

### Supplementary File for Figure 4

Quantification of NFAT5 movement from the cytoplasm to the nucleus during salt stress

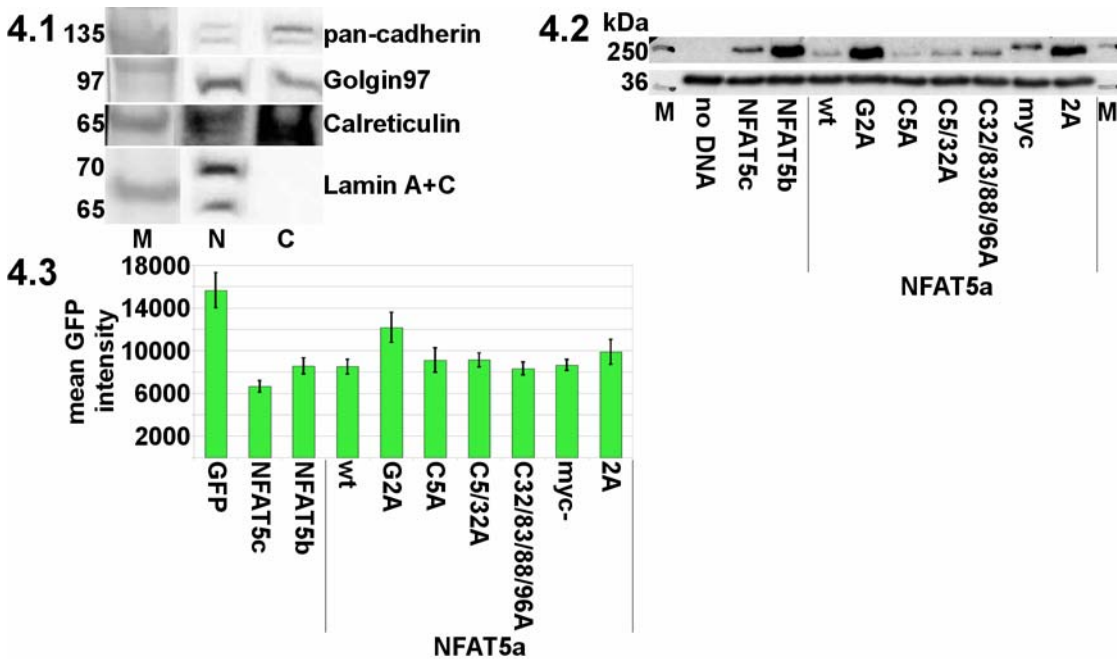
Supplementary figure 4.1: Data on separation of nuclei and cytoplasm

Supplementary figure 4.2: Expression analysis of all constructs by Western blot

Supplementary figure 4.3: GFP expression in single cells via FACS analysis

Supplement for image processing methodology: Computer-based image analysis methodology for the quantification of distribution of labelled NFAT5 forms with regard to subcellular compartments (for fixed cells and for live cell videos)

### Supplementary Figure 4



**Supplementary figure 4.1: Separation of nuclei and cytoplasm**

We followed the procedure as described <sup>1</sup>. The fractions were tested for sufficient separation using the following antibodies pan-Cadherin (PM), Golgin97 (Golgi), Calreticulin (ER) and LaminA+C (Nuclear envelope)(M = HighMark). Besides the LaminA+C bands that are only visible in the nuclei fraction, all other fractions contain Golgi, ER and PM bands. This indicates that the fractions are not clean enough to quantify NFAT5 constructs using this method before and after salt stress. (M – Marker, N – Nucleus, C – cytoplasm). See Supplementary Figure 4.1 above.

**Supplementary figure 4.2: Expression analysis of all constructs by Western blot**

Expression analysis of NFAT5 constructs in HeLa cells: Whole cell extract was prepared as described in Supplement-Methods.pdf. After SDS-Page electrophoresis and Western blotting, GFP antibody was used for detection of protein yield. GAPDH was used as loading control. Equal amounts of GAPDH indicate that similar amount of sample was loaded. The expression of NFAT5b/c and the myristoylation deficient mutants G2A, 2A and myc- of NFAT5a is, as a trend, higher compared to wild-type NFAT5a and the mutants C5A, C5/32A and C32/83/88/96A. See Supplementary Figure 4.2 above.

