Ready, set? Go! Microscopy and image analysis of cell signalling in cell migration
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ANALYSIS OF NUCLEO-CYTOPLASMIC SHUTTLING OF THE PROTO-ONCOGENE SET/I2PP2A

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SUMMARY

SET/I2PP2A is a nuclear protein that was initially identified as an oncogene in human undifferentiated acute myeloid leukemia, fused to the nuclear porin Nup-214. In addition, SET is a potent inhibitor of the phosphatase PP2A. Previously, we proposed a model in which the small GTPase Rac1 recruits SET from the nucleus to the plasma membrane to promote cell migration. This event represents an entirely novel concept in the field of cell migration. Now, fluorescent versions of the SET protein are generated to analyze its nucleo-cytoplasmic shuttling in live cells. Our studies showed that under steady-state conditions a fraction of the SET protein, which is primarily localized in the nucleus, translocates to the cytosol in an apparently random fashion. SET exiting the nucleus was also seen in spreading as well as dividing cells.

We designed an image analysis method to quantify the frequency of nuclear exit of the SET proteins, based on 4D confocal imaging. This straightforward method was validated by analysis of SET wild-type and mutant proteins. This showed that the frequency of nuclear exit of a Ser-9 phosphomimetic mutant (S9E) is enhanced compared to wild type SET or a S9A mutant. Thus, we have developed a novel method to analyze the nucleo-cytoplasmic shuttling of the proto-oncogene SET dynamics in live cells. This method will also be applicable to monitor dynamic localization of other nuclear and/or cytoplasmic signaling proteins.

Keywords

SET (I2PP2A), nucleo-cytoplasmic shuttling, image analysis
INTRODUCTION

Directional cell migration requires tight control of localized signalling by small GTPases of the Rho and Ras families (1-4). These GTPases act as switches and can cycle between an ‘off’-state, when bound to GDP, and an ‘on’ state, when bound to GTP. GDP-bound inactive RhoGTPases associate to a chaperone, RhoGDI (guanine nucleotide dissociation inhibitor) which is a cytosolic protein. GTPase activation by GEFs (Guanine Nucleotide Exchange Factors) is paralleled by dissociation from the GDI and translocation from the cytosol to the plasma membrane where most, if not all, signalling occurs (5,6). Finally, internalisation of membrane domains (7), GAP (GTPase Activating Proteins)-stimulated GTP hydrolysis (8), as well as regulated ubiquitylation and degradation of active GTPases (9) serve to terminate GTPase signalling.

Activated RhoGTPases can bind, as a result of a conformational change that involves the switch I and switch II regions, to effector proteins such as PAK, p21 activated kinase, that in turn stimulate more downstream signalling pathways (ERK, JNK, p38MAPkinase). In addition, Rho GTPases are known for their regulation of the dynamics of the actin cytoskeleton. Whereas RhoA promotes actomyosin-based contraction, CDC42 and Rac1 stimulate Arp2/3 mediated actin polymerization in a highly localized fashion, which results in cell protrusion (10). In addition, RhoGTPases are known to signal to the nucleus to drive cell division and oncogenic transformation (11,12). Activation of nuclear signaling was originally demonstrated by studies on RhoA-dependent activation of the transcription factor SRF (serum-response factor) (13). In later studies, RhoGTPases and their regulators were found to be firmly linked to the regulation of tissue morphogenesis, cell proliferation and ageing as well as transformation and tumorigenesis (11,14).

RhoGTPases are characterized by an effector domain in the N-terminus and a hypervariable region in the C-terminal portion that also harbors the CAAX box, which, after post-translational modification, carries the lipid anchor. Focusing on the RhoGTPase Rac1, our lab has identified a series of interacting proteins that bind the hypervariable region and appear to act as regulators, rather than effectors. Among these, we previously identified the nuclear oncogene SET/I2PP2A (inhibitor 2 of protein phosphatase 2A) as a novel Rac1 binding protein (15). SET is a versatile protein implicated as a template activating factor (TAF-1) in adenovirus replication (16,17). It has also been identified as an inhibitor of histone acetyltransferase (INHAT (18)), an inhibitor of granzyme-A-activated DNAse (GAAD, (19,20)), and as a potent inhibitor of PP2A (I2PP2A (21)). The fusion of SET and CAN (Nup214) was originally identified as a chromosomal translocation (6;9) in acute myeloid leukemia (22,23). SET was found to contribute to Rac1-induced cell migration (15,24), likely as a result of its modulation of kinase signalling pathways. The apparent discrepancy of a nuclear protein implicated in Rac1 signalling at the plasma membrane was solved when it was found that Rac1 activity promotes nuclear exit and membrane targeting of the SET protein, in particular when in its phosphorylated state (15). This initial result was based on analysis of SET localization in fixed cells, in the presence or absence of Rac1 mutant proteins.

