 0 min (bands A and D), 2.5 min (bands B and E), or 5 min (C and F) and the αNFAT2-antibody (clone 7A6) from different manufacturers (bands A-C = manufacturer 1, bands D-F = manufacturer 2) was compared. The antibody of manufacturer 1 showed a better overall performance, binding with the higher affinity even to fixed samples (compare bands A-C and bands D-F). (b) The αNFAT2-antibody (clone D15F1) from a different manufacturer was used. This antibody showed only a poor performance in unfixed samples (band A) and the epitope was masked upon fixation. Therefore, no binding of the antibody could be detected after fixation (bands B and C).

Figure 3: Examples of chromatin of good and poor shearing quality from Jurkat cells assessed by gel electrophoresis. The Jurkat cells were fixed and sheared for different time periods. Fixation was done for 0 min (bands A and D), 2.5 min (bands B and E), or 5 min (C and F). Shearing was performed either for 10 min (bands A-C) or 20 min (bands D-F). Chromatin of good shearing quality is characterized by a DNA fragment size of 200-500 bp which can be detected as a smear in the respective region (bands A and B). Chromatin of poor quality can be recognized either by an almost complete or complete absence of the DNA smear due to an insufficient amount of starting material used (band C) or by a smear in a smaller or larger size region because of inappropriate shearing conditions (bands D-F).

Figure 4: ChIP of Jurkat cells assessed by qRT-PCR. (a) NFAT2 binds to *LCK* and the *IL-2* promoter in Jurkat cells. The relative enrichment of *LCK* and *IL-2* promoter regions precipitated with the NFAT2 antibody is shown as analyzed by qRT-PCR. Three independent ChIP-experiments are shown as mean ± SEM (Student's t-test, $*P < 0.05$; $**P < 0.01$ $***P < 0.001$). (b) DNA from samples with poor quality sheared chromatin was used. No relevant binding of NFAT2 to the target sequences could be detected. A similar picture can be detected if there was no fixation or the incubation time with the NFAT2 antibody was insufficient. Three independent ChIP-experiments are shown as mean ± SEM (Student's t-test, $*P < 0.05$; $**P < 0.01$ $***P < 0.001$).

Figure 5: ChIP of primary human CLL cells assessed by qRT-PCR. (a) NFAT2 specifically binds to the *LCK* and *CD40L* promoters in primary human CLL cells from patients with an indolent course

of the disease. The diagram shows the relative enrichment of *LCK* and *CD40L* promoter regions precipitated with the NFAT2 antibody as analyzed by qRT-PCR. Three independent ChIP-experiments are shown as mean ± SEM (Student's t-test, *P < 0.05; **P < 0.01 ***P < 0.001). (b) DNA from patient samples with poor quality sheared chromatin was used. No binding of NFAT2 to the respective target sequences could be observed. Three independent ChIP-experiments are shown as mean ± SEM (Student's t-test, *P < 0.05; **P < 0.01 ***P < 0.001)

Table 1: Schedule for pipetting the chromatin immunoprecipitation. The chromatin immunoprecipitation was performed by preparing the reactions as indicated in the table.

Table 2: Schedule for pipetting the qRT-PCR. The qRT-PCR was performed by preparing the reactions as indicated in the table.

DISCUSSION:

The critical steps of performing a successful ChIP assay are the selection of an appropriate antibody and the optimization of the chromatin shearing process[25]. The selection of the αNFAT2 antibody proved to be particularly challenging during the development of this protocol. While there are several αNFAT2 antibodies commercially available and the majority of these works fine for western blotting and other applications, clone 7A6 was the only antibody which could be successfully used for ChIP[7]. Even supposedly identical 7A6 antibodies from different manufacturers exhibited significant differences with respect to their performance in ChIP. A major challenge is the potential disruption of target protein epitopes by the fixation process used in XChIP protocols[25].

The chromatin shearing procedure is also critical for acquiring appropriate results in XChIP. A fragment size of 200-500 bp as assessed by agarose gel electrophoresis was found to be optimal in this NFAT2 ChIP protocol[7]. Inappropriate shearing conditions resulting in a shortage of DNA starting material or DNA fragments of smaller or larger size typically lead to suboptimal results when performing this assay. Suboptimal shearing was also observed when using frozen and rethawed PBMCs. Hence, thawing of PBMCs resulted in a lower yield of DNA from cells as well as an increase in excessive shearing of DNA-fragments.

