Ready, set? Go! Microscopy and image analysis of cell signalling in cell migration
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Citation for published version (APA):
Lam, B. D. (2013). Ready, set? Go! Microscopy and image analysis of cell signalling in cell migration

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GENERAL INTRODUCTION
AND SCOPE OF THIS THESIS

Humpty Dumpty, Set on a wall,
Humpty Dumpty had a great fall;
All the King’s horses and all the King’s men,
Couldn’t Set Humpty Dumpty up again.

- Walter Crane (Mother Goose’s Nursery Rhymes, 1877)
“Can a biologist fix a radio?” was a question that Lazebnik asked when he was studying apoptosis (Lazebnik, 2002). The reductionist approach of cell biology is: taking cells apart, study the components and their individual interactions, and then putting everything back again. But are we able to fix the cell in this way if something is broken?

Since the Human Genome Project was finished, most of the parts that constitute a cell are known. Firstly the DNA sequences that were derived directly from the results of the project and secondly the proteins that arise from the translation of the coding regions. Prediction of open reading frames results in approximately 21,000 protein-coding genes, which is far less then originally predicted, but there is still no agreement on the exact number of genes due to the large number and size of these open reading frames. Many of these genes code for multiple protein products and thus there is also no final consensus yet on what defines a ‘gene’ (Baetu, 2012). Even less is known about protein interactions in space and time in highly concentrated (100mg/ml protein and total 400mg/ml macromolecule), crowded, environments such as the cytoplasm and the various compartments within a living cell (Gierasch and Gershenson, 2009; Kuhn et al., 2011).

All of live consists of cells, everything smaller than a cell is not alive and cells are only produced from other cells. This is the cell theory, first posed in the 19th century by Theodor Schwann, Matthias Schleiden, and Rudolph Virchow, thereby generating public attention for a finding by Robert Remak (Mazzarello, 1999). The first place to start investigating the smaller components that constitute live in an integrated context is therefore the living cell.

In the development of the cell theory, microscopy has played an important role. In 1665 Robert Hooke, using very primitive microscopes, discovered ‘cells’ in cork (Micrographia), and in 1676 Antoni van Leeuwenhoek, who made much better microscopes, described ‘animalcules’ (little animals) in various preparations, including the bacteria that make up the biofilm of dental plaque. Also Jan Swammerdam has contributed importantly to early microscopy with his observation of red blood cells in 1658.

According to Bruce Alberts (known for his work in science public policy and as an original author of Molecular Biology of the Cell), we have underestimated the complexity of cells. He states that a new generation of cell biologists should approach the cell as being a collection of protein machines, not as a collection of individual proteins. This requires an engineering point-of-view which is more quantitative than is common in most of cell biology (Alberts, 1998). “We need to focus more on how information is managed in living systems and how this brings about higher-level biological phenomena”, writes Paul Nurse in ‘Life, logic and information” (Nurse, 2008). Complexity results in emergence, system properties that are not obviously inherent from the individual components. Cell migration is one of those emergent phenomena to study.

As it was in the past, microscopy is still the tool of choice for detailed analysis of cellular components and cellular behavior. Developments in single-cell analysis have produced better imaging probes to visualize individual proteins and advanced microscopy, including super-resolution techniques to study single molecule dynamics in living cells (Maxmen, 2011). Furthermore, microscopy is likely to become the ‘vital cornerstone’ of systems biology, facilitating the attempt to study complex and dynamic cellular systems at high resolution, both in space and time. Image analysis is thereby providing the information from which multi-parametric quantitative data can be extracted for use in cell models (Lock and Stromblad, 2010).
CELL MIGRATION AND THE RHO GTPASES

Cell migration is a widely used mechanism in cell biology, essential for processes such as embryonal development, wound healing and the immune response. Cells migrate by making protrusions at the leading edge of the cell and contractions at the rear of the cell (Ridley et al., 2003). This is a result of an integration of internal and external signals which activate complex signal transduction cascades. These give rise to highly dynamic and localized remodeling of the actin and microtubule cytoskeleton, cell-cell and cell-substrate interactions. Understanding these processes requires a multidisciplinary approach combining biochemical, biophysical and mathematical modeling of signaling networks and filaments to whole cells (Carlsson and Sept, 2008).

