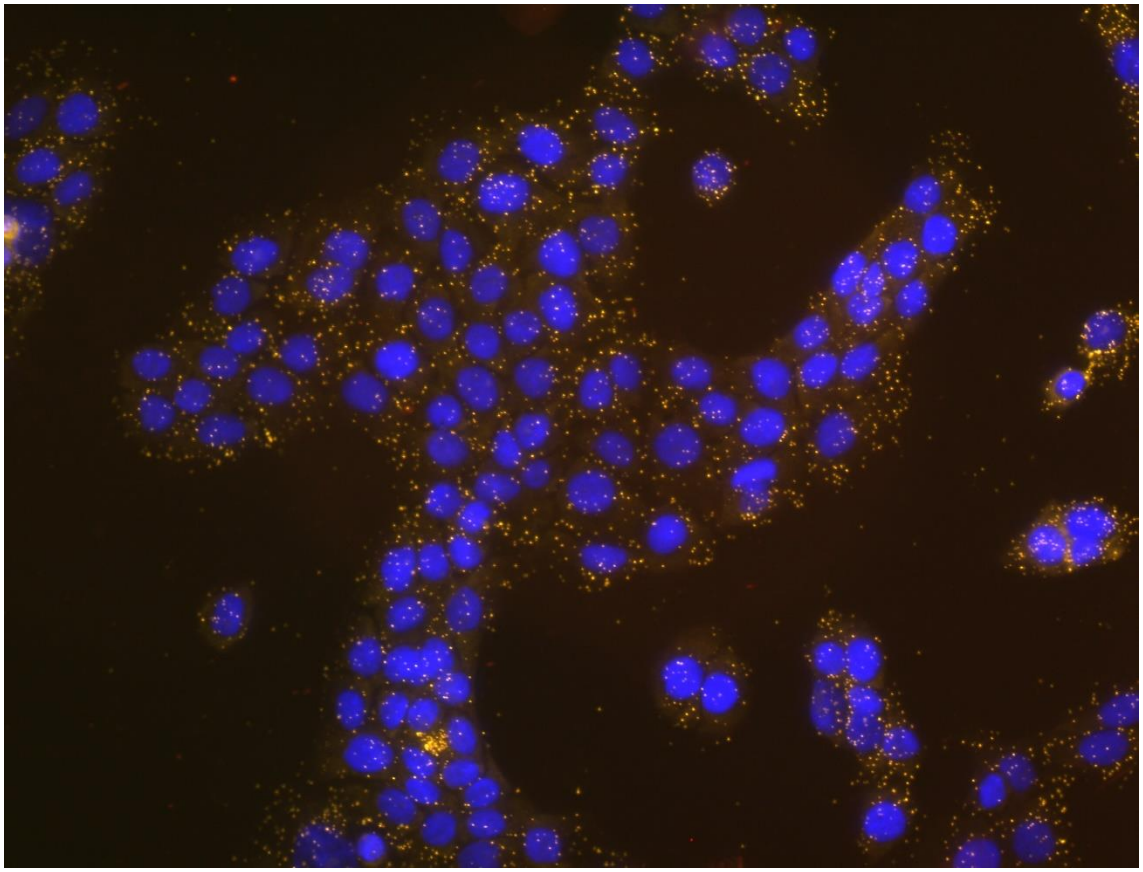
Protein tyrosine phosphatase interacting protein 51 during migration in HaCat cells

Inaugural dissertation
to obtain the Degree of Doctor of Medicine
at the Faculty of Medicine
at the Justus-Liebig-University Gießen



Submitted by
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Gießen 2021

Survival comes and goes with migration!

This thesis shows the importance of the Protein Tyrosine Phosphatase Interacting Protein 51 in migrating cells. Migrating cells are essential for wound healing - alias survival - or, on the other hand, are taking a responsible part of a carcinogen progress during metastatic invasion – alias death. Therefore, it is utmost of interest to discover more in detail the involved proteins and the protein-protein-interactions.

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Declaration of authorship (English)

I, Eva Katharina Schubkegel, hereby declare that this graduate thesis and the work presented in is my own work and has been generated by me as the result of my own original research. In addition, I do solemnly swear that neither I nor anybody else has submitted this thesis or parts of it beforehand to obtain credits elsewhere. It was done mainly or wholly while in candidature for the doctoral degree at the Justus-Liebig University in Giessen, Germany, by the Faculty of Anatomy. I have clearly marked and acknowledged all quotations, references, secondary literature and other sources which are listed in the references. The same applies to all charts, diagrams and illustrations as well as to all Internet sources. None of this work has been published before submission or no parts of this work have been published previously. Moreover, I consent to this thesis being electronically stored and sent anonymously in order to be checked for plagiarism. I am aware that this thesis cannot be evaluated and may be graded as a fail if the declaration is not made.

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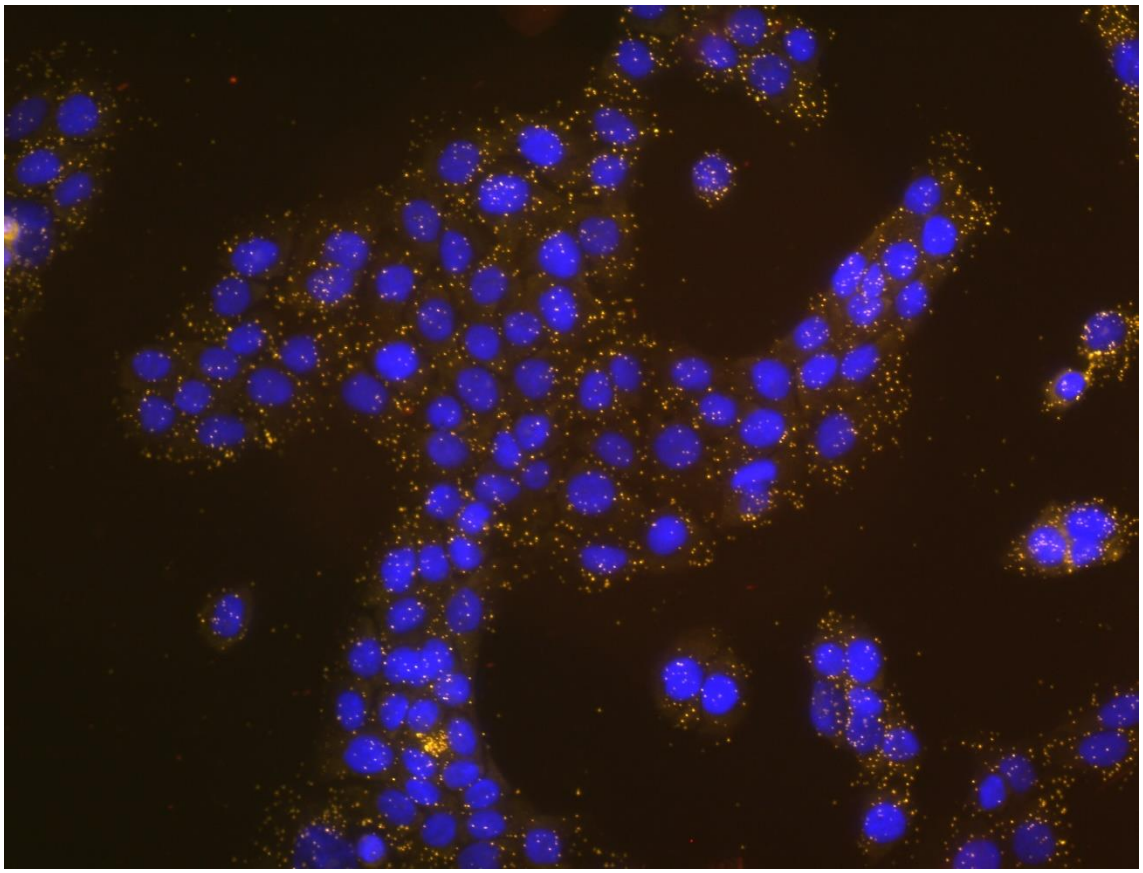
„Willst du dich am Ganzen erquicken, so musst du das Ganze im Kleinsten erblicken.“

Johan Wolfgang von Goethe



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1 Introduction

The human skin protects the body in many ways: it serves as a mechanical barrier against bacteria, toxins, chemical compounds and UV radiation. It is made up of two layers, the epidermis and dermis and it preserves the body temperature, protecting from minor traumata and abrasions during daily activities.
The multicellular epidermis is about 0.1mm thick and has four sublayers – stratum germinativum, stratum spinosum, stratum granulosum and stratum corneum. The cells are attached to each other by tight junctions, desmosomes and hemi-desmosomes. The stratum germinativum is responsible for the continuous cell proliferation, to replace the shredded cornified keratinocytes. On their way to the stratum corneum, the cells differentiate finally. During this process, their characteristic cell organelles, such as the nucleus and mitochondria, dissolve and the cells become keratinized [50]. Due to its barrier function, any injury to the epidermis has to be repaired immediately. As wound healing is an indispensable process, the mechanisms of this process is subject to many scientific studies. Amongst other experimental setups, wound-healing studies are performed with keratinocytes. A prerequisite for wound healing is the ability of keratinocytes to migrate. Thus, keratinocytes also represent a model system for studies of molecular mechanisms and signal transduction in migration.