The availability of a broad variety of ChIP kits and ChIP-grade antibodies is challenging, but also offers the possibility to use the technique in a wide context. Thus, a diversity of transcription factors can be investigated in different cell types. For example, other members of the NFAT family (NFAT1 and NFAT4) have been investigated recently using ChIP[35-37].

The ChIP kit used here also had to be modified to suit our needs. First, the time of fixation was adjusted to avoid masking of the epitope recognized by the used antibody and to enable optimal shearing. The volume of buffers used for lysis and shearing was also changed to reduce the loss of cells during the different washing steps. Additionally, the shearing conditions were optimized to obtain DNA fragments in the range of 200-500 bp. The protease inhibitor and the IgG-control antibody were exchanged with other commercially available reagents as they proved to be comparable and more cost-effective. The step involving the carrier provided by

the manufacturer was omitted as it interfered with the precipitation of the DNA. In the end, the amount of buffer used to elute the DNA was adapted to yield a sufficient volume to be used in the qRT-PCR.

There are several other kits available from various companies and there is also the possibility to perform the ChIP without a kit. However, testing and establishment are very challenging, as there are many factors to be considered, like the compatibility of analyzed cells and proteins with different fixation and shearing methods. The mentioned kit was used as it was well-established in our laboratory.

A major restriction of this method is its limited applicability to the large-scale investigations in intact model organisms because appropriate antibodies have to be identified or generated for each individual transcription factor. The analysis of genes which are expressed only at low levels, in a small number of cells or during a restricted time window can also be challenging using ChIP.

While ChIP is the gold standard to demonstrate a direct interaction of a given transcription factor with its respective target genes in intact eukaryotic cells[25], there are a number of other techniques to investigate an interaction between proteins and DNA. EMSA is a useful method to detect low-abundance DNA binding proteins in cell lystates[24]. It can also be used to characterize the binding affinity of a particular protein through a systematic DNA probe mutational analysis. A major advantage of EMSA in comparison to ChIP is the fact that it is generally substantially less time-consuming to establish the respective assay. DNA pull-down assays, microplate capture and detection assays and reporter assays are other techniques to analyze protein-DNA interactions[23].

More recent developments have made it possible to apply the ChIP assay to genome-wide approaches by its combination with the microarray technology (ChIP-on-chip)[38-40] or the next-generation sequencing (ChIP-Seq)[41-43].

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DISCLOSURES:

The authors have nothing to disclose.

