

Supplement Methods

Supplementary description of experimental detail: cloning and constructs, cell culture, qPCR and rtPCR, Western blot procedure and antibodies, *in vivo* and *in vitro* myristoylation and palmitoylation, fixing cells and microscopy, quantitative analysis of confocal microscopy pictures, live cell imaging

Cloning and constructs

All NFAT5 constructs are based on a plasmid generously provided by Joan Ferraris containing NFAT5c. Constructs for *in vitro* labeling assays (myristoylation): A PCR reaction with the primers `ctcgagaagcgtaatacgcactactatagggagaccaccatgggcggtgcttgagctc` and `gtcgacaaaggagccagtcgaagttgttc` resulted in NFAT5a with an upstream T7 promoter and introduced restriction sites for Xho1 (5') and Sal1 (3'). The PCR product was cloned into pGEM-T. T7-NFAT5a was cloned via Apa1/Sal1 into a self-constructed plasmid with a GST-tag (pSK+GST) downstream of its MCS (GST-stop amplified by PCR from pGEX-5X-1 and inserted via Sac1/Sac1 into the pSK+-vector). Using the Quickchange XL site-directed mutagenesis kit (Stratagene) and the primers `gggagaccaccatggccggtgcttgagctcc` and `ggagctgcaagcaccggccatggtgtctccc`, we introduced a G2A mutation. Both constructs had to be shortened for the *in vitro* transcription translation to a total length of 541 AA. The wild-type was amplified via PCR using the following primers `ctcgagaagcgtaatacgcactactatagggagaccaccatgggcggtgcttgagctc` and `gaattctcttttctgctgttacttc` introducing XhoI and EcoRI out of the T7-NFAT5a-GST into pGEM-T. Afterwards the construct was reintroduced in the pSK+GST vector using XhoI/EcoRI resulting in T7-NFAT5a(AA1-541)-GST. The G2A mutant was cloned using the Hind3/BamH1 restriction sites in the following constructs: T7-NFAT5a(AA1-541)-GST and the T7-NFAT5a(G2A)-GST. Ligation resulted in T7-NFAT5a(G2A)(AA1-541)-GST.

NFAT5a(AA1-123)(G2A)(C5A)-HA and NFAT5b/c-HA were cloned amplifying NFAT5a(G2A)(C5A)/b/c-GFP as templates with the primers gaattcatgggcggtgctgcagctc and gcagaattcatgggcggtgctgc and direct ligation into the pEGFP-HA vector using EcoR1/Sal1. GFP constructs wild-type and mutants for localization studies: NFAT5a-GFP was cloned from NFAT5a-GST into pEGFP-N3 via Xho1/BamH1. NFAT5a(G2A)-GFP was cloned the same way starting out from NFAT5a(G2A)-GST. To generate NFAT5a(C5A)-GFP we performed a PCR using the primers gtcgacaaaggagccagtcgaagttgtc and ctcgagaagcgtaatacgaactcactatagggagaccaccatgggcggtgctgccagctcctttacc with NFAT5a in pGEM-T as a template and cloned it into pGEM-T Easy (NFAT5a(C5A) in pGEM-T easy). To finally generate NFAT5a(C5A)-GFP we replaced the N-terminus of NFAT5a(G2A)-GST with the one of NFAT5a(C5A) in pGEM-T easy via Xho1/Sac1. For NFAT5a(C5/32A)-GFP, we introduced the C32A mutation into NFAT5a(C5A)-GFP using the QuikChange® *Multi-Site Directed Mutagenesis* Kit (Stratagene) with the primer tgcaagtggagagcgctcctcagccgtggg. With the primers acagtcggatgtccgccaggatgaggggctggattggaat and ctgagcagagcgccagtatgtggatggagg, we added the mutations C83A, C88A and C96A resulting in NFAT5a(C5/32/83/88/96A)-GFP. Later, we cured the C5A mutation with the primers ctcgagatgggcggtgctgcagctcctttaccacctctt and aagagtggtaaaggagctgcaagcaccgccatctcgag resulting in NFAT5a(C32/83/88/96A)-GFP. NFAT5a_2A-GFP was constructed by mutation with site directed mutagenesis kit (Stratagene) from Plasmid NFAT5a-GFP with the forward primer ggagaccaccatggcaggcggtgcttg and its reverse. myc-NFAT5a-GFP was a gift from Cristina Lopez-Rodriguez.