Suitable cell lines for such functional studies are non-malignant cells representing normal cell signalling. In 1988, Fusenig (et al.) [5] cultured cells from the healthy surroundings of a primary malignant melanoma from a 62-year-old man which spontaneously immortalized. The aneuploid human keratinocyte cell line grows in low calcium and under 37° C temperature. The cells differentiate quite quickly and grow in monolayers. Lower calcium concentrations support the cells to create an adherent monolayer of proliferating keratinocyte single cells [5].
Scratching monolayers is a standard method to study the mechanisms of wound healing and cell migration. Cells are grown to confluency and the monolayer is scratched in a standardized way. Thus, the effects of different agonists on cell migration can be followed in a time-dependent manner. Keratinocytes, adjacent to the gap, can be analysed and compared to the results seen for keratinocytes in the middle of the still continuous monolayer.

1.1 Migration

The migration of cells in the human body is an important mechanism to support healing or to avoid inflammatory progresses. Immediately after an injury of the epidermis, the wound-healing process begins with a coagulation cascade, followed by the forming of a fibrin clot [26]. Hours later, the keratinocytes migrate into the wounded area to restore the intact morphology of the epidermis [39]. In the last number of years, the understanding of keratinocyte migration has been improved tremendously, but there are still open questions, especially concerning signal transduction in the regulation of migration.

In 1996, Lauffenburger and co-workers [44] investigated the physical locomotion across two-dimensional substrata as a coordinated process of *"morphological polarization with membrane extension, formation of cell-substratum attachments, contractile force and traction and release of attachments*". To migrate, cells form an asymmetric structure and reorganize their cytoskeletal components, such as filaments. Following on from this, molecular rearrangements of chemosensory receptors take place in response to migratory stimuli, and lamellipodia and filopodia concentrate and polarize at the cell front on the adhesive expansion region [44] The reorganisation and restructuring of the cytoskeleton filaments are regulated by the activation of specific actin-binding proteins, of signal transductions pathways of membrane receptors, of kinases and phosphatases proteins.

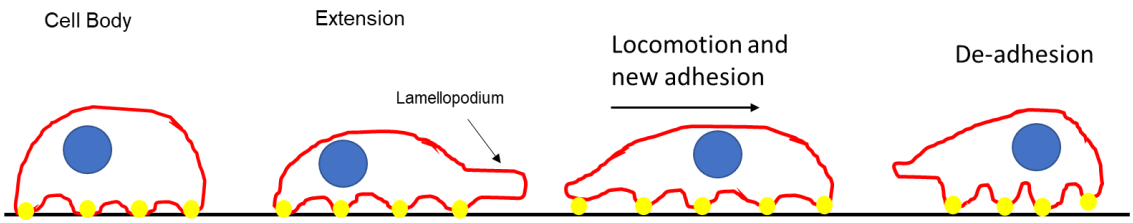


Figure 1: Schematic drawing of migrating cells.

The transduction pathways regulating migration are the well-known TNFα pathway and G-protein coupled signalling, including the Rho family GTPases, such as Rac, which regulate the actin cytoskeleton reorganisation by the formation of the membrane ruffles and the production of lamellipodia [52] [20]. Epidermal growth factor (EGF) receptors and the insulin receptor (IR) are also involved in the locomotion and proliferation of keratinocytes [2] [53].

The inactive epidermal growth factor receptor (EGFR) monomeric, if activated by growth factor ligands, generates an active homodimer [80]. This dimerization stimulates the activity of the intracellular tyrosine-kinase part, followed by the autophosphorylation of several tyrosine residues which activates the signalling of downstream proteins via MAPK (mitogen-activated-protein-kinase) pathway, which works as an “on”- “off” cascade. If EGFR is auto-phosphorylated, receptor-associated proteins (SOS, GBR2) activate the small GTPase Ras, which activates Raf. This protein activates through MEKs (MEK1, MEK2) the Erk1/2 protein complex, which carries the signals into the nucleus for the respective gene activation during the proliferation process [30] (Figure 2).

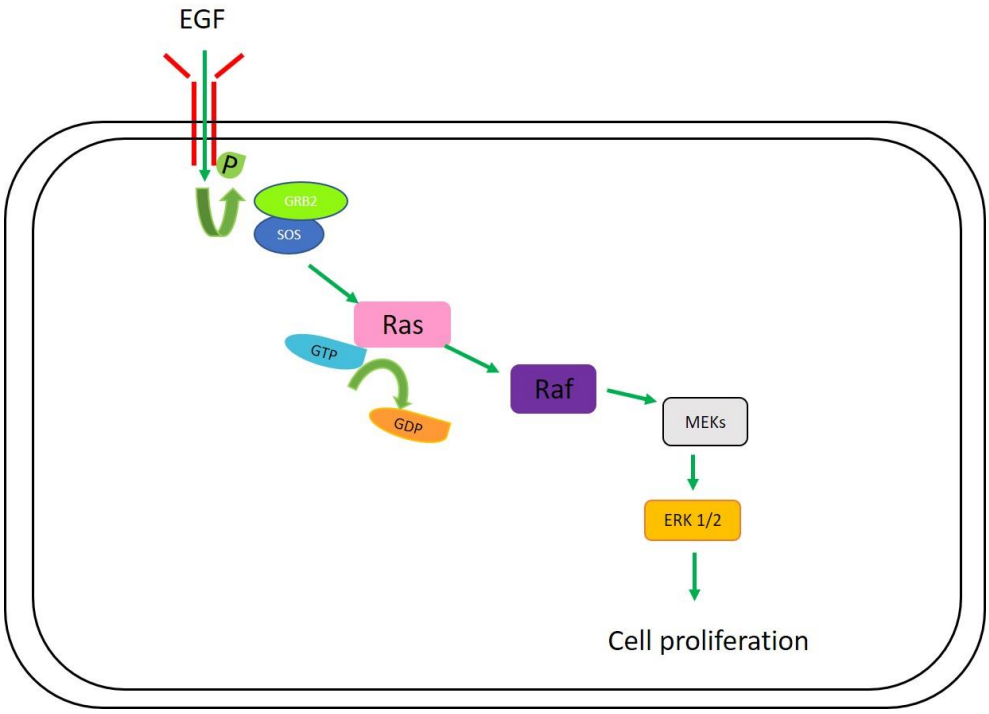


Figure 2: EGFR - MAPK signalling cascade.

Furthermore, the focal adhesion kinase (FAK) gets activated by the EGF. The FAK is part of the focal adhesion complex, which includes the proteins Scr, CAS and praxillin. The tyrosine phosphorylated FAK activates Scr kinase, which in turn activates CAS and praxillin. CAS binds to Crk and the protein dedicator of cytokinesis-180 (DOCK180) gets activated. As a consequence, Rac gets activated. Rac is a regulatory protein through the WASP (Wiskott-Aldrich Syndrome Protein (WAVE)) protein of the cytoskeleton reorganisation [59].