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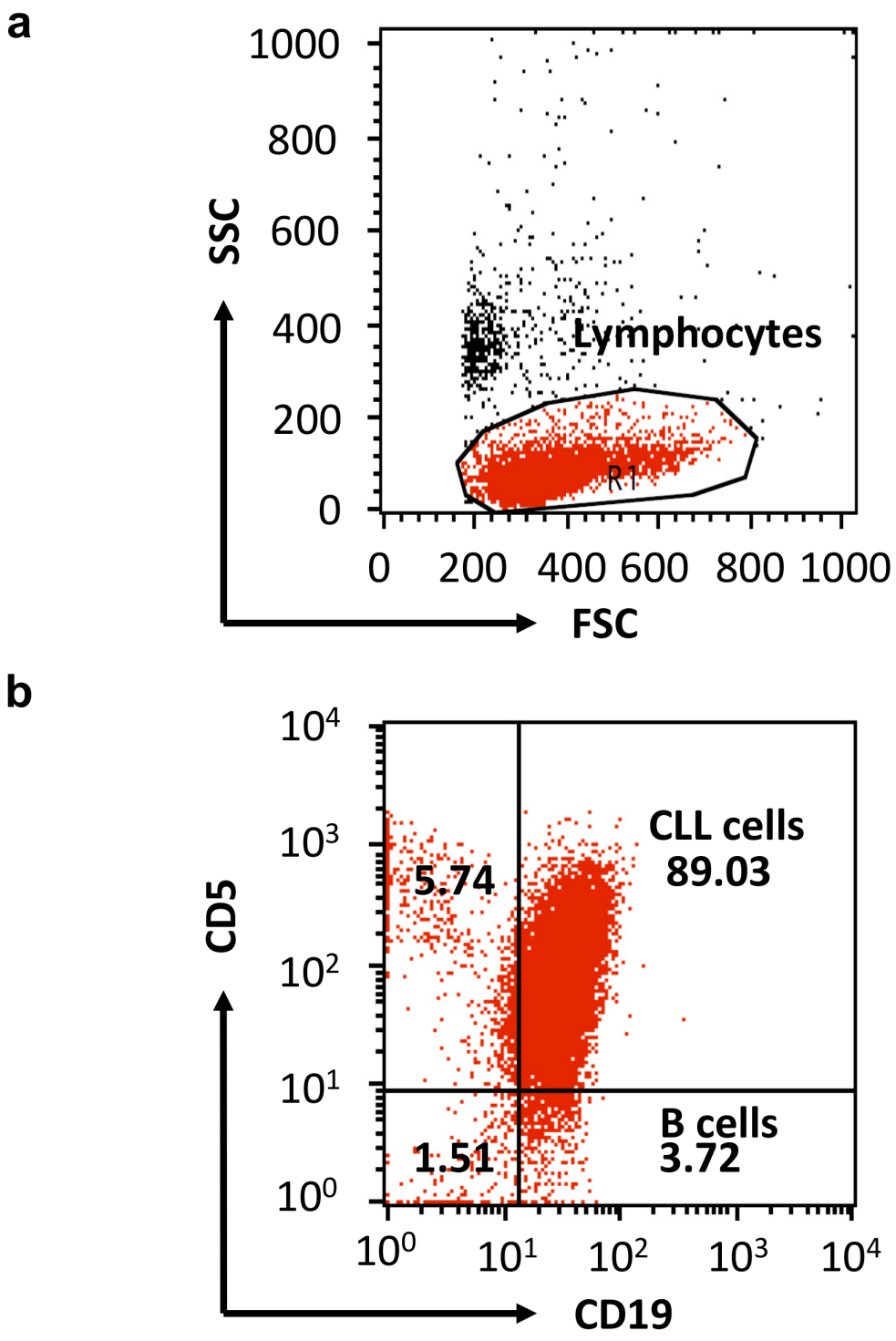