We introduced an HA-tag via Sal1/BamH1 followed by a stop codon between the ORFs of NFAT5a(G2A)(C5A) and the GFP tag of the plasmids using the oligos

tacagtcgactaccctacgacgtgcccgactacgccgtctagggatccta and
taggatccctagacggcgtagtcgggcacgtcgtagggtagtcgactgta. The results are NFAT5a(*G2A*)(*C5A*)-
HA.

APMAP(1-61)-GFP was constructed by RT-PCR from TT cell cDNA using the primers gatctcgagatgagcggagggcggacggg and gatggatcctcccagcagcatcatggctcc and directly cloned into pEGFP-N3. APMAP(1-61)-NFAT5a-GFP, APMAP(1-61)-NFAT5b-GFP and APMAP(1-61)-NFAT5c-GFP was constructed by ligating the PCR of APMAP(1-61)-GFP with primers gatctcgagtcctcccagcagcatcatggctcc and gatgctagcatgagcggagggcggacggg into the plasmid NFAT5a/b/c-GFP using XhoI/NdeI.

All cloning products were checked for correctness via sequencing according to the sequences published under the accession numbers NM_173215 (NFAT5a), NM_138713 (NFAT5b), NM_006599 (NFAT5c), and NM_020531 (APMAP).

Cell culture

HeLa and HEK293 cells were used as standard cell lines and cultured in DMEM + 4500 g/L glucose + 10% FBS (GIBCO) + 2 mM L-Glutamine (GIBCO) + 1 Unit/ml Pen-Strep (GIBCO). For introduction of plasmids into cells Lipofectamine Plus™ Reagent or Lipofectamine2000 (Invitrogen) were used according to the manual. DNA was incubated 24 hours.

qPCR and rtPCR

Total RNA was purified from HeLa cells with Trizol Reagent (Invitrogen) according to the manual. RNA concentration was determined using NanoDrop. cDNAs were synthesized

using Maxima First strand cDNA synthesis kit for RT-qPCR (Fermentas). Real-time PCR was run using Maxima SYBR Green qPCR Master mix (Fermentas) on Rotor-Gene Q PCR cycler (Qiagen). PCR condition were 95°C for 10 min, 40 cycles of 95°C for 10 sec, 55°C for 15 sec, 72°C for 20 sec.

A serial dilution test of the primers was done to determine the threshold use for Ct calculation. Triplicates of each sample were analyzed by DDCt method.

For sequencing the PCR products an rt-PCR was done using the cDNA protocol from above and iDNA 5x Mastermix for PCR. PCR products were separated by electrophoresis on 2% agarose gels with SYBR-Safe (Invitrogen). The bands were cut and Quick Gel extraction Kit (Quiagen) was used for purification. The PCR products were ligated into pGEMT and sequenced.

The following primers were used for qPCR and RT-PCR: GAPDH fwd 5' GAGTCAACGGATTTGGTCGT 3', rev: 5' TTGATTTTGGAGGGATCTCG 3', segment -|X fwd 5' GATTTGCCTCTGAAGCAGGGAG 3', rev 5' CCTTGCTGTCGGTGACTGAGG TAG 3', exon 2|B fwd 5' ATTCTCTGAAGTTACACCCATC 3', rev 5' CTCACCACGGCTTGTCTGACTC 3', exon 4|D fwd 5' ACCACCTCTTCCAGCCCTACCA 3', rev 5' TGTGCCTCTTCGGTGTTGATG 3'

Western blot procedure and antibodies

Whole cell extract preparation: Cells were seeded, transfected and grown in 6-well plates (Nunc). Cells were scraped in 1x Sample-Buffer (0.01 M Tris-HCl pH6.8, 2 % Glycerol, 0.3 % SDS, add fresh 100 mM DTT). Samples were cooked at 95°C for 5 min. Lysate was

loaded. Antibody dilution and incubation was performed according to the manufacturer protocols. Primary antibodies for Western detection: Anti-GST-HRP Conjugate (Amersham), mouse anti-HA BEAM (ETC), mouse anti-GFP (Roche) and GAPDH (14C10) Rabbit mAb (Cell Signaling). As secondary antibody, we used goat anti-mouse-HRP conjugated (Santa Cruz). For Western blot detection ECL Plus™ Western Blotting Detection Reagent (Amersham) was used.