Additionally, FAK is involved in the reorganisation of stress fibres within the locomotion centre, and are thus crucial to the spreading process and creation of adhesive contacts [2]. The phosphorylated FAK and the FAK complex are pivotal for the regulation of downstream pathways in cell spreading, cell movement and stability of focal adhesion and, as a consequence, for cell survival (Figure 3).

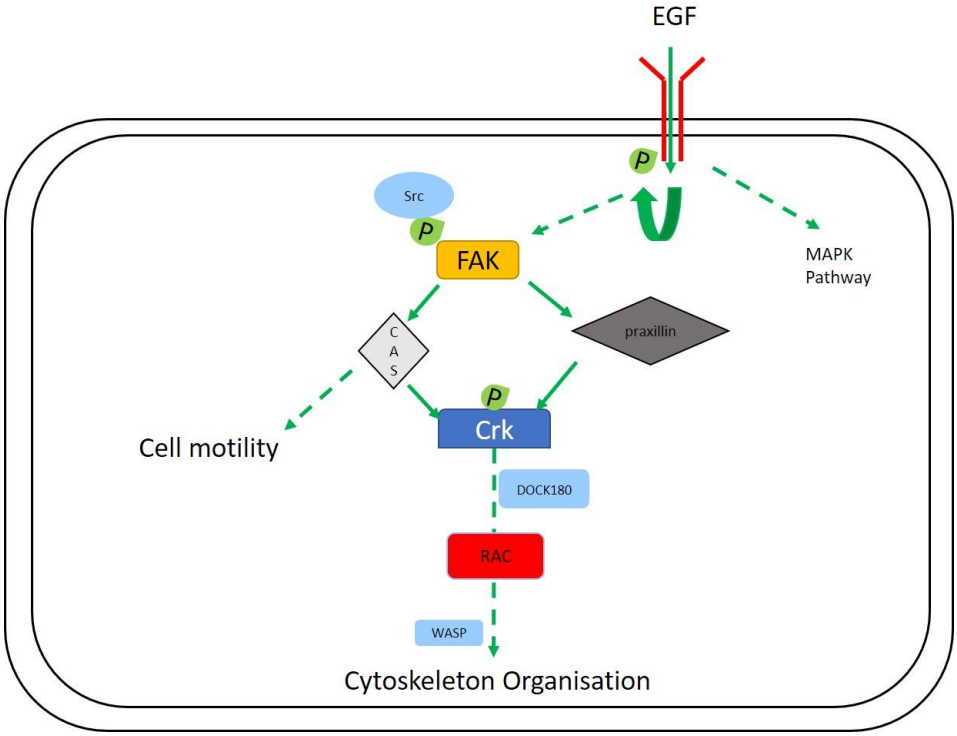


Figure 3: The EGF, FAK and Rac pathway.

Interestingly, FAK also gets activated by the TNFα pathway and by insulin. Insulin, by binding to the receptor, activates pathways which play key roles in growth and the metabolism [56]. It is known that insulin activates the MAPK pathway. The downstream part of the insulin signalling is not exactly known, but the IRS (insulin receptor substrate) members play a key role in the activation of the Akt and the Ras/Raf pathway is involved in the proliferation processes. IRSs probably activate Rac to induce migration [34], and additionally, the protein kinase c (PKC), which plays a critical role in stress fibre formation [24]. To elaborate, insulin regulates the formation of the IRS-1, PKCα and δ, Caveolin-1 and the Integrin α6β4 complex [64]. This complex, including the integrin component, is important to the integrin function and behaviour of keratinocytes during migration.

Choma (et al.) [16] described the importance of integrin (α3β1) in promoting the stabilization of the leading lamellipodia. He assumed that Rac interacts with the α3β1 integrin during this process. Based on these observations, Rac`s possible interaction partners play a crucial role in the investigation of the formation of the cytoskeleton (Figure 4).

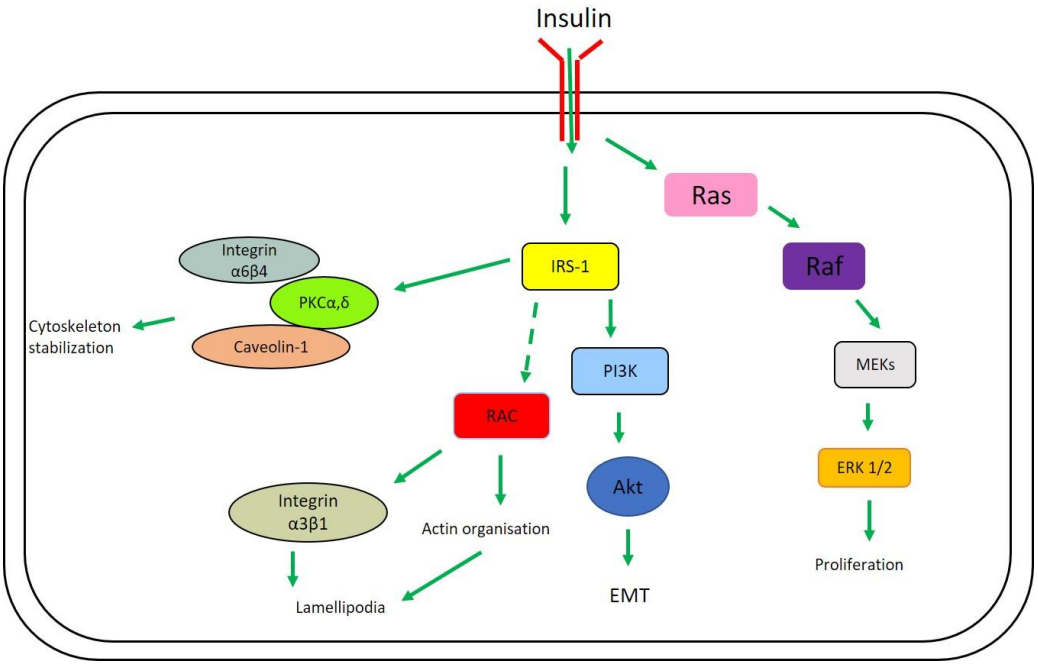


Figure 4: IR downstream signalling.

On the other hand, the MAPK pathway is also activated by the Tumor necrosis factor α (TNFα) through one of the Tumor necrosis factor receptors (TNFR1 and TNFR2). TNFα/ TNFR signalling induces many different signal cascades, depending on the type of cell, which can trigger cell death or migration during the inflammatory response. For example, TNFα induces the early activation of MAPK and, therefore, mediates the signal cascades, resulting in cell migration [25].

TNFα also triggers the snail and β-catenin pathway through the NFκB and AKt intracellular signal transducer for the activation and invasion of tumour cells [79]. Subsequently, the cells proliferate to stabilize the cytoskeleton for adhesion and movement (Figure 5).

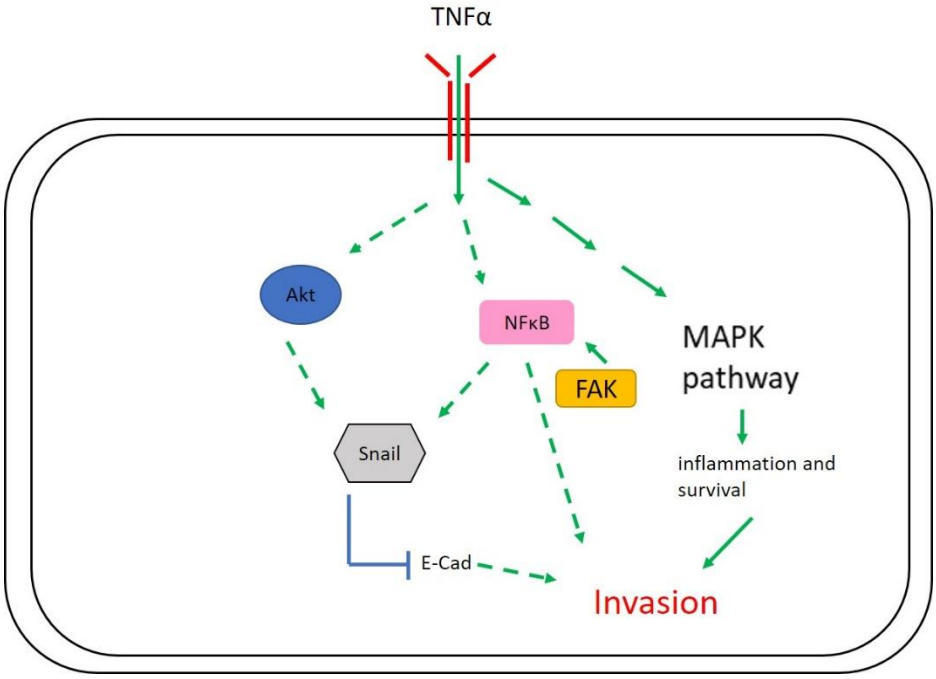


Figure 5: TNFα pathway involved in invasion.