Figure 1

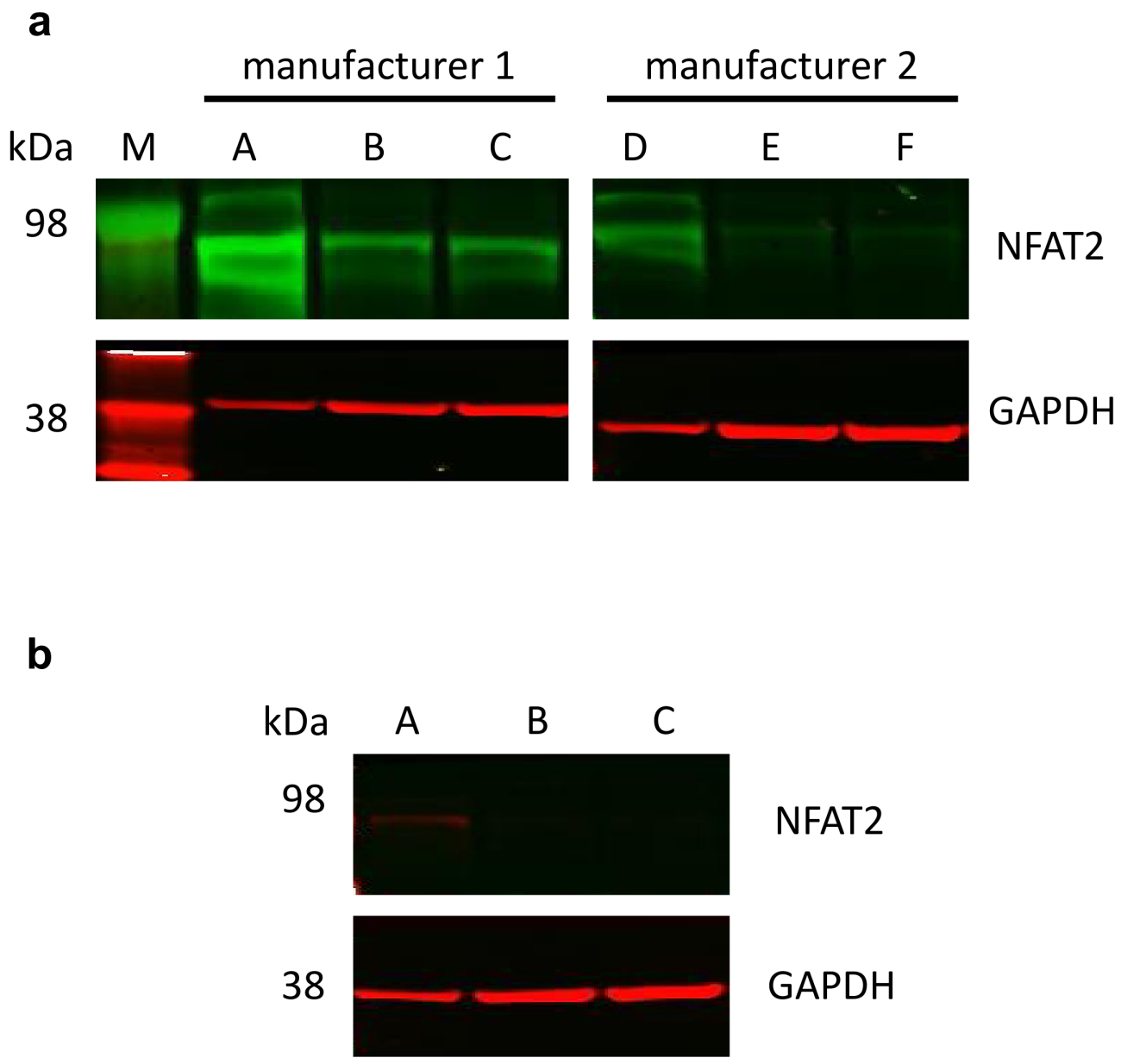

Figure 2

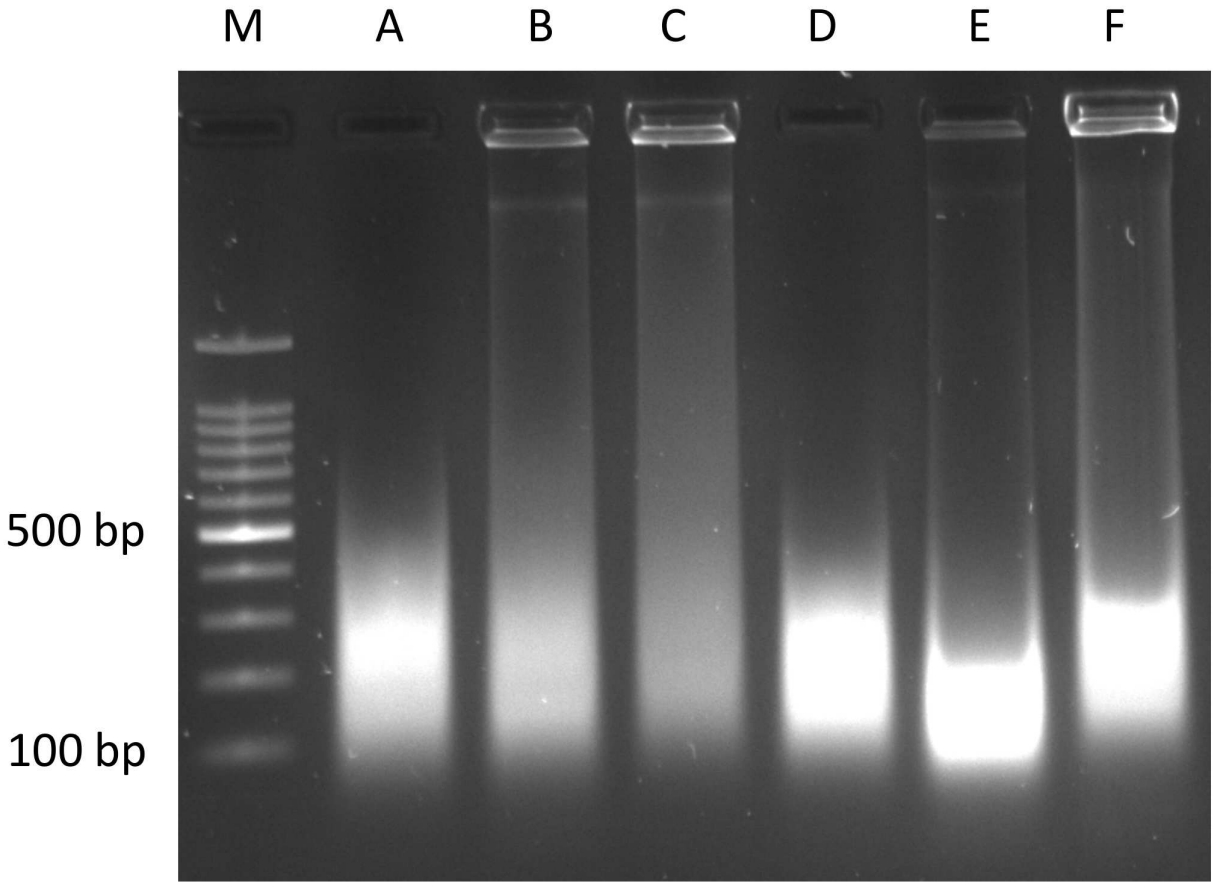

Figure 3

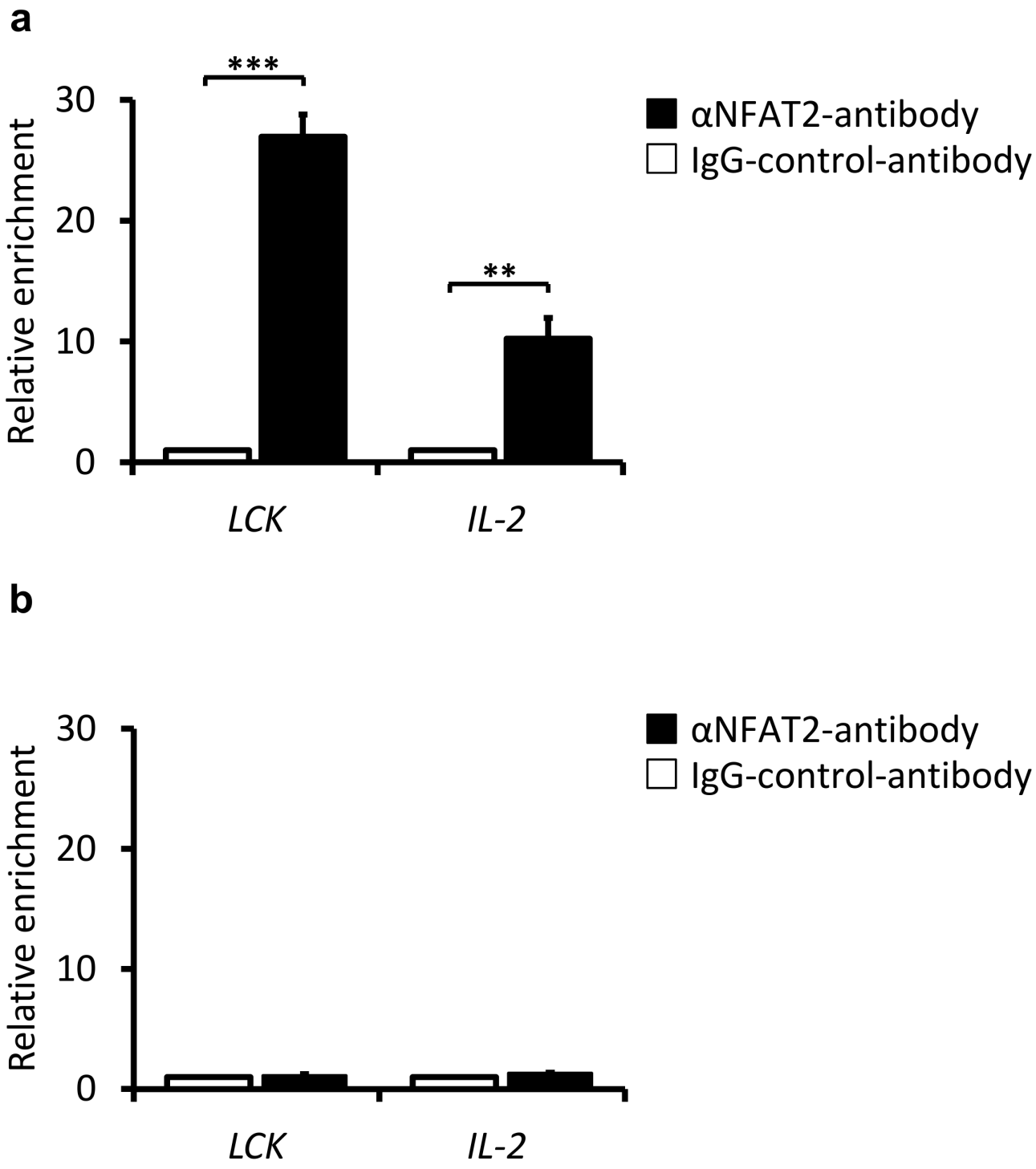

Figure 4

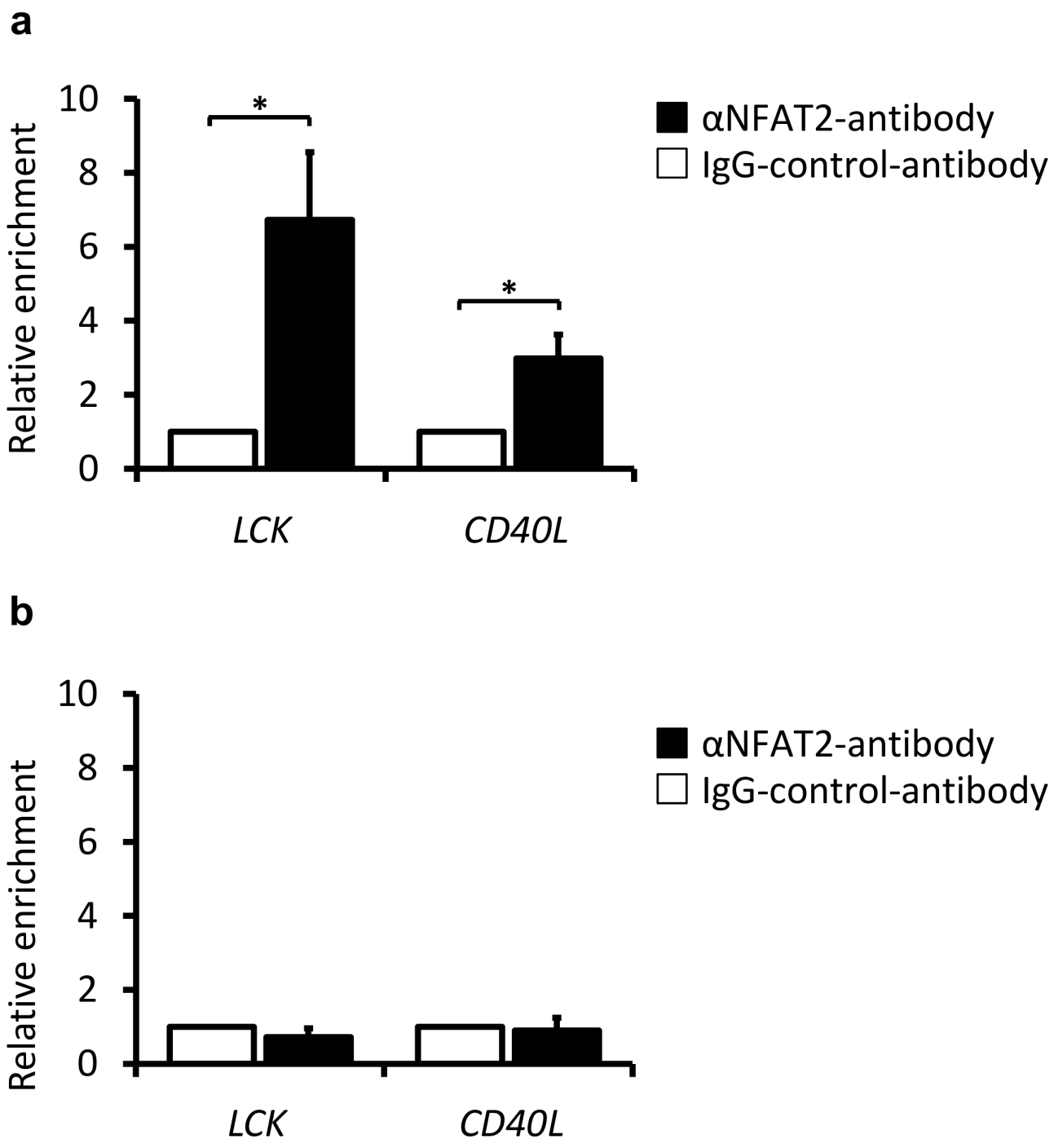

Figure 5

Table 1

item	volume (µL) per IP
5% BSA	6
100 x protease inhibitor	3
5 x ChIP buffer 1	56
sheared chromatin	15
Protein A coated magnetic beads	20
ChIP seq grade water	170-x
antibody (αNFAT2 or IgG-control)	x (1.09 µL or 1 µL)
total	270

Table 2