Separation of nuclei and cytoplasm

Cells were seeded, transfected and grown in 6-well plates (Nunc). Cells were washed with 1x PBS and scraped 200 µl 1x PBS, transferred to micro centrifuge tubes and spin down for 2 min at 1000 x g at 4°C. The pellet was resuspended in 200 µl Buffer A (10 mM HEPES pH7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M Sucrose, 10% Glycerol, 1% A-Iodoacetamide, 1 mM PMSF and complete were added fresh) + 0.1% Triton X-100. The sample was left on ice for 15 min. After that it was centrifuged at 1300 x g for 4 min at 4°C. The supernatant (cytoplasmic fraction) was transferred to a new micro centrifuge tube and kept on ice. The pellet (nuclear fraction) was washed 2x with Buffer A. The nuclear fraction was resuspended in 200 µl Buffer A. To both samples 4x NuPage Sample-Buffer (Invitrogen) was added to achieve a 1x concentration. 100 mM DTT was added per sample. Samples were cooked at 95°C for 5 min. 25 µl lysate was loaded.

SDS-Page Gels: Either self made SDS-Page or precast NuPAGE® Bis-Tris gels (Invitrogen). Percentage and buffers were optimized for achieving the best resolution. The following buffers were used: Tris-Glycine-SDS (0.025 M Tris, 0.192 M Glycine, 0.1 % SDS), NuPAGE® MOPS SDS Running Buffer (for Bis-Tris Gels only) (20X) (Invitrogen) and NuPAGE® MES SDS Running Buffer (for Bis-Tris Gels only) (20X). Western transfers to

nitrocellulose membranes (BioRad) were done either wet or semi-dry. For self-made gels Tris-Glycine Buffer (0.025 M Tris, 0.192 M Glycine) was used as transfer buffer. For NuPAGE® Bis-Tris gels NuPAGE® Transfer Buffer (20X) according to manual was used. Antibody dilution and incubation was preformed according to the manufacturer protocols. Primary antibodies for Western detection: Anti-GST-HRP Conjugate (Amersham), mouse anti-HA BEAM (ETC), mouse anti-GFP (Roche) and GAPDH (14C10) Rabbit mAb (Cell Signaling). Secondary antibodies used: Goat anti-mouse- and goat anti-rabbit-rabbit-HRP conjugated (Santa Cruz). For Western blot detection ECL Plus™ Western Blotting Detection Reagent (Amersham) was used.

In vivo and in vitro myristoylation and palmitoylation

The in vitro assay for protein N-myristoylation is a modified version of the prenylation assay published previously ^{1,2}. All tags were C-terminal and used for immunodetection. The proteins were expressed using the The TNT® T7 Quick Coupled Transcription/Translation System (Promega) according to the manual.

For *in vitro* myristoylation 20 µCi [9,10-³H]- Myristic acid (Perkin Elmer) were spin dried till almost dry in a vacuum centrifuge. The TNT reaction was used to resuspend the myristate. SDS-sample buffer was added directly to samples. They were cooked for 5 min at 95°C and were loaded run on a SDS-Page gel and blotted to nitrocellulose membranes. The purification step of the fusion protein with glutathion beads was omitted.