Interestingly, actins are also known to play a crucial role during cell migration, cell development, cell division and gene expression. There are six different isoforms [33] of actin known in human cells, such as alpha 1 & 2, beta and gamma 1 & 2. The beta type especially, which lies in the cytosol or next to the cytoskeleton, plays an important role during locomotion. The β-actin forms filaments which are rapidly assembled and disassembled during the migration process, according to the needs of the cell. The monomer of β-actin, also known as G-actin, polymerizes when ATP (adenosine triphosphate) [59] bands and constructs the F-actin. This F-actin is the important component of the microfilaments.

The actin microfilaments form two ends, one barbed and one pointed end. Both ends add G-actin proteins to increase their length, but on the barbed end, the expansion is faster. During the cell motility, the end elongates while the other contracts. Bunnell (et al.) [13] in 2011 stated that the β-actin in particular takes part in the regulation of the G-pool during the filament organisation (figure 6a and 6b).

In consequence, the flexible organisation and reorganization of the cytoskeleton components are essential during migration, therefore β-actin regulates the assembly. Up to now, the interactome of different cytoskeletal proteins during the assembly and disassembly of the microfilaments is not yet fully evident (Figure 6 a,b).

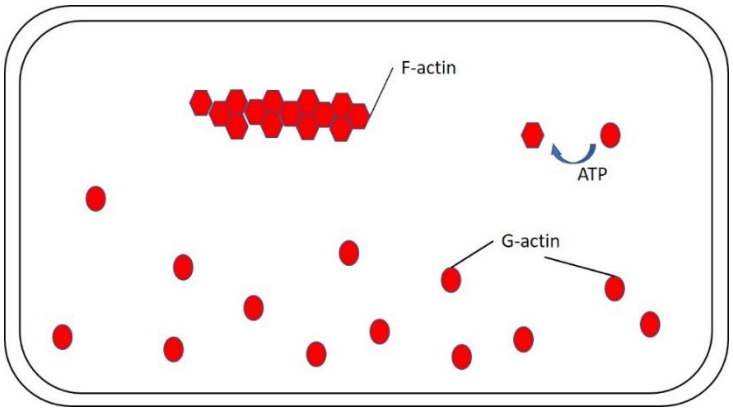


Figure 6 A: Construction of F-actin through polymerisation of G-actin.

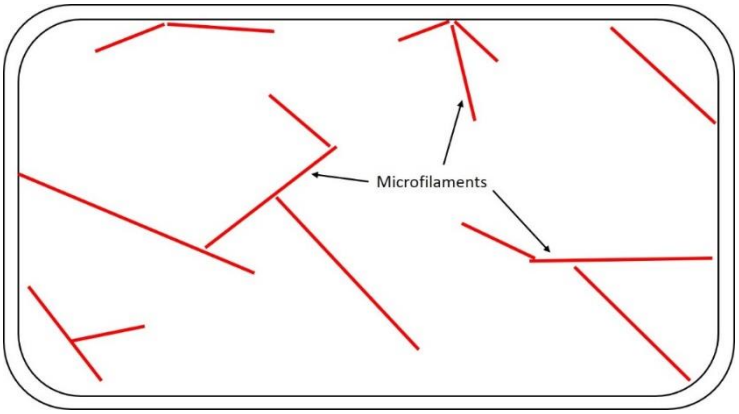


Figure 6 B: Microfilaments as cytoskeleton components.

The increased proliferation and movement of cells can also be seen as an invasive process, comparable to the invasion by metastasizing cells.

Thus, cell migration can be seen as a response to inflammation or as a tumourigenesis occurrence. Both are highly important processes, which make it necessary to study the underlying protein-protein interactions and to disclose therapies supporting wound healing or blocking metastatic invasion. Moreover, a case study in 2020 [69] demonstrated that heart failure in COVID infected patients is linked either to transient viraemia or alternatively to migrated infected macrophage from the lung. In the light of this pandemic, it is extremely important to investigate probably target proteins such as the PTPIP51 during migration.

1.2 Protein tyrosine phosphatase interacting protein 51

The protein tyrosine phosphatase interacting protein (PTPIP51, synonymes: RMD3, FAM82C; FAM82A2 and TCPTP-interacting protein 51) [8] [59], was identified in 2001 by Porsche and colleagues [61] in a yeast two-hybrid screen to identify potential interacting partners for the non-receptor protein tyrosine phosphatase 1B (PTP1B) and the T-cell protein tyrosine phosphatase (Tc-PTP).

The PTPIP51 is known to regulate crucial cellular processes. In mitosis, migration, differentiation, appoptosis and communication between organelles, PTPIP51 acts as a scaffold protein [10].

The gene of the PTPIP51 protein is located at chromosome 15 (15q15.1)(Fig.:7). 12 out of 13 exons encode the protein. The gene spans 19,373 basepairs and encodes a protein of 470 amino acids (aa) (Swiss Prot Q96TC7) with a calcuated molecular mass of 52,118 kDa [68]. Stenzinger (et al.) described several calculated isoforms with molecular masses of 30, 38, 45 and 52 kDa [66].

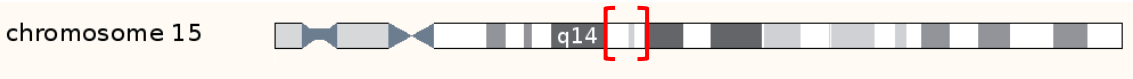


Figure 7: The PTPIP51 gene location on chromosome 15q15.1 [59, 68]

Interestringly, RMD3 is used as a synomen for PTPIP51 and describes the regulation of microtubular dynamics, which point to its function during mitosis. Brobeil and colleagues concluded in 2012 [11] that PTPIP51 forms a complex with the proteins Nuf-2 and CGI-99 at the equatorial region, which leads to the necessary organisation of the microtubule during mitosis.

Tissue expressed patterns of PTPIP51

In 2005, Stenzinger (et al.) [67] analysed the expression pattern of PTPIP51 in various tissues samples. Reproductive tissues, specific in primary spermatocytes, which develop into elongated spermatids in mice, rats and humans, as well as in oocytes at different stages, revealed PTPIP51. In skeletal muscle, PTPIP51 is restricted to type II muscle fibers. For example, all ciliated cells in the uterine tube, trachea, efferent ductules of the testis and ependyma of the brain express PTPIP51 in microtubuli and the basal bodies. Even in neuronal tissue (axons of sensory neurons and axons of motorneurons) PTPIP51 is found.

Additionally, hippocampal neurons express PTPIP51, probably involved in memory processes, as reported by Brobeil and colleagues [12].
A specific expression was reported in liver parenchyma where the PTPIP51 protein was seen in hepatocyte nuclei and in distinct non-parenchymal cells, which were indentified as stellate cells, Kupffer cells and natural killer cells [68]Furthermore, the PTPIP51 protein is also expressed in cells forming the biliary tree [68].
Orieux (et al.) [58] reported a stage dependent PTPIP51 expression in photoreceptors of the retina.

It is noteworthy that PTPIP51 was identified in epidermal tissue specificially associated with the suprabasal keratinocytes, whereas basal cells solely display PTPIP51mRNA.
Every investigated cell line expresses PTPIP51 e.g. glucagon-producing cells (INR1G9), human placental cells (BeWo), Jurkat T-lymphoma cells, cardiomyocytes (HL1) and acetylcholine-producing cells (NS20 Y). Among the tested human keratinocytes, HaCaT cells were also tested postive for PTPIP51.