Since previous detection of SET translocation were based on end-point assays, we wished to chart SET translocation dynamics in live cells over prolonged periods of time. We therefore performed extensive live-cell imaging studies recording spontaneous nucleo-cytoplasmic shuttling of YFP-tagged SET wild type and -mutant proteins during overnight imaging.
experiments. In addition to recording this translocation by taking Z-stacks in time (4D imaging), we aimed to quantify the translocation behaviour of SET. However, current image analysis methods, for instance aimed at intensity fluctuations of nuclear protein expression (25), do not readily allow the extraction of quantitative data on nucleo-cytoplasmic shuttling. We therefore developed and validated a novel image analysis protocol to analyse SET nucleo-cytoplasmic shuttling in a quantitative fashion in live cells.

MATERIALS AND METHODS

Cell culture and transfection
HeLa cells were maintained at 37°C and 5% CO2 in Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco) enriched with 10% heat inactivated Fetal Calf Serum (FCS, Life Technologies, Breda, The Netherlands) and 100 U/mL penicillin and streptomycin. Cells were passed by trypsinization. Where indicated, cells were allowed to adhere to glass coverslips that were coated with 10µg/ml fibronectin (Sigma) in PBS for 1 h 37°C and washed, prior to cell seeding. HeLa cells were transfected using TransIT®-LT1 Transfection Reagent (Mirus Bio) according to the manufacturers’ recommendations (ratio TransIT:DNA = 3:1), and allowed to produce protein for at least 24 hours.

Expression constructs
SET-mutants were cloned from the myc-tagged vector variants, described previously (15), into the pEYFP(C1) vector, using BamHI and EcoRI restriction enzymes. The sequence was checked using forward primer: 5’-GAGATCGAATTCTTCGGCGCAGGCGGCCA-3’ (Tm=58°C) annealing in SET, and reverse primer: 5’-CTACAAATGTGGTATGGC-3’ (Tm=52°C) annealing in YFP. H2B-mCherry was obtained from AddGene (Plasmid 20972) and included in the experiments as a nuclear marker. H2B is, like SET, a DNA binding protein and the distribution in the nucleus of the two proteins is similar, allowing proper comparison of the images (26,27).

Pull-down assay
Peptides were synthesized on a peptide synthesizer (Syro II) using Fmoc solid phase chemistry at the peptide synthesis facility of the Netherlands Cancer Institute. Peptides encoded a biotinylated protein transduction domain (Biotin-YARAAARQARAG) followed by the 10 amino acids proceeding the CAAX domain of the indicated RhoGTPase peptides. The control peptide (ctrl) is the biotinylated protein transduction domain. Peptide pull-downs were performed as described previously (28). In short: cells were lysed in NP-40 lysis buffer (50mM TRIS/HCl pH 7.5, 100mM NaCl, 10mM MgCl2, 10% glycerol and 1% NP40) supplemented with protease inhibitors (Complete mini EDTA, Roche, Almere, The Netherlands), centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was then incubated with the indicated RhoGTPase C-terminal peptides (5 µg) in the presence of excess streptavidin-coated beads (Sigma) at 4°C for 1 hour while rotating. After washing the beads with NP-40 buffer, proteins were eluted by boiling in SDS-sample buffer and analysed by SDS-PAGE and western blotting.
SDS-Page and Western Blotting
Cells were washed with PBS (supplemented with 1mM CaCl₂ and MgCl₂) and subsequently lysed in NP40 buffer (comprising 50 mM Tris-HCl, 100 mM NaCl, 10 mM MgCl₂, 10% glycerol and 1% NP-40). Lysates were taken up in SDS sample buffer and ran on a 12% polyacrylamide gel. Subsequently, proteins were transferred to a nitrocellulose membrane (Whatman) using a BioRad blotting system at 100V for 1 hour. The blot was blocked with 5% milk powder (ELK, Campina) in TBST (Tris-Buffered Saline Tween-20) and the SET protein was detected using Goat-anti-I2PP2A antibody (Santa Cruz SC-5655) or Mouse-anti-GFP (Clontech 632381). Next, the blots were washed 3 times for 10 minutes in TBST and subsequently incubated with HRP-coupled secondary antibodies (Pierce, dilution 1:5000) in TBST for 1 hour at RT. Finally, blots were washed 3 times with TBST for 20 minutes each and subsequently developed by ECL (GE Healthcare, Hoevelaken, The Netherlands).