**Supplementary figure 4.3: GFP expression in single cells via FACS analysis**

FACS analysis was carried out to determine the mean GFP intensity per cell for each construct (determined over 50000 events, see Supplement-Methods.pdf). In general the expression of one construct in a cell population varies from cell to cell (broad Gaussian distribution). The G2A mutant of NFAT5a appears as a clear outlier with about 50% higher expression than other constructs. At the same time, the differences between the other constructs are much less prominent. See Supplementary Figure 4.3 above.

Divergence between the FACS and the Western blot analysis of expression can be expected since one method measures intensity per cell and the other the total amount of expression per plate. To conclude, the results indicate that especially the myristoylation deficient form G2A of NFAT5a is expressed at a markedly higher level than other constructs. Therefore, mathematical normalization of expression of microscopy data is necessary.

**Supplement for image processing methodology: Computer-based image analysis methodology for the quantification of distribution of labelled NFAT5 forms with regard to subcellular compartments**

**Quantitative analysis of confocal microscopy pictures**

For analysis of GFP localization in the cell,  $n$  images of single cells were acquired denoted by  $\{I_1(x,y), I_2(x,y), \dots, I_n(x,y)\}$ , where  $(x,y)$  are the coordinates in spatial domain. Then, nuclei and cells are segmented using EGVD algorithm<sup>2</sup>. Then, the cells were selected based on the transfection rate and health of the cell. The cell segment and nucleus segment were denoted as  $\omega_i^{cell}$  and  $\omega_i^{nucleus}$ . Background regions  $\Omega_{background}$  (regions not occupied by cells or non-transfected cells) were also determined during segmentation. The whole set of images (25-30 cells per condition) can be downloaded from the authors' WWW page associated with this work: [http://mendel.bii.a-star.edu.sg/SEQUENCES/NFAT5\\_2011/](http://mendel.bii.a-star.edu.sg/SEQUENCES/NFAT5_2011/).

It was necessary to apply background correction since the GFP value at the background was not zero. The mean value of the background was obtained based on:

$$\mu_{background} = \frac{\int_{\Omega_{background}} I(x,y) dx dy}{\int_{\Omega_{background}} dx dy} \quad (2)$$

Without background correction, the total concentrations of GFP in a given cell and nucleus are calculated as follows:

$$M_{cell}^{No\ Correction}(i) = \int_{\omega_i^{cell}} I(x,y) dx dy \quad (3)$$

$$M_{nucleus}^{No\ Correction}(i) = \int_{\omega_i^{nucleus}} I(x,y) dx dy \quad (4)$$

The total concentration of GFP in a given cell with background correction is given by:

$$M_{cell}(i) = \int_{\omega_i^{cell}} I(x,y) dx dy - \mu_{background} \times \int_{\omega_i^{cell}} dx dy \quad (5)$$

$M_{cell}(i)$  shows the total amount of GFP in this given cell. The total concentration of GFP in the corresponding nucleus is:

$$M_{nucleus}(i) = \int_{\omega_i^{nucleus}} I(x,y) dx dy - \mu_{background} \times \int_{\omega_i^{nucleus}} dx dy \quad (6)$$

### ***Bioimaging analysis of live cell images***

Each video was screened for healthy and sufficiently GFP expressing cells before the start of the mathematical analysis. To analyze NFAT5 translocation, the set of acquired videos contains  $n$  GFP images captured at different times  $t_i$  ( $i = 1, 2, 3 \dots n$ ). We denote these  $n$  images by  $\{I(x, y, t_1), I(x, y, t_2), I(x, y, t_3) \dots I(x, y, t_n)\}$ , where  $(x, y)$  are the coordinates in spatial domain and  $t_i$  is the coordinate in time domain. The whole set of movies can be downloaded from the authors' WWW page associated with this work: [http://mendel.bii.a-star.edu.sg/SEQUENCES/NFAT5\\_2011/](http://mendel.bii.a-star.edu.sg/SEQUENCES/NFAT5_2011/).

The nuclei and cells were segmented using a touch screen laptop and “Segmentation Editor” of the Fiji package (<http://pacific.mpi-cbg.de/wiki/index.php/Fiji>). The boundary of each nucleus/cell was drawn manually. Since Fiji “Segmentation Editor” has the function of 3-D interpolation, it was not necessary to draw the boundary at each time point. Drawing of the boundary in an interval of 3-7 slices depending on the shape of the nuclei/cells was sufficient. In order to correct for the background, the region of the background and the cells without transfection were also drawn. The mean values of these regions were used to correct the measurements.