More studies analysed the PTPIP51 expression in malignant tissues, such as prostate carcinoma [40], myeloid leukemia and in glioblomastoma [51]. Even in keratinocyte carcinomas (human basal cell carcinomas BCCs) and squamous cell carcinomas (SCCs), the expression of PTPIP51 was altered [41]. As stated before, this led to the assumption of a functional implication of PTPIP51 in cellular processes, such as proliferation, differentiation, migration and apoptosis.
Additional projects have analysed the mechanism of PTPIP51 involvement in these cellular processes. Brobeil and colleagues [10] published a review in 2016 on PTPIP51 protein being a scaffold protein which forms multiple protein complexes, thus cross-linking many pathways.
Interestingly, since its first description, PTPIP51 was assumed to be regulated by its phosphorylation level. As reported in the manuscript of Stenzinger and colleagues (2009), the amino acid sequence in PTPIP51 reveals different possible tyrosine and serine phosphorylation sites [68]. Up to now, two key regulatory sites of PTPIP51, tyrosine 176 and serine 212 residue, have been identified [7] [8] [12]
Brobeil and co-workers demonstrated that the functions of PTPIP51 are mediated through protein-protein interactions [8][10], these being regulated by the tyrosine/serine phosphorylation status of PTPIP51. Today the regulation by phosphorylation of the tyrosine 176 residue [7] [11] [12]e.g. by Src kinases, is well understood.

When phosphorylated, PTPIP51 does not interact with the MAPK pathway on the Raf1 level via 14-3-3. Dephosphorylation by PTP1B on the tyrosin 176 residue leads to an activation of MAPK signalling [68]. Beside Tyr 176 as a potential phosphorylation site, serine 212 can be phosphorylated by various serin kinases [8], which activate interactions with different signal transduction pathways. Additionally, in 2018 Dietel (et al.) [19] also investigated the protein kinase A (PKA) phosphorylates the serine residue 46 of PTPIP51 and stimulates the MAPK binding via Raf-1 and 14-3-3 β.

Among the potential tyrosine kinases acting on Tyr 176, two further kinases of the Src family, namely c-Src and Lyn, were identified [6]. The altered phosphorylation status can affect the spindle apparatus during the formation of microtubuli by CGI-99 and Nuf-2 [1]. In addition, PTPIP51 is an interacting partner of the IR and the EGFR. Both interactions depend on PTPIP51 dephosphorylation at the tyrosine 176 residue (Figure 7) [1] [3] [6] [8] [12] [68]. The interaction is possible if the PTPIP51 is dephosphorylated by PTP1B at the tyrosine 176 site and phosphorylated at the serine 212 residue [1] [10]. Raf-1 and 14-3-3 are part of the MAPK pathway and, therefore, PTPIP51 can affect that pathway [81]. So far, the phosphorylation status of PTPIP 51 seems to be the switch for an on-off effect in different protein-protein interactions.

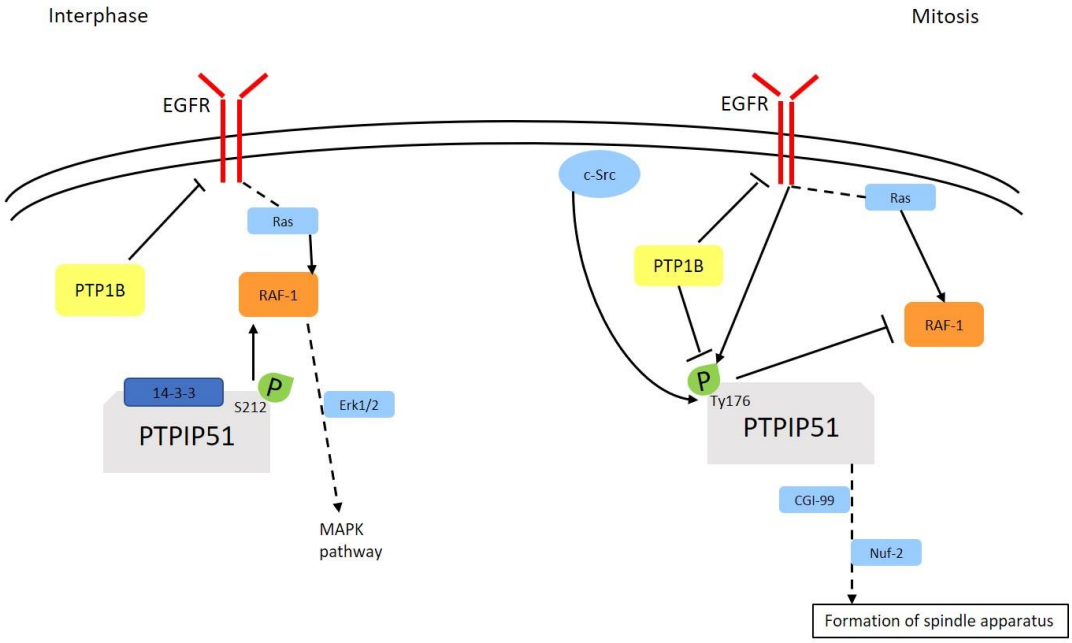


Figure 8: PTPIP51 interactions in the EGFR pathway regulated by different phosphorylation statuses during different stages in cell cycle.

Currently, the effects of PTPIP51 on signal transduction are not investigated to their full extent. The involvement of PTPIP51 in the MAPK pathway has been analysed in more detail. Interestingly, when 14-3-3 is bound, PTPIP51 forms a complex with Raf1 and consequently modulates the MAPK pathway. This complex depends on the phosphorylation status of the Tyr176 residue, if phosphorylated the PTPIP51/14-3-3/Raf1 complex dissociates [7]. When the complex is bound, PTPIP51 activates Raf1 which phosphorylates MEK1/2, which in turn activates Erk1/Erk2. The Erk1/Erk2 substrates transfer the signal into the nucleus and consequently regulate the transcriptional activity of the corresponding genes [30]. These known potential transcription factors, such as NFκB and its inhibitor (IκBa), as well as GSK3β (glycogen synthase kinase 3 β) are verified interaction partners of PTPIP51 [10] [9]. Additionally, GSK3β is known as an interacting partner via the snail pathway, which leads to the blocking of the E-cadherin and, in consequence, dissolving of the cytoskeleton binding [79].

Yet, its role in cytoskeletal remodelling and migration remains enigmatic. The MAPK signalling plays a pivotal role in migration and wound healing. Interestingly, Brobeil (et al.) [12] reported a reverse inhibition of the PTPIP51 interaction with Raf-1 if the EGF forcefully activates the EGFR. In addition, PTPIP51 forms a complex with proteins of the NFκB pathway. The regulator protein of the NFκB pathway, IKK, which is the inhibitor of the kappa B (IκB kinase =IKK), gets activated by the activated TNF receptor and the IKK phosphorylates IκBα. The activated IκBα forms a protein complex with another NFκB protein, the RelA [68] and with PTPIP51 [10]. As a consequence, TNFα decreases the PTPIP51 complex binding, followed by a possible suppression of other NFκB pathway members.

It is noteworthy that Bobrich and co-workers [3] reported a direct interaction between the IR and PTPIP51. The IR phosphorylates PTPIP51 at its tyrosine 176 site, which leads to modifying the MAPK pathway activity via Raf-1 and 14-3-3β [8]. Insulin is known to promote wound healing via chemotactic migration [48]. These facts make it interesting to investigate PTPIP51 expression during insulin treatment and migration.

To sum up, PTPIP51 promotes the formation of the spindle apparatus via the Nuf2/ CGI-99 pathway [11]. Yet, there are also known interactions with different isoforms of dynein. Dynein is an important protein partner within transport processes (eg. cytoplasmic dynein as a microtubule motor protein) [28].

1.3 Aim of the study

Several receptors, such as TNFR, IR and EGFR, can induce migration, if activated. Their downstream signalling can be regulated by PTPIP51. Thus, it is of the utmost interest to analyse the role of PTPIP51 in the process of migration. PTPIP51 exerts its regulatory function by forming protein complexes with various signalling partners. So, important signalling hubs, which control cytoskeleton reorganisation, such as actin polymerisation and the migration promoting protein Rac-1, are examined for their possible interaction with the regulatory protein PTPIP51. Due to the important role of FAK during actin organisation, the study investigates a probable interaction with PTPIP51. To study migration, scratch assays applied to human keratinocytes (namely HaCaT) monolayers are used. The expression of PTPIP51 and its interaction profile is recorded in resting and migrating keratinocytes under different conditions to identify a possible future target for manipulating wound healing or preventing tumourigenesis and metastatic invasion.