item	volume (µL) per qRT-PCR
immunoprecipitated DNA	9
qRT-PCR Mix	10
primer forward (*LCK* /*CD40L* /*IL-2*)	0.5
primer reverse (*LCK* /*CD40L* /*IL-2*)	0.5
total	20

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 X PBS	Sigma Aldrich	D8537	
1.5 mL tube shaker Themomixer comfort	Eppendorf	5355 000.011	Can be substituted with similar instruments
10X Bolt Sample Reducing Agent	Thermo Scientific	B0009	
20X Bolt MES SDS Running Buffer	Thermo Scientific	B0002	
37 % Formaldehyde p.a., ACS	Roth	4979.1	
4X Bolt LDS Sample Buffer	Thermo Scientific	B0007	
Anti-NFAT2 antibody	Alexis	1008505	Clone 7A6
Anti-NFAT2 antibody	Cell Signaling	8032S	Clone D15F1
Anti-NFAT2 antibody ChIP Grade	Abcam	ab2796	Clone 7A6
big Centrifuge	Eppendorf	5804R	Can be substituted with similar instruments
CD19-FITC mouse Anti-human	BD Biosciences	555412	Clone HIB19
CD5-PE mouse Anti-human CD5	BD Biosciences	555353	Clone UCHT2
Density gradient medium Biocoll (Density 1,077 g/ml)	Merck	L 6115	
DNA LoBind Tube 1.5 mL	eppendorf	22431021	
FBS superior	Merck	S0615	
Flow Cytometer	BD Biosciences	FACSCalibur	Can be substituted with similar instruments
Halt Protease and Phosphatase Inhibitor Cocktail (100X)	Thermo Scientific	78440	
iBlot 2 Gel Transfer Device	Thermo Scientific	IB21001	
iBlot 2 Transfer Stacks, nitrocellulose, regular size	Thermo Scientific	IB23001	
iDeal ChIp-seq kit for Histones	Diagenode	C01010059	
Ionomycin calcium salt	Sigma Aldrich	I3909	