After the cell and nucleus at different  $t_i$  were segmented, the cell segment and nucleus

segment were denoted as  $\omega_{t_i}^{cell}(t_i)$  and  $\omega_{t_i}^{nucleus}(t_i)$ . Background regions with and without non-transfected cells were denoted by  $\Omega_{background}$ .

It was necessary to apply background correction since the GFP value at the background was not zero. The mean value of the background and cell background was obtained based on:

$$\mu_{background}(t_i) = \frac{\int_{\Omega_{background}} I(x, y, t_i) dx dy}{\int_{\Omega_{background}} dx dy} \quad (7)$$

Since these values are to be constant at different times  $t_i$ , we used their average over time as background correction denoted by  $\mu_B$ .

Without background correction, the total amounts of GFP in a given cell or nucleus are calculated as follows:

$$M_{cell}^{No\ Correction}(t_i) = \int_{\omega_{t_i}^{cell}} I(x, y, t_i) dx dy \quad (8)$$

$$M_{nucleus}^{No\ Correction}(t_i) = \int_{\omega_{t_i}^{nucleus}} I(x, y, t_i) dx dy \quad (9)$$

The total amount of GFP in a given cell with background correction is given by:

$$M_{cell}(t_i) = \int_{\omega_{t_i}^{cell}} I(x, y, t_i) dx dy - \mu_B \times \int_{\omega_{t_i}^{cell}} dx dy \quad (10)$$

$M_{cell}(t_i)$  shows the total amount of GFP in this given cell at time  $t_i$ . The total concentration of GFP in corresponding nucleus is:

$$M_{nucleus}(t_i) = \int_{\omega_{t_i}^{nucleus}} I(x, y, t_i) dx dy - \mu_B \times \int_{\omega_{t_i}^{nucleus}} dx dy \quad (11)$$

For curve smoothing purposes, we assumed that the amount of GFP remained constant inside a given cell during the time of recording the video and the values  $M_{nucleus}(t_i)$  were

proportionally adjusted based on the average value of total GFP in the cell during recording of the video.

To calculate the relative brightness of GFP in the given nucleus and cell, the following formulas were used:

$$\mu_{GFP}^{nucleus}(t_i) = \frac{M_{nucleus}(t_i)}{\int_{\omega_i^{nucleus}} dx dy} \quad (12)$$

$$\mu_{GFP}^{cell}(t_i) = \frac{M_{cell}(t_i)}{\int_{\omega_i^{cell}} dx dy} \quad (13)$$

Essentially, the value computed in (13) is only a function of time due to possible area changes of the cell under osmotic stress after the total amount of GFP per cell was assumed to be constant (see above). The relative brightness of GFP in the given nucleus and cell is used to demonstrate the translocation of NFAT5, which is calculated using:

$$\gamma_{nucleiV.S.Cell}(t_i) = \frac{\mu_{GFP}^{nucleus}(t_i)}{\mu_{GFP}^{cell}(t_i)} \quad (14)$$

$\gamma_{nucleiV.S.Cell}(t_i)$  stands for the ratio of average GFP concentration in nucleus and cell. This value will increase, if more GFP translocates into the nucleus. In order to compare the relative change of  $\gamma_{nucleiV.S.Cell}(t_i)$ , the initial status of  $\gamma_{nucleiV.S.Cell}(t_1 = 0 \text{ min})$  was taken as a reference:

$$\tilde{\gamma}_{nucleiV.S.Cell}(t_i) = \gamma_{nucleiV.S.Cell}(t_i) - \gamma_{nucleiV.S.Cell}(t_1) \quad (15)$$

## ***References***

1. Estrada-Gelonch A, Aramburu J, Lopez-Rodriguez C. Exclusion of NFAT5 from mitotic chromatin resets its nucleo-cytoplasmic distribution in interphase. *PLoS One*. 2009;4:e7036.
2. Yu W, Lee HK, Hariharan S et al. Evolving generalized Voronoi diagrams for accurate cellular image segmentation. *Cytometry A*. 2010;77:379-386.