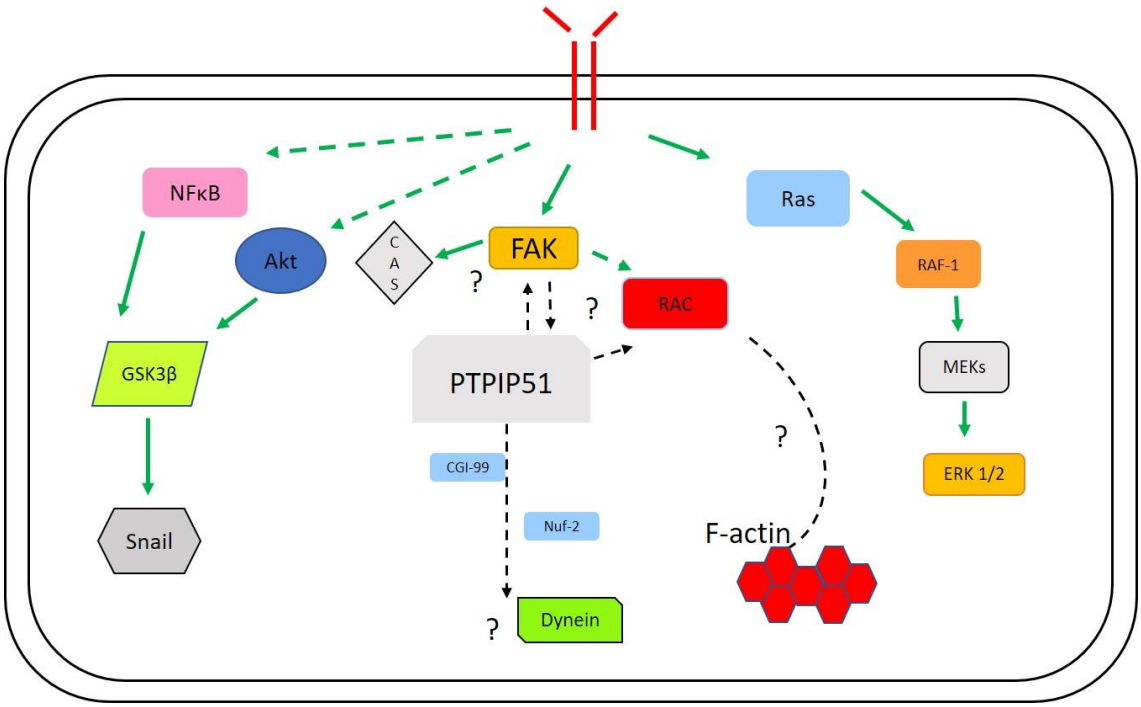


Figure 9: Possible interaction partners of PTPIP51: FAK, Rac-1, ß-actin.

2 Material and methods

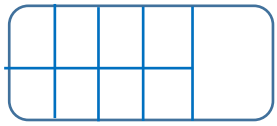
2.1 Methods

Cell culture

In this study, all experiments were performed with the HaCaT cell line, which was kindly provided by Dr. Teschemacher (Department of Pharmacology, Justus-Liebig-University, Gießen, Germany) with the permission of Dr. Fusenig (DKFZ, Heidelberg, Germany) [5, 12]. Before dissemination, culture bottles and culture slides were coated with fetal calf serum (FCS). HaCaT cells were cultured in flasks in a humidity chamber at +37°C and in a 5% CO_2 atmosphere (see Table 1). For the experiments, 25,000 cells per well were seeded on culture slides and grown until they reached near confluency. For treatment, the culture medium was replaced by a medium supplemented with the corresponding effector. Insulin experiments were performed in a concentration of 5µg/ml and EGF experiments were performed in a concentration of 1µg/ml. LDC-3 was applied in concentrations of 5µM, 50µM, 100µM diluted in culture medium. Additionally, scratched wells were treated with increasing concentrations of effectors.

Figure 10: Object slides used in this study:

Culture slide



Culture slide with scratch

Duolink Proximity Ligation Assay (DPLA)

The Duolink II PLA Probe kit was used for the detection of the in situ interactions between PTPIP51 and the examined interaction partner (see Table 2 and user manual [49]). A signal is exclusively generated if both PLA probes have bonded and both proteins are in a proximity closer than 40 nm. Each signal marks an interaction between both proteins [23].

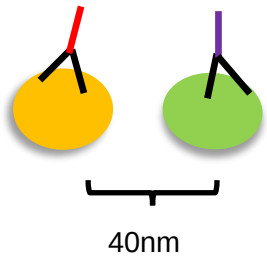
Fixation of the cells:

To conclude the fixation experiment, the medium was dripped off the wells and each well was washed three times for 10 minutes with 300µl PBS buffer under a continuous rotating movement of the culture slides (Swip Akku by Edmund Bühler, ID: 1045389) in 90/min horizontal circulations. After washing, excess buffer was removed manually and the cells were fixated by 300µl methanol per well (Sigma-Aldrich/Lot# SZBD142SV). Following on, the culture slides were incubated in a deep freezer (-20°C) for about 10 minutes. Then the methanol was allowed to run off and the slides were dried at room temperature.

PLA probes and primary antibodies:

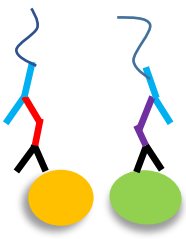
The fixated cells were pre-incubated with 50µl blocking agent (DPLA-kit) in a pre-heated humidity chamber for 30 minutes at + 37°C to avoid unspecific binding of the primary antibodies. PTPIP 51 and either Rac-1, FAK and β-actin, (see Tab.2) were diluted to a suitable concentration in the antibody solution. For each well, 100µl of the antibody solution were added after the blocking step. The slides were incubated overnight in a humidity chamber under constant movement (ST 5 CAT/neoLab®/ID: 2017039).

Figure 11: Primary antibody reaction.



To detect mouse and rabbit antibodies, Duolink II PLA probes were diluted 1:5 in antibody solution or in PBS buffer and 10 µl was applied to each well. The culture slides were incubated for 1 hour at +37°C in a pre-heated humidity chamber.

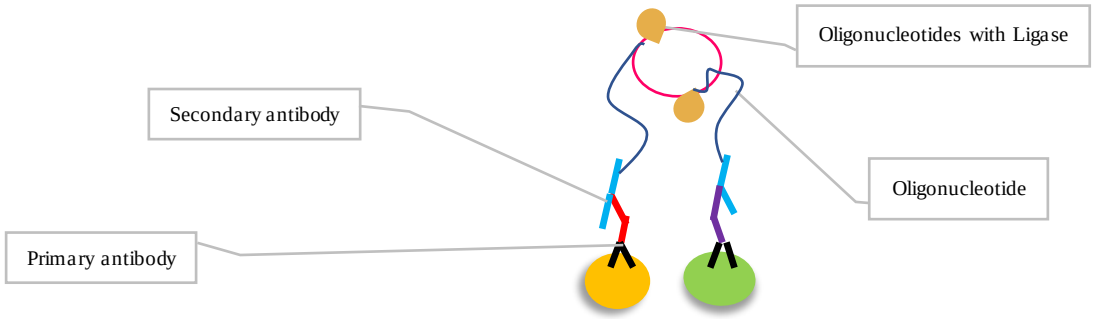
Figure 12: Secondary mouse and rabbit PLA probe reaction.