IRDye 680LT Donkey anti-Rabbit IgG (H + L), 0.5 mg	LI-COR Biosciences	926-68023	
IRDye 800CW Goat anti-Mouse IgG (H + L), 0.1 mg	LI-COR Biosciences	925-32210	
LI-COR Odyssey Infrared Imaging System	LI-COR Biosciences	B446	
LightCycler 480 Multiwell Plate 96, white	Roche	4729692001	Can be substituted with other plates in different real-time PCR instruments
Lysing Solution OptiLyse B	Beckman Coulter	IM1400	
M220 AFA-grade water	Covaris	520101	
M220 Focused-ultrasonicator	Covaris	500295	
Magnetic rack, DynaMag-15 Magnet	Thermo Scientific	12301D	Can be substituted with similar instruments
MEM Non-Essential Amino Acids Solution 100X	Thermo Scientific	11140050	
Microscope Axiovert 25	Zeiss	451200	Can be substituted with similar instruments
microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm	Covaris	520045	
Neubauer improved counting chamber	Karl Hecht GmbH & Co KG	40442012	Can be substituted with similar instruments
NH4 Heparin Monovette	Sarstedt	02.1064	
Nuclease-free water	Promega	P1193	
NuPAGE 4-12% Bis-Tris Protein Gels, 1.0 mm, 15-well	Thermo Scientific	NP0323BOX	
Odyssey® Blocking Buffer (TBS) 500 mL	LI-COR Biosciences	927-50000	
Penicillin/Streptomycin 100X	Merck	A2213	
PerfeCTa SYBR Green FastMix	Quanta Bio	95072-012	
PMA	Sigma Aldrich	P1585	

Primer *CD40L* promotor region forward	Sigma Aldrich		5'-ACTCGGTGTTAGCCAGG-3'
Primer *CD40L* promotor region reverse	Sigma Aldrich		5'-GGGCTCTTGGGTGCTATTGT -3'
Primer *IL-2* promotor region forward	Sigma Aldrich		5'-TCCAAAGAGTCATCAGAAGAG-3'
Primer *IL-2* promotor region reverse	Sigma Aldrich		5'-GGCAGGAGTTGAGGTTACTGT-3'
Primer *LCK* promotor region forward	Sigma Aldrich		5'-CAGGCAAAACAGGCACACAT-3'
Primer *LCK* promotor region reverse	Sigma Aldrich		5'-CCTCCAGTGACTCTGTTGGC-3'
Rabbit mAb IgG XP Isotype Control	Cell Signaling	# 3900S	Clone DA1E
Real-time PCR instrument	Roche	LightCycler 480	Can be substituted with similar instruments
Roller mixers	Phoenix Instrument	RS-TR 5	
RPMI 1640 Medium, GlutaMAX Supplement	Thermo Scientific	61870010	
Safety-Multifly-needle 21G	Sarstedt	851638235	
SeeBlue Plus2 Pre-stained Protein Standard	Thermo Scientific	LC5925	
Shaker Duomax 1030	Heidolph Instruments	543-32205-00	Can be substituted with similar instruments
small Centrifuge	Thermo Scientific	Heraeus Fresco 17	Can be substituted with similar instruments
Sodium Pyruvate	Thermo Scientific	11360070	
ß-Mercaptoethanol	Thermo Scientific	21985023	
Tris Buffered Saline (TBS-10X)	Cell Signaling	#12498	
Trypan Blue solution	Sigma Aldrich	93595-50ML	

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May 27, 2018

Dear editors,

please find included the revised version of our manuscript "ChIP-assay to identify novel NFAT2 target genes in chronic lymphocytic leukemia" by Alexander Fuchs et al. to be considered for publication in the Journal of Visual Experiments. We have now revised the manuscript again according to your comments from May 23, 2018. All changes made to the previous version of the manuscript are highlighted in green.

Please find our detailed response below.

Editorial comments:

1. The editor has formatted the manuscript as per the journal's style. Please retain the same.

   Response: The format has been retained.

2. Please address all the specific comments marked in the manuscript.

   Response: All the specific comments marked in the manuscript have now been addressed.

3. Unfortunately, there are a few sections of the manuscript that show significant overlap with previously published work. Though there may be a limited number of ways to describe a technique, please use original language throughout the manuscript. Please see lines: 33-34, 39-40, 62-63, 88-89.

   Response: The respective sections have been rephrased.

4. Please remove embedded tables from the manuscript and upload it separately as .xls/xlsx file. Two individual files. Please include the table legend in the figure/table legends section.

   Response: The respective tables have been removed from the manuscript and are now added as separate .xls/xlsx files. The table legend is now included in the figure/table legends section.

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   Response: The manuscript does not contain any figures from previous publications.

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Please do not hesitate to contact us if there should be any additional questions.

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