Imaging, processing and data analysis
The fusion protein YFP-SET was expressed in HeLa cells by transfection, followed by overnight imaging in a 24-wells glass bottom plate (Zell Kontakt). Microscopic images were taken by a Zeiss LSM 510 confocal microscope, connected to a Zeiss Axiovert 200 microscope body with a Zeiss EC Plan-Neofluar 40x/1.3 Oil DIC objective. To excite YFP, the 514 nm line from an argon laser was used. Z-stacks consisting of five images (512x512 pixels) were taken at several locations (FOVs) within the well at five-minute intervals for a period of 15 hours, using the Multiple Time Series plug-in for the Zeiss LSM software ZEN 2008. Afterwards the images were processed and analysed using ImageJ v1.44f (29,30). For each condition, six fields of view (FOV) were analysed. The exit events per FOV were summed and corrected for the number of cells (approximately 5-10 ROIs/cells per FOV), so the averages per cell as indicated in the figure are from six replicates. Data clearance and processing was done using Microsoft Excel 2002. Statistical testing was done using GraphPad Prism 4. Figures were assembled using Adobe Photoshop CS4.

We measured the mean gray value (MGV) of the nuclear signal before (MGV_before) and after (MGV_after) an exit event in cells where the nuclear signal is not saturated. The ratio MGV_after/MGV_before is a measure for the percentage of the signal that remains in the nucleus. 1-(MGV_after/MGV_before) is then the percentage of SET that exits the nucleus.

RESULTS
SET-Rac1 binding
To allow analysis of SET/I2PP2A nucleo-cytoplasmic shuttling in live cells, fusion proteins of SET and SET mutants with YFP were generated. These proteins were all expressed to equal levels in HeLa cells (Figure 1A). We previously showed using fixed-cell assays that Rac1 activity enhanced the cytoplasmic localization of wild-type SET. In addition, we have shown that SET, in which Ser residue at position 9 is mutated to glutamic acid to mimic phosphorylation, promotes its accumulation in the cytoplasm, as compared to wild-type SET or a S9A mutant (15). To test whether SET phosphorylation would affect its capacity to bind to the hypervariable C-terminus of Rac1, YFP-fusion proteins of wild type (wt) SET, of a mutant SET(A9) that cannot be phosphorylated and the phosphomimetic mutant SET(E9) were expressed. By means of
a pull-down assay the binding to these proteins to the Rac1 C-terminus was tested. The data show that YFP-SETwt as well as YFP-SET(A9) and YFP-SET(E9) all bind equally well to the Rac1 C-terminus (Figure 1A) suggesting that SET phosphorylation does not affect Rac1 binding. Binding to the ctrl peptide was minimal, demonstrating the specificity of the interaction, in line with our earlier findings (15).

To document SET translocation from the nucleus to the cytosol in live cells, we co-transfected with YFP-SET with mCherry-Histone 2B (as a nuclear marker) and imaged the transfected cells overnight with a time interval of 5 minutes. Under these conditions, the localisation of YFP-SET is primarily nuclear, colocalizing with mCherry-H2B (Figure 1B). However, at random moments, a portion of the SET protein was found to exit the nucleus towards the cytoplasm in a seemingly spontaneous fashion. The fraction of the nuclear SET protein exiting ranged from 19-80% and averaged 38.7 ± 6.1 % (mean ± SEM, n=11). Typical still images from one of the movies are shown with the arrow marking the exit events (Figure 1B, Supplemental movie S1). The analysis also shows that SET exiting the nucleus is not a rapidly reversible event and the translocated portion of the SET protein remained cytoplasmic for >15 hours (see also below).