For *in vitro* palmitoylation, palmitate had to be activated with CoA. [9,10-³H]-Palmitoyl-CoA was generated following the protocol described earlier ^{3,4}. 500 µCi of [9,10-³H]-Palmitic acid (Promega) was vacuum dried till almost dry followed by adding the following reaction

mixture 100 μ l 2x buffer (20 mM Tris-HCL, pH7.4, 10 mM MgCl₂, 2 mM DTT, 0.2 mM EGTA), 10 μ l 100mM ATP, 5.72 μ l 35 mM LiCoA, 60 μ l Acyl-CoA synthetase. Reaction was carried out at 30°C for 25min. This mixture was stored at -18°C. Per reaction around 37 μ Ci were added to the TNT reaction mix. The labeled TNT products were immune-precipitated with 30 μ l agarose beads conjugated to rabbit-anti HA (Santa Cruz) for 1 hour at 4°C. Beads were washed 3 times after incubation and beads were boiled for 5 min at 95°C in 15 μ l 1x SDS-sample Buffer. Samples were run on SDS-page gels and blotted to nitrocellulose membranes.

In vivo labeling assays were carried out as described involving an immune-purification step based on the HA tag ^{1,2}. 10 cm plates were used for transfection and labeling. For myristoylation and palmitoylation experiments it was necessary to spin dry the solution till nearly dry and mix them with 15 μ l DMSO and DMEM + PSG before adding it to the cells. 250 μ Ci [9,10-³H]-Myristic acid per sample were used for myristoylation and 500 μ Ci [9,10-³H]-Palmitic acid for palmitoylation. Medium was added around 4 hours after transfection of the cells and was incubated over night. Cells were washed with 1x PBS and lysed in 500 μ l CHIP Buffer. Samples were sonicated for 5 min in an ultrasound water bath with ice. Immune-precipitation was done using 25 μ l Dynalbeads M-280 Sheep anti-mouse and mouse anti-HA BEAM (ETC) for 1 hour at rt. Beads were washed 3 times after incubation and beads were boiled for 5 min at 95 °C in 35 μ l 1x SDS-sample Buffer. Samples were run on SDS-page gels and blotted to nitrocellulose membranes.

The membranes were first scanned for radioactive label incorporation using a Thin Layer Chromatographic Scanner (TLC-scanner) for 20 min per lane. The scanner registers all radioactive events within a specified window as counts. The resulting graph is the length of

the sample lane (x-axes) vs. the counts detected (y-axes). After the scan was completed the membranes were incubated with a protein-tag specific anti-body (Anti-GST-HRP Conjugate (Amersham), mouse anti-HA BEAM (ETC)). Besides achieving higher experimental accuracy by testing protein amounts and radioactive anchor incorporation in one and the same experiment, this technique has also the advantage of higher sensitivity and saving time (20 min per lane). This allows quicker variations of experimental parameters because of less waiting time between them.

Fixing cells and microscopy

Cells were seeded at a density of 5×10^4 cells/well on ethanol-flamed 15mm cover slips placed in 12-well plates approximately 12 hours prior to transfection. The inhibitors 100 μ M 2-Bromo-palmitate (Sigma) and 5 μ g/ml BrefeldinA (Calbiochem) were incubated with the cells for around 12 hours (added at the change of medium after transfection) For salt stress experiments, NaCl was added to a final concentration of 350 mOsmol per well and incubated for 1 hour immediately before fixing the cells. Cells were washed with cold 1x PBS and fixed with 2% formaldehyde in 1x PBS for 15 mins. Cells were blocked with 5% BSA and 0.3% Triton-X in 1x PBS for 1 hour. The antibody incubation steps were done in 1% BSA in 0.3% Triton-X 1x PBS for 1 hour at room temperature. For staining of cytoplasm, rabbit anti-beta-tubulin (Abcam) and goat anti-rabbit IgG Alexa 546 (Invitrogen) was used. For localization studies, mouse anti-PDI (Abcam) and mouse anti-Giantin (Abcam) was used as primary antibodies against ER and Golgi respectively while secondary antibody was goat anti-mouse IgG Alexa 546 (Invitrogen). Plasma membranes were stained with wheat germ agglutinin Alexa 555 (Invitrogen) following the suppliers protocol. Three 5 minutes washing steps using 0.3% Triton-X in 1x PBS were incorporated between all incubation steps. Cover slips were inverted onto glass slides with VECTASHIELD, Hard SET Mounting Medium with DAPI

(VECTOR LABORATORIES). The microscopy platform LSM 510 META upright and LSM 5 LIFE Duo Scan by Carl Zeiss were used for image acquisition at 63x magnification.