Classically, the ‘main switches’ of cell migration are the small GTPases of the Rho family. These were originally identified as such in two ‘landmark’ papers by Anne Ridley and Alan Hall in Cell (Ridley et al., 1992; Ridley and Hall, 1992), showing that Rac1 and Rho regulate different aspects and structures within the actin cytoskeleton. RhoGTPases act as ‘switches’, cycling between an ‘off’ (i.e. GDP-bound) and an ‘on’ (i.e. GTP-bound) state. The intrinsic GTPase activity for most small GTPases is very low, allowing the protein to maintain one of the binary states over longer periods of time. The switch from the off – to – on state and vice versa is controlled by GEFs and GAPs (Bos et al., 2007). GEFs exchange the bound GDP for a GTP, inducing a rather large conformational change, which turns the GTPase switch to ‘on’. GAPs lower the activation energy for GTPase activity, thereby promoting GTP hydrolysis, turning the molecular switch back to the ‘off’ state. In the GTP-bound (active) state, the GTPase is able to relay extracellular signals to downstream effectors, depending on its own sub-cellular localization as well as that of the interaction partners, which are in turn regulated at multiple levels by localization, phosphorylation, etc. (Symons and Settleman, 2000).

The initial hypothesis regarding the role of Rac1 and RhoA in cell motility has been that Rac1 predominantly acts at the migrating front of the cell and RhoA at the contracting rear (Ridley et al., 2003). However, recent research has uncovered a more subtle interplay between Rho and Rac1, for instance in the formation of membrane ruffles in the leading edge of cells. For example, an unexpected RhoA activity was also detected in the leading edge of cells, contrasting the prevailing dogma (Pertz et al., 2006). Thus, these studies have provided a more complicated picture of Rho GTPase signaling (Pertz et al., 2006; Hodgson et al., 2008; Machacek et al., 2009). For a more extensive overview of the regulation of Rac1 signaling, including an overview of the specific role for its hypervariable C-terminus, see Chapter 2 of this thesis.

SET/I2PP2A

As detailed further in Chapter 2, the Rac1 hypervariable region has been found over the past 10-15 years to associate to a growing number of proteins and protein complexes, that are all in different ways relevant for activation, signaling as well as inactivation and degradation of Rac1 (van Hennik et al., 2003; Klooster et al., 2006; ten Klooster et al., 2007; Nethe et al., 2010; van Duijn et al., 2010; de Kreuk et al., 2011; Nethe et al., 2012; Saci et al., 2011; Modha et al., 2008). One of these interaction partners of Rac1 that was found in our laboratory is the proto-oncogene SET (ten Klooster et al., 2007). SET was first discovered in acute undifferentiated leukemia (AUL)
as a fusion protein with the nucleoporin CAN (Nup214) (von Lindern et al., 1992). The group which found this chromosomal translocation first identified the protein tyrosine kinase ABL as a fusion to CAN. Subsequently, this ‘new’ gene was called after the brother of ‘Abel’ in the Bible, which is ‘Seth’ (von Lindern et al., 1992). The SET-CAN fusion protein disorganizes nuclear export by mislocalizing the exporter hCRM1 (Saito et al., 2004) and this probably inhibits proliferation and differentiation of human promonocytic U937T cells (Kandilci et al., 2004).

Next to ‘protein SET’, the protein is also known as TAF-1, template activating factor in adenovirus replication (Nagata et al., 1995), INHAT, inhibitor of acetyl transferase (Beresford et al., 2001), IGAAD, inhibitor of granzyme A activated DNAse (Fan et al., 2003) and I2PP2A, because it is a potent inhibitor of phosphatase 2A (Li et al., 1995; Li et al., 1996). Furthermore, SET regulates p53 acetylation (Kim et al., 2012) and it was suggested that it regulates the Akt/PTEN pathway which plays an important role in embryonal development and cell migration (Leopoldino et al., 2012). However, as SET is such a multifunctional protein, it is hard to judge how overexpression or knockdown of the protein will influence different intracellular pathways simultaneously, bypassing or obscuring physiological signaling. Yet, as shown by us and others, SET is a highly dynamic protein that can translocate from the nucleus to the cytosol and vice versa. In line with this, SET appears to exert relevant functions on many pathways in both the nucleus (e.g. transcriptional regulation) as well as the cytosol (e.g. inhibition of PP2A).

Protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase, composed of three subunits (Perrotti and Neviani, 2008). It is a ubiquitous protein, amongst others involved in dephosphorylation of G-protein coupled chemokine receptors, inactivation of Akt, regulation of Rac activity and modulates EGF-receptor signaling (Basu, 2011). Phosphatases have initially been overlooked in (cancer) cell signaling, but they play an important role in controlling both the amplitude and duration of MAP-kinase signaling (Hornberg et al., 2005). Furthermore, it has become clear recently that phosphatases also are part complex signaling cascades in which successive phosphatases regulate each other (Jailkhani et al., 2011).

NUCLEO-CYTOPLASMATIC SHUTTLING

The nuclear pore complex (NPC) is a macromolecular complex with a diameter of 120 nm. The pore itself has a functional diameter of 50 nm, spanning the nuclear membrane with a depth of 90 nm. It is constructed from 30 different proteins, including the natively unfolded FG (phenylalanine – glycine)-repeat proteins that form the permeability barrier, in which the phenylalanine is acting as a molecular ‘peddle’ and the glycine facilitates flexible movement. The NPC is a highly dynamic, crowded environment. The complex is constantly loaded with cargo while providing selectivity for cargo based on unfolded proteins. Moreover, multiple nucleoporins have been found to have additional functions, for instance as transcription factors (Grunwald and Singer, 2012).

Current knowledge suggests that changes in the nuclear envelope environment can propagate alterations in cell signaling, as well as control transcription, cell migration and polarity, genome stability and cell division. Recent findings support the view that the nuclear envelope is a site where many aspects of cell function are coordinated. Nuclear envelope components have
vital roles in this environment as well as — through dynamic spatial and temporal regulation — other sites in the cell. For example the LINC-complex links the cytoskeleton to the nucleus; overexpression of lamin A results in down-regulation of E-cadherin and ERK-phosphorylation regulates Nup214 decreasing its interaction with importin-β (Chow et al., 2012). There is increasing evidence that many types of cytoskeletal proteins are not only present in the cytoplasm, but also localize to the nucleus, suggestive for their direct involvement in the transmission of nuclear signaling and the regulation of nuclear functions (Kumeta et al., 2012). Also focal adhesion proteins such as zyxin, paxillin and the tyrosine kinase Abl are found in the nucleus where they contribute to the regulation of gene expression (Hervy et al., 2006). SET was originally found as a fusion protein to CAN (Nup214), a nuclear porin, but it is unknown if SET is involved in regulation of the nuclear pore complex.

**MICROSCOPY AND IMAGE ANALYSIS**

The main tool used for the research described in this thesis is confocal laser scanning microscopy. With this technique, a sample is scanned pixel by pixel using a laser beam steered by galvanic mirrors. Emission fluorescence light is passed along a dichroic mirror and an emission filter onto a photomultiplier tube located behind a pinhole. The pinhole blocks all the out-of-focus light from hitting the detector, enabling optical slicing of the specimen. To extract quantitative data from such microscopic images, image analysis is needed. Recently, some intriguing examples are shown, also in the field of cell signaling and cell migration. One example is the work of Claire Waterman (Kanchanawong et al., 2010), who used iPALM (interferometric Photo-Activated Localization Microscopy) to study the integrin adhesome. In PALM, photoactivation is used to gain resolution by recalculating the center of a diffraction-limited spot from one single fluorescent molecule. Using interference of the signal from two axial objectives via dichroic mirrors onto three cameras makes it possible to precisely measure the z-position of the diffraction-limited spot. In this way the orientation of talin within integrin clusters could be addressed, which was debated based on previous conflicting, biochemical data. Another example comes from the work of Klaus Hahn and co-workers, who used GTPase biosensors to quantify the coordinated activation of RhoA, Rac1 and Cdc42 in a 0.9 µm wide peripheral area at cell protrusions (Machacek et al., 2009). By calculating the correlation between GTPase activity and edge protrusion, they could construct a model involving sequential activation of Rho, Rac1 and Cdc42 which was different from the consensus in the field as described in the previous paragraph “Cell migration and the Rho GTPases”.