It is unclear what signal triggers SET translocation towards the cytoplasm in resting cells. In spontaneously dividing cells, it was found that SET exits the nucleus when the cells round up,
possibly because the nuclear membrane is broken down during mitosis (Figure 2A). After cell division SET exiting the nucleus is again seen in one of the two daughter cells, in parallel with the post-division cell spreading (Supplemental movies 2 and 3). In the other cell, SET remains nuclear indicating that the translocation event is transient and reversible and not the result of an artefactual loss of nuclear membrane integrity.

In our previous work, it was found that expression of an activated version of Rac1 promotes nuclear exit of SET (15). To test if specific conditions during which Rac1 is activated, can promote SET translocation, YFP-SET expressing cells were seeded on fibronectin-coated coverslips in order to activate Rac1 (31) and SET localization was monitored. These experiments showed that SET indeed translocates from the nucleus to the cytoplasm during the initial spreading phase (Figure 2B). This is also in line with the exit of SET observed after cell division, when the divided cells spread again. In summary, SET shows nucleocytoplasmic shuttling in a random fashion.

**FIGURE 2.** YFP-SET shuttling in cell spreading and cell division (A) SET translocation from the nucleus to the cytoplasm before and after cell division (arrows indicate nuclear exit). Scale bar, 50µm. (B) Live-cell imaging of HeLa cells spreading on fibronectin. During cell spreading, a fraction of YFP-SET translocates from the nucleus to the cytoplasm (arrows indicate nuclear exit). The lower panels depict images which are background subtracted, contrast-stretched and where bins are shown in colours, corresponding to the indicated lookup table (LUT).
in resting cells. In addition, both cell spreading on fibronectin as well as spreading after cell division are accompanied by SET exiting the nucleus, correlating with Rac1 activation.

4D Image analysis and quantification

The above analysis, as well as our initial studies regarding SET translocation, was based on the imaging of fixed (15) and live cells (Figure 1, 2) in the absence of any quantification of the translocation event. In an attempt to design a method to quantify SET translocation frequency, a method was developed, based on 4-D image analysis obtained by confocal imaging for prolonged periods of up to 16-18 hours.

The flow-diagram of the image analysis procedure is depicted in Figure 3 using one of the time-lapse movies of spontaneous SET translocation as an example. Cells were transfected with YFP-SET and imaged on a Zeiss LSM confocal system, equipped with a chamber in which humidified air (5% CO2, 37°C) was circulating, creating a stable and even distribution of temperature for the duration of the experiment. Anticipating a slight focal drift during the assay, five slightly overlapping confocal images along the z-axis were taken (optical slice of 3µm and a total depth of 12µm) at every timepoint, thus creating a 4D imaging file (Figure 3A).

The first step is the splitting of the multichannel z-stacks for each channel, in most experiments comprising the YFP-SET and the mCherry H2B (Figure 3A). Subsequently, we generated a maximum intensity projection of the confocal z-slices for each timepoint to convert the 4D image series back to a ‘2.5 D’ image series \((x,y,t)\) (Figure 3B). Next, Gaussian blurring is used to remove the noise and an automatic triangle threshold method is applied (32-34). This triangle method finds the maximal distance between the histogram and a line between the highest peak and the lowest value in the histogram (Figure 3C). The threshold is set on the intercept of this maximal distance line and the x-axis of the histogram (i.e. the split point). As the procedure uses the minimal and maximum values in the histogram, a background subtraction and linear contrast stretch are not required. This part of the procedure results in a stack of thresholded images (Figure 3D).

During most recordings, the cells show a drift in \(x\) or \(y\) direction that precludes direct extraction of data from a Region of Interest (ROI), defined at the start of the image acquisition. In addition, cells at the periphery of the image tended to either move in or out the field of view, thus giving rise to potential artefacts. To solve these problems, the image stacks are led into a translation and rotation procedure (rigid body transformation). The rigid body transformation is an existing algorithm that is used to align MRI-scans (35). Figure 3E shows a side view of an extreme example of cells drifting in the \(x\)-\(y\) plane over time. The first and last slices are indicated by arrows 1 and 2 respectively and the corresponding images are shown below the graph and are indicated E1 and E2. After applying the rigid body transformation, the drift is corrected (Figure 3F). Images of the first and the corrected last plane (arrows 3 and 4, respectively) are included as Figure F3 and F4.