Ligation and amplification:

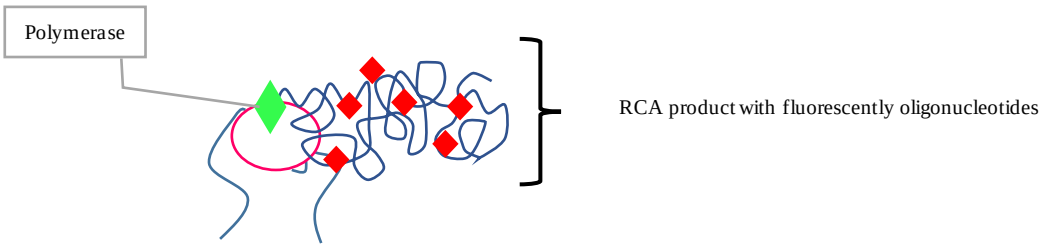
The slides were washed twice with Buffer A (see Tab 1) for 5 minutes under gentle rotation (Swip Akku by Edmund Bühler, ID: 1045389 in 90/min horizontal circulations). The ligation stock was diluted with high purity water in a concentration 1:5. After the drying process, the ligase was added in a 1:40 dilution to the ligation medium and then the ligation-ligase solution (10µl) was applied carefully. The slides were incubated in a pre-heated humidity chamber for 30 minutes at +37°C. The ligase will form a closed circle by hybridizing PLA probes, if the two proteins are closer proximity than 40 nm.

Figure:13 Ligation of the two PLA probes forming a closed circle.



The ligase reaction was stopped and the slides were washed twice with Buffer A for 2 minutes. The signal was amplified with fluorescence labelled oligonucleotides using the amplification stock diluted 1:5 in highly purified water. The reaction was started by adding the polymerase (1:80). The 10µl amplification-polymerase solution was added to each sample. The culture slides were incubated for 100 minutes in a pre-heated humidity chamber at +37°C. The ligated circle forms a template for the rolling-circle amplification (RCA) reaction, using the PLA oligonucleotides as primers. The fluorescent capabilities of a certain part of these specific oligonucleotides will mark the RCA product, resulting in a distinct fluorescent dot under the microscope, each dot representing one interaction.

Figure:14 Rolling circle amplification and fluorescence labelling.



The following steps were performed in the dark. The slides were washed twice with Buffer B for 10 minutes. Following on, the slides were washed in Buffer B (1:100 in high purity water) for 1 minute. Nuclei were visualized by DAPI (working solution, Table 1). The slides were washed for 10 minutes with the PBS buffer and dried at room temperature then the cover slips were mounted with Mowiol (listed above).

Polyclonal PTPIP51 Antibody

The polyclonal PTPIP51 rabbit antibody was produced as described by Stenzinger (et al.) 2005 [67] and Brobeil (et al.) 2012 [11]:
The cDNA sequence encoding aa 131-470 of the PTPIP51 protein was inserted into the BamHI and HindIII sites of the plasmid pQE30 and expressed as His6-tagged protein in a transferred into the protease-deficient Escherichia coli strain AD202. The protein was purified by chromatography through a Ni-Agarose gel column [61]. Rabbits were immunized with 0.5mg of the purified protein in 0.5 ml RIBI adjuvant [38] by the boosted injection method (booster injections with 0.5 and 0.3 mg on days 14 and 21, respectively). After achieving immunity to the pTPIP51 protein, the antiserum was collected on day 28 according, to the method described by J B Olmsted [37], to produce monospecific antibodies. Subsequently the monospecific antibodies were purified and a SDS (sodium dodecyl sulphate) electrophoresis was performed, blotting 2mg of the purified antigen. The protein band was marked with Ponceau solution and extracted. The membrane was blocked with 1% low fat milk powder in phosphate-buffered saline solution and incubated together with the antiserum. After extensive washing, the antibodies were diluted with 0.2 M glycine (pH 2.0) for 2 minutes, followed by immediate neutralization with 1 M Triethanolamine. The specificity of the PTPIP51 antibody was tested by ELISA and by immunoblotting of the isolated purified recombinant protein staining bands with 52 kDa, 34 kDa and 30 kDa. Immunoblotting of homogenates from porcine spleen tissue revealed bands of 48 kDa, 40 kDa, and 29 kDa [18]. The antibody bonds to the EGFP fusion PTPIP51 protein expressed in HEK293 [19]. Preabsorbing the PTPIP51 antibody against its antigen completely abolished the immune reaction in all tested tissues.

Immunohistochemistry

Immunohistochemistry was performed as previously described by Koch et al [42]. The cells were fixed (see also 3.2.3). Nonspecific binding sites were blocked with the blocking solution as described above. The primary antibodies were diluted in PBS 0,1M 7,4 pH with bovine serum albumin (5%), normal goat serum (5%) and cold-water fish skin gelatine (0,1%) (see Table 1). The primary antibody solution (100µl) was applied to each well and the samples were incubated overnight in a humidity chamber under constant movement. After washing the slides with the PBS buffer three times for 10 minutes, the samples were incubated with a corresponding secondary antibody and 5% DAPI solution in PBS at room temperature for 1hour in the dark. Thereafter, the slides were washed three times with the PBS buffer and covered with Mowiol and analysed.

Fluorescence Microscopy

The fluorescence reactions were visualized and documented by the Axioplan 2 fluorescence microscope, equipped with Plan-Apochromat lenses (Carls Zeiss, Jena, Germany) for photo documentation.
For the visualization of the secondary antibody Alexa Fluor 488 anti-mouse IgG, an excitation filter with a range of 460-500nm and an emissions filter with a spectrum of 512-542 was used. The secondary antibody Alexa Fluor 555 anti-rabbit IgG antibodies were visualized with an excitation filter with a range of 530-560 nm and an emissions filter with a spectrum of 565-640 nm.
To illustrate the RCA product of Duolink II Ligation, an excitation/emission filter for Cy3 in the respective wavelengths was used [49].

Invert Reflected Light Microscopy

To visualize native cells e.g. in culture bottles and in culture slides an invert reflected light microscope (Leica DM IRB, ID: 2034888, Germany) equipped with a 5x/0.12 N PLAN objective with a tube lens prism module fixed at 1.6x was used. The scratches were measured by an ocular (10x/25) containing a metric scale.

Semi quantification of Immunofluorescence Intensities

Intensity correlation analysis was carried out by using the Image J tool (v 1.46r) [21] within an appropriate plug-in package for intensity correlation analysis of the Wright cell imaging facility [1, 45].

Fluorescence analysis of Duolink® Ligation Assay

The fluorescent dots, representing the interactions, are documented and evaluated by the Duolink Image tool [57]. The images were imported and the ImageTool analysed the signals with a fixed cytoplasm- and product size. The data gained was analysed by Graph Pad Prism 6 [29]

2.2 Compounds

LDC-3

The small molecule LDC-3 was synthesized by the Lead Discovery Center GmbH, Dortmund, Germany. Brobeil (et al.) [9] [10], investigated LDC-3 as a molecule which strongly enhances the affinity of the PTPIP51 to MAPK-binding, regardless of its phosphorylation status. In these studies, LDC-3 seems to force PTPIP51 into its MAPK protein complex and stabilizes the interactions of PTPIP51 with 14-3-3 and Raf-1. The forcing and stabilizing effect to PTPIP51 protein complexes indicates LDC-3 is an interesting binding molecule to the wound-healing pathways.

Insulin

The peptide hormone, produced by beta cells of the pancreatic islets, insulin is a very important anabolic hormone. When insulin binds to its receptor, the monomers form a homodimer which promotes the autophosphorylation of the receptor followed by the phosphorylation of proteins inside the cell, also known as insulin receptor substrates (IRS). The phosphorylation initiates a signal transcription cascade which activates other kinases and transcription factors within the MAPK pathway. Through binding phosphatidylinositol 3-kinase (PI-3), the IRS-PI-3 kinase complex activates other proteins inside the cell, which activates further important downstream processes, such as lipogenesis and gluconeogenesis [31]. Bobrich (et al.) [3] investigated the IR (insulin receptor) as a new interacting partner with PTPIP51 in 2013. Subsequently, insulin is an interesting compound for treatment of the HaCaT cell during the scratch experiments.