A third example is an approach to use FLIM-FRET (Fluorescence Lifetime Imaging Microscopy – Forster Resonance Energy Transfer) for imaging the Raichu-Rac1 probes by Enrico Gratton (Digman et al., 2008). Raichu probes measures the activity of Rac1 by sensing the interaction of Rac1 with a portion of the Rac1 effector PAK included as a ligand in a single-chain construct (YFP-PAK-Rac1-CFP-tail) (Itoh et al., 2002; Nakamura et al., 2005; Nakamura et al., 2006; Nakamura and Matsuda, 2009). The donor lifetime shortens when the energy is transferred to the acceptor, which can be measured in the time domain by single photon counting, or in the frequency domain by frequency modulation of the excitation light. The Phasor-analysis is a way to simplify lifetime plots by plotting them as vectors to quickly quantify
the contribution of different fluorochrome species in a single pixel. This saves computation time because it is not required to do a multi-exponential fit on the lifetime decay curves.

Finally, Victor Small suggested in 2010 that lamellipodia might not be as much branched as we used to think (Urban et al., 2010; Small, 2010). The technique he used to prove this conclusion was electron tomography, but to address this question further, super-resolution techniques will be useful. In 2012, the same group used correlated light and electron microscopy (CLEM) to find that branched actin is found further from the edge of migrating cells, probably acting to initiate and maintain a network of filaments (Vinzenz et al., 2012). In these various ways, new technology can provide new insights in existing dogmas. These examples show that the combination of microscopy and image analysis is fruitful to further developments, to break the diffraction limit (as in super-resolution techniques), to address spatio-temporal signaling behavior in a cellular context and to resolve features that cannot be seen by eye (as in FRET analysis and tracking of actin cables).
**SCOPE OF THIS THESIS**

In this thesis, two proteins are investigated in detail. This is firstly the small GTPase Rac1, a central regulator of cytoskeletal dynamics, cell adhesion and migration. Secondly, we studied the proto-oncogene SET which we previously found to associate to the hypervariable region (HVR) in the Rac1 C-terminus. SET is not the only protein that binds to the Rac1 HVR. In fact, we and others have found >10 different proteins that bind to Rac1 through this domain. This is unexpected, as the Rac1 HVR, which harbors a polybasic region was initially suggested to act as a charge-dependent membrane association domain. In **Chapter 2**, we present a review of the various Rac1 C-terminal interaction partners and their function in the cell, supporting the view that also Rac1 is indeed “more than a simple switch” (Symons and Settleman, 2000).

In **Chapter 3**, we describe the dynamics by which SET is recruited from the nucleus into the cytosol. To analyse the spontaneous nucleo-cytoplasmatic shuttling dynamics of SET, we have developed an image analysis method based on live-cell imaging of SET, fused to a fluorescent protein (e.g. YFP). Using this approach, we show that a fraction of nuclear SET shows spontaneous shuttling to the cytosol. In addition, we show that a phosphomimetic mutation at position Ser9 in the SET N-terminus increases the number of times a fraction of SET exits the nucleus. This data provides quantitative support for previous findings and also validates the new image analysis method. Moreover, this analysis provides more insight in the spatiotemporal behavior of this protein.

Having established that SET can readily translocate from the nucleus, we invested in identifying compounds or stimuli that would promote the nuclear exit of SET. The results of these experiments are presented in **Chapter 4**. Here we analyze, using the newly developed image analysis method, the dynamic interplay between SET and Rac1 in live cells. Moreover we describe our finding that the immunosuppressant FTY720 (also known as Fingolimod) induces rapid nuclear exit of SET, concomitant with nuclear exit of Rac1.

Previous work from our group has indicated that SET, once in the cytosol, could cooperate with Rac1 in the induction of cell motility. In **Chapter 5**, we further investigated the relevance of cytoplasmatic SET in cell migration, by making use of a SET mutant with a mutation in the cryptic nuclear localization signal. In addition, we have used additional image analysis approaches for the quantification of cell migration, both using a scratch assay and by kymograph analysis of membrane ruffles to further define the contribution of non-nuclear SET to cell motility.

In **Chapter 6** we address the question whether the position of the SET-binding region in Rac1, the Rac1 C-terminus is important for Rac1 activation and signaling by constructing a GFP moiety in front of the HVR of Rac1. This way, the HVR is dislocated relative to the more N-terminal effector domain and the core G-domain in general. These experiments showed that Rac1 with a dislocated, but otherwise normal HVR no longer localizes properly and shows impaired signaling. These findings underscore the more recent notion that the hypervariable C-terminal domains do in fact form an integral part of Rho-like GTPases, controlling not only membrane anchoring, but also activation by GEFs as well as the capacity for downstream signaling. Finally, in **Chapter 7**, the findings described in this thesis are briefly summarized and discussed.
REFERENCES