After alignment, several ROIs are selected from each field of view (FOV) with one single cell per ROI. Cells that move out of the field of view are not included in the analysis, because the area of analysis becomes smaller in time as a result of cellular movement, without being related to SET distribution (crossed out in Figure 3F). Cells that went through cell division during the experiment are also not included in the analysis, as a second nucleus in a FOV would give rise to a doubling of the area measured.
FIGURE 3. The image processing and analysis. The image processing procedure starts with splitting the multichannel z-stacks per channel (A) followed by the generation of maximum intensity projections (B). (C,D) Each image in the time-series is Gaussian blurred and thresholded according to the triangle algorithm. The triangle method first searches for the maximum of the histogram (C,1), draws a straight line between this maximum and the maximum pixel value (C,2). It next finds the maximal perpendicular distance between a point on this line and the histogram itself (C,3). The bit value of the histogram at the point of maximal distance is the splitpoint (threshold, C,4). The thresholded image is depicted in D. Scale bar, 50µm. (E,F) Next, stacked images are rotated and translated using the StackReg plugin designed for ImageJ (35). The slices at the arrows in Figure 3E and F are shown as xy-planes in panels marked E1, E2, F3 and F4 respectively. (G) Subsequently, several regions of interest (ROIs) are selected from each field of view (FOV) and the area occupied by each object in a ROI is quantified using ImageJ and the data is transferred to GraphPad Prism. (H) After manual curation (see text) the size of the area is plotted against time and the finite difference of the area is calculated and plotted (I). Changes in occupied surface area above a threshold (defined using H2B-Cherry, dotted line in I) are counted as nuclear exit events. (J) For each FOV, the exit events are summed, and the averages of six FOV per condition are calculated and plotted.
Single frames from four different regions of the cells in Figure 3D are shown in Figure 3G. The area of each ROI is quantified as a function of time, using ImageJ 1.44f (Figure 3H). In addition to the measurement of the area occupied by YFP-SET, the center of mass is tracked and the slice number is added to each measurement in a separate column. At this stage, objects smaller than 100 pixels are discarded, to filter out signal from, e.g., cellular debris. In this step it is still possible that multiple objects are captured in one ROI, due to movement of other cells into the field of measurement. This is solved by drawing the wide-margin ROI around the object (i.e. the cell) of interest and discarding the objects that have pixels touching the border of the ROI. If there are any additional objects left, the largest area is manually selected for further analysis after data transfer.

The collected data is transferred to GraphPad Prism, in which duplicate measurements are selected manually. The largest area of YFP-SET per slice was chosen for further analysis. This selection of the data is required to prevent an overestimation of exit events later on in the analysis. Finally, the measured YFP-SET area (in absolute number of pixels) of a single cell is plotted against time, as a function of the time interval between slices, as depicted in Figure 3H.

To quantify SET nuclear exit events, the difference quotient of the area plot is calculated using the formula below in which $A$ is the area, $t$ is time and $s$ is the slice number of the stack.

$$\frac{dA_s}{dt} = \frac{A_s - A_{s-1}}{t_s - t_{s-1}}$$

In this way, the difference between two consecutive time points is calculated for each time point in every ROI (Figure 3I). Based on the signal of mCherry-H2B included as an internal control for the image acquisition, a difference larger than an arbitrarily set threshold, for which the fluctuations in the H2B signal were used (see below), is classified as an exit event (the dotted line in Figure 3I). These fluctuations in the H2B signal arise from the fact that cells do move and to a certain extent change shape in the course of the experiment. As a result, also the nucleus changes shape, giving rise to small fluctuations in the recordings. Finally, the number of exit events of YFP-tagged SET per FOV are summed and represented in a bar graph (Figure 3J). Since an exit event involves only a fraction of the expressed SET protein that resides in the nucleus, it is possible to record multiple consecutive exit events.

Having established the above method for quantification of SET nuclear exit and its translocation to the cytoplasm, the method was applied to a series of 4D recordings of YFP-SET wt, co-expressed with the nuclear marker mCherry-H2B, in which nuclear exit of SET had been observed. Figures 4A and 4B represent the area analysis of the mCherry H2B and the YFP-SET-wt in the same cell, recorded for a period of over 20 hours. A second example is shown in Figure 4C and 4D. In these examples, it is clear that whereas the SET protein shows a large rise in occupied area corresponding to its exit from the nucleus, the H2B protein shows no dramatic change in occupied area and remains confined to the nucleus. The temporal fluctuations in area occupied by H2B or SET proteins are represented as the finite difference of the area in the corresponding right panels. The H2B trace in Figure 4A shows some increase, but this fluctuation did not exceed 1000 pixels. This value was therefore chosen as an arbitrary limit above which a nuclear exit event was counted. Using this threshold for the SET-transfected cells, one peak in Figure
4B and three peaks in Figure 4D were counted as individual exit events (marked by arrows). The graph of Figure 4B corresponds to the cell which is depicted in the inset of Figure 1B. See also corresponding supplemental movie 1.