Quantitative analysis of confocal microscopy pictures

Per construct and condition 10 images were taken in two separate experiments. Pictures were segmented and cells were selected by the following criteria: (i) cells must look healthy, (ii) cells must be transfected, (iii) the whole cell must be in the image and (iv) cells must be recognized as individual cells by the segmentation software. For each construct and each condition a different number of cells were chosen about 25-30 cells.

Complete list of number of cells selected - no salt: no DNA (32), GFP control (37), NFAT5c-GFP (26), NFAT5b-GFP (28), NFAT5a-GFP (wt) (23), NFAT5a(G2A)-GFP (28), NFAT5a(C5A)-GFP (21), NFAT5a(C5/32A)-GFP (27), NFAT5a(C32/83/88/96C)-GFP (22), NFAT5a(2A)-GFP (25), myc-NFAT5a-GFP (21).

Complete list of number of cells selected - salt stress: no DNA (37), GFP control (33), NFAT5c-GFP (23), NFAT5b-GFP (31), NFAT5a-GFP (wt) (22), NFAT5a(G2A)-GFP (30), NFAT5a(C5A)-GFP (24), NFAT5a(C5/32A)-GFP (28), NFAT5a(C32/83/88/96C)-GFP (30), NFAT5a(2A)-GFP (20), myc-NFAT5a-GFP (26).

All images, including an image showing which cells were selected, can be found on our homepage http://mendel.bii.a-star.edu.sg/SEQUENCES/NFAT5_2011/. Mathematical procedures for processing image data are described in Supplement-Figure-4.pdf.

Flow cytometry analysis

For FACS analysis cells were seeded, grown and transfected in 6-well plates. 24 hours after transfection cells were washed with 2 ml cold 1x PBS. To bring cells into suspension the wells were incubated with 200 μ l 0.125% Trypsin/Versene pH7.0 \pm 0.3 for 5 min at 37°C and 5% CO₂. Cells were resuspended in 2 ml 1x PBS. After counting the cells using a hemocytometer, they were pelleted at 200 x g for 5 min at 4°C. The cell pellet was resuspended in cold 1x PBS with 0.02% EDTA to reach a cell density of 1 x 10⁶ cells/ml. 1 ml of the cell suspension was filtered and stored for analysis using 12 x 75 mm Round-Bottom Tubes with Snap Caps containing 35 μ m nylon mesh cell strainers (BD Falcon™). Cells were analyzed using a Becton-Dickinson (BD) LSR II with FACSDiva software in cooperation with the Biopolis Shared Facilities Flow-Cytometry Unit. 50 000 events were analyzed for their GFP content. No Gating was needed. Three independent sets of samples were analyzed. The mean GFP intensity was calculated using the following formula:

$$\bar{X} = \left(\sum X_i \right) / n$$

X stands for the GFP intensity in the population of GFP containing cells $i=1$ to n .

Live Cell Imaging

Around 1x10⁵ cells HeLa cells were seeded on ethanol-flamed 24mm cover slips and incubated overnight. After 12 hours cells were transfected and after another 12 hour incubation cells were transferred to cover slip chambers before mounting onto the Nikon TI-E inverted microscope platform. The Nikon TI-E inverted microscope with a heated stage, humidified CO₂ chamber and motorized X, Y and Z stage used for these experiments were courtesy of SBIC-Nikon Center, Singapore. Images were taken using the GFP channel (protein expression and localization) and the DIC for each cell. Per cover slip 4 different locations were chosen for imaging. After an initial image of each side was taken ($t = 0$ min),

DMEM medium was gently withdrawn and salt solution was added. Thereafter, Images were acquired every 1 min for 30 min. Cells were stained with 15 μ l of 10 μ g/ml diluted Hoescht 33342 dye (nucleus) at the end of the 30 min image capturing process. A separate image was taken including the stained nuclei. The dye could not be used throughout the process because of an increased cell mortality rate. The experiment was repeated 4 times. In total for each construct 16 videos were made. Not all videos could be used. Screening criteria included (i) cells need to look healthy throughout the whole video, (ii) Cells must be in the picture fully throughout the video. Mathematical procedures for processing image data are described in Supplementary File4.

References

References

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