Finally, the method was validated by analysing the distribution and translocation of YFP-SET wt and comparing this with the phosphodeficient YFP-SET-A9 and phosphomimetic YFP-SET-E9 mutants. Previous, fixed-cell assays had indicated that SET phosphorylation might promote nuclear exit, based on the analysis of cells transfected with the A9 and E9 mutants (15). The time-lapse analysis for these SET mutants was repeated at least three times, with true duplicates for each experimental condition. This resulted in six independent experiments for each condition (WT, A9 and E9) that were used to calculate an average figure representing the number of SET nuclear exit events per FOV per experimental condition (Figure 5). The phosphomimetic mutant (E9) showed a significantly higher frequency of exit events (about 3-4 fold) as compared to the wild type SET protein. The non-phosphorylatable mutant (S9A) shows no significantly different number of exit events compared to the wt SET.

In conclusion, we have analysed nucleo-cytoplasmic shuttling of the proto-oncogene SET and have developed a method that allows quantification of 4D live-cell imaging in an unbiased fashion. The method was validated using the established SET mutants and will be applicable not only for future studies on the regulation of SET nucleo-cytoplasmic shuttling but also for the analysis of other proteins that shuttle between the nucleus and the cytoplasm.

**DISCUSSION**

The current study presents a method to analyse, in an objective and quantitative fashion, the nucleo-cytoplasmic shuttling of the phosphatase inhibitor SET/I2PP2A. SET is a ubiquitously expressed oncogene that localizes to the nucleus as well as to the cytosol (19,36) of different cell types. The two known SET isoforms (SET A and SET B) are members of the nucleosome assembly protein (NAP) family. The SET NAP domain is flanked by an N-terminal region containing regulatory phosphorylation sites, and a C-terminal acidic region (37). We recently found that the small GTPase Rac1 binds to SET, a finding that was recently confirmed by another laboratory (24). Rac1 requires its hypervariable region to bind to the NAP domain in SET (15). Moreover, SET, as well as its target PP2A, have also been detected in a proteomic screen for proteins associating to a signalling module comprising PAK1- (p21 Activated Kinase, a Rac1 effector protein) and βPIX- (a Rac1 activator) (38). Thus there are several indications that SET is linked to Rac1 signalling. Since we previously found that Rac1 activity promoted accumulation in the cytosol and that SET phosphorylation on Ser 9 is required for this to occur, a method was developed to analyse the dynamics of SET translocation in live cells.

After having confirmed that YFP-tagged SET (wt and Ser 9 mutants) binds with equal efficiency to Rac1, SET localisation was monitored in live-cell experiments. It was found that in resting cells, monitored for prolonged periods of time (up to 20 h), a fraction of the nuclear wt SET protein seemingly spontaneously translocates from the nucleus to the cytosol. We have also observed in cells that were co-transfected with YFP-SET and CFP-Rac1G12V, that upon spontaneous translocation of SET, the pool of Rac1 that resided in the nucleus, also translocated to the cytosol (data not shown). These findings will be elaborated on in a forthcoming
FIGURE 4. Analysis of spontaneous SET nucleo-cytoplasmic shuttling (A) Analysis of mCherry-H2B fluorescence area and corresponding finite difference as a function of time for one region of interest (ROI, i.e. one cell). A limit of difference area value of 1000 is indicated by the dashed line, (based on the maximal fluctuation in the H2B-mCherry signal in this experiment, H2B diff). (B) Corresponding traces of YFP-SET of the same cell as in A. In this cell, one exit event is recorded (arrow). (C) The H2B plot for a second ROI (i.e. cell). (D) Traces of YFP-SET corresponding to the H2B traces in (C). In this cell, three exit-events (arrows) are recorded.
manuscript. In non-dividing cells, the increased cytoplasmic YFP-SET signal remained stable over periods of hours indicating that the translocated proteins did not readily shuttle back to the nucleus. The number of such exit events under resting conditions varied per cell and did not appear to correlate with any specific morphological event. To quantify this behaviour, a method for image analysis was designed that would allow us to better describe the nucleocytoplasmic shuttling of SET.

The method we developed is based on 4D fluorescent imaging of live cells, followed by an image analysis procedure in which reduction of the background, corrections for drift in X, Y and Z directions and selecting ROIs containing single cells are the key first steps. Next, changes in the area covered by the YFP-SET signal over time are measured, followed by the introduction of an area limit which defines a nuclear exit event and which is based on signal fluctuations of the co-transfected H2B protein, included as a nuclear marker. The procedure thus allows quantification of the number of occasions (‘exit events’) during which a significant portion of nuclear SET enters the cytoplasm.

The results show that the method is straightforward and could be validated. It can be seen in the various movies that, whereas the signal for the H2B protein (Figure 4 A, C) does show some fluctuations as a result of morphological changes of the nucleus, these are clearly distinct from the nuclear exit of a portion of SET, which diffuses readily through the cytosol. As a result, the H2B protein can be used to set an arbitrary threshold which discriminates nuclear translocation from morphology-induced fluctuations in the occupied area.

Despite the fact that many proteins are known to shuttle between the cytosol and the nucleus and vice versa, there are only a few methods designed to analyse this in live-cell recordings. Nuclear translocation is usually imaged using fixed cells and quantified by visual inspection or using an intensity profile along the nucleus and cytosol (39). Alternatively, live-cell imaging has been used, and quantification of protein dynamics has been performed using FRAP studies, such as for beta-catenin (40). This method is useful for continuously shuttling proteins but less amenable to spontaneous shuttling as studied here. Recently, a method was published

FIGURE 5. Frequency of exit in SET mutants. The different SET proteins, WT, YFP-SET E9, YFP-SET Ser9 and YFP-SET-A9 were expressed in Hela cells and SET localisation was recorded by 4D imaging. Nucleo-cytoplasmic shuttling of YFP-SET proteins was documented over a 20 hour period and images were processed by the method detailed in Figure 3. On the Y-axis, the normalised number of exit events is shown, relative to the YFP-SET wt protein, which is set at 100%. Student’s t-test was performed between two of three conditions; the difference of SET(S9E) with SET(WT) was significant in the 95% confidence interval.
which measures the signal intensity of a nuclear oscillator protein in time-lapse microscopic images within the confined region of the nucleus (25). This method could perhaps also be used to quantify nuclear transport. For this, the procedure would have to be adjusted to measure the entire cell including the nuclei. By plotting the ratio between nuclear and cellular signal intensity, comparable graphs can be extracted from the recordings similar to our method. But also here, the setting of a threshold which defines nuclear exit is required. In addition, definition of the cells’ entire surface using a putative cytosolic marker may not be trivial.

In addition, a more general tracking method was published recently by Padfield et al. (41). This method is mainly used for analysis of high-throughput experiments, but might turn out to be useful in other applications as well, because it tracks complex behaviour such as mitosis, cell movement, and the entering and exiting the field of view. In addition, the method is able to measure and correct for microscope defocusing and stage shift, which is very useful. Other methods to analyse spatio-temporal signalling events include FRAP (Fluorescence Recovery After Photobleaching) and kinetic analysis, but those techniques are more suitable for the analysis of nuclear protein dynamics; for example the dynamics of polymerases, transcription factors and histones, as reviewed in (42). Those methods are not readily applicable for the analysis of nuclear export or -import. In conclusion, the current method is a straightforward procedure to quantify the dynamics of nucleo-cytoplasmic shuttling of fluorescently tagged proteins in living cells.

Finally, our image analysis procedure was validated using time-lapse recordings of wtSET and the A9 and E9 mutants. In agreement with our earlier findings, the E9 mutant showed a relatively high number of nuclear exit events, up to seven per cell over a ten hour period. The A9 and wt SET proteins remained mostly nuclear, in line with earlier findings. This data therefore shows that the method provides similar data for SET translocation in live cells as obtained using fixed cells, and adds additional relevant information regarding the number of nuclear exit events and the duration of the translocation. Current studies in our lab involve the use of this method to study the induction of SET nucleo-cytoplasmic shuttling by extracellular agonists. Since there are as yet few methods to quantify shuttling of protein to and from the nucleus in live cells, we propose that this method is not specific for SET and may well be used for the analysis of other proteins that show induced or spontaneous translocation between the nucleus and the cytosol.

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