EGF

The epidermal growth factor is a single protein consisting of 53 amino acid residues and three intramolecular disulphide bonds between the six cysteine components. EGF was discovered by Cohen in 1962 and he found it during experiments with mouse salivary gland extract that induced the opening of eyelids and eruption of teeth in new-born mice, so he named it the tooth-lid factor [17]. In addition, urogastrone, which was isolated from human urine and later on identified as human EGF, has similar characteristics to mouse EGF [15, 36, 78]. Up to now, many studies were performed about EGF, EGF receptor characteristics and EGF family members, such as transforming growth factor α (TGFα), heparin-binding EGF like growth factor (HB-EGF), amphiregulin (AR) and betacellilin (BTC) [78]. When binding to its receptor, EGF stimulates the proliferation of the DNA through MAPK pathway. Up to now, this target (MAPK pathway) is an interesting source to investigate during the development of cancer [35].

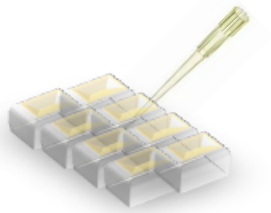
EGF plays a crucial role during the proliferation process, cell growth and, in addition, it is important for migration (as described above) during the early phase of the wound-healing process. These characteristics of EGF make it interesting for usage as a compound in this study.

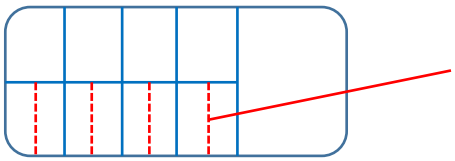
2.3 Scratch protocol

An *in vitro* scratch assay [46] was used to measure cell migration and the effects of cell-cell and cell-matrix interactions. Additionally, the scratch protocol was performed to investigate effects in intracellular processes during wound healing *in vitro* and to capture images at the beginning and at regular intervals during cell migration. In this study, the scratch assay was performed in HaCaT cells to investigate the protein-protein interactions in the pathways with PTPIP51 and its respective interaction partners which link to the cytoskeleton pathways during cell migration.
The protocol was performed similar to the protocol used by Chun-Chi Liang [46], but there were several modifications introduced, such as the Duolink and immunohistochemistry assays after the scratch assay.

Procedure:

1. HaCaT cells were cultured as described above and were seeded on the culture slides with 25,000 cells per well.
2. Cells were cultured until they were near to confluency.
3. Each well was vertically scratched by a 200 µl pipette tip.





4. Detached cells were removed by washing the wells three times with culture medium (Tab. 1).
5. Culture slides were treated with EGF, insulin and LDC-3 in different concentrations diluted with culture medium.
6. The gaps were measured by means of the inverted reflected-light microscope and by an ocular (10x/25) containing a metric scale (see 2.1).
7. The slides were incubated in a humid chamber at +37°C and in a 5% CO_2 atmosphere.
8. The gap was repeatedly measured at defined timed intervals.
9. When one or more gaps were closed, the treatment was stopped and cells were fixed (see above).
10. Duolink or immunohistochemistry investigation started (see above).

After the fixation of the cells, the gaps were measured again to control if cells had been lost during the fixation process.

2.4 Material

The materials and their suppliers used in this study are given in Table 1 and Table 2.

Table 1: Material and suppliers

Product/Solution	Manufacture	#Lot/ #CAT/ Art.-no.	Dilution/Solution components
Trypsin EDTA solution	Sigma Life Science	# SLBP1360V	
Culture medium	Gibco or Biochrom GmbH Sigma Life Science	#618-70-010 or # FG1215 #P11-010 #084M3281	RPMI (Rosswell Park Memorial Institute) 1640 medium *Supplemented with:* Penicillin (1%) Fetal calf Serum (10%)
Buffer PBS	Merck Roth	#A829973 #4984.3 #3957.1	2,72g KH_2HPO_4 14,24g $Na_2HPO_4x2H_2O$ 9g NaCl
Buffer A	Roth Sigma Life Science Merck Milli-Q-Anlage	#3957.1 #48552 #P2287-500ML #HC765757 #4004320	8,8g NaCl 1,2g TRIS base 0,5ml Tween 20 HCl 1000ml high purity water
Buffer B	Roth	#3957.1	5,84g NaCl 24,23g TRIS base, HCl 1000ml high purity water
Mowiol	Calbiochem	#475904	2.4g Mowiol 6ml high purity water 12ml 0.2 M TRIS base 6g Glycerol
Duolink II® In Situ kits (DPLA)	Sigma-Aldrich Olink Bioscience (inclusive user manual, which is described in 3.2.2)	 #A61006.1 #A60502 #A53602.2.2	(=PLA-Probe and Blocking solution) anti-Rabbit Minus anti-Mouse Plus Detection Reagents (=Ligation stock and Ligase, Amplification stock and Polymerase
Falcon® products		#2095 #354108	Small tubes Culture slides

Product/Solution	Manufacture	#Lot/ #CAT/ Art.-no.	Dilution/Solution components
Tips	Starlabgroup TipOne®		20µl, 200µl, 1000µl
Pipettes by	CellStar®		5ml, 10ml, 25ml
Nalgene Cryo-tubes		#500-0020	
Nunc Easy Flask™ 75cm^2		#148093	(Culture bottle)
DAPI 4',6-Diamidin-2'-phenylindol-dihydrochlorid	Cell biology Boehringer Mannheim	# 236276	Stock solution: high purity water and DAPI pulver Concentration: 1-5mg/ml Working solution: Methanol and stock solution: Concentration 1µg/ml

Table 2 List of antibodies used for DPLA and immunohistochemistry and compounds:

Antibodies	Immunogen	Antibody source	Dilution	Manufacturer
PTPIP51	Human recombinant PTPIP51 protein encoding amino acids (aa) 13-470	Rabbit polyclonal	1:500	Prof. HW Hofer, Biochemical Department, Univerity Konstanz, Germany
FAK(H-1)		Mouse monoclonal	1:100	Santa Cruz Biotechnology #C0713
Rac1	clone 23A8	Mouse monoclonal	1:200	Millipore #2585802
Anti-β-Actin		Mouse monoclonal	1: 500	Sigma Aldrich # A5441
Alexa Fluor anti-mouse IgG 488		Goat	1:800	Life technologies # A11029
Alexa Fluor anti-rabbit IgG 555		Goat	1:800	Life technologies #A21428
EGF	Epidermal Growth Factor human, recombinant in E.coli		1:1000	Sigma Aldrich # E9644
Insulin	Human recombinant	Insulin solution Human	1:2000	Sigma Aldrich # 19278

3 Results

The aim of this study was to analyse the involvement of PTPIP51 in the regulation of migration. Therefore, PTPIP51 interactions with signal proteins of migration were investigated as described in the introduction. The interaction partners Rac-1, β-actin and the FAK, which are all part of the regulatory processes in proliferation and migration, were addressed.

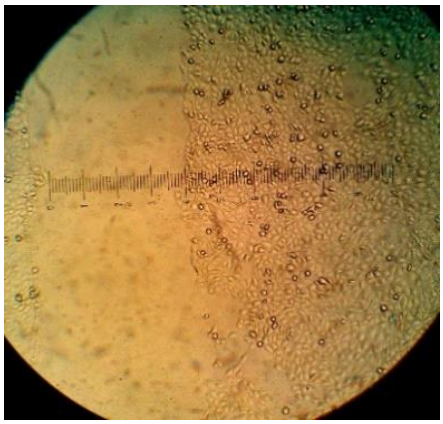
The study was performed with a spontaneously immortalized human keratinocyte cell line (HaCaT). These cells were grown as an adherent monolayer. Migration velocities and the associated interactomes were analysed with scratches of intact monolayers to induce migration and re-epithelialization. The corresponding PTPIP51 interactome was investigated by the Duolink Proximity Ligation Assay. The interactions were analysed in cells from the ***migration zone (sc)*** and, in addition, in cells from the ***proliferation zone*** in the neighbourhood of the scratch. These cells were not directly affected by the scratch, but were influenced indirectly (***non-scratched*** cells/ ***n-s***). Concerning the scratch protocol, the respective control groups were taken from the unscratched monolayers within separate wells named as (***zero scratched*** group/ ***0)***. Additional control groups (in this study addressed as ***(control)***) consisted of cells which were taken from the migration zone (sc), the proliferation zone(n-s) and the zero zone (0) which were not exposed to any reagent *but* were grown only in ***medium***. Apart from this experimental setup, a second approach analysed the effects of different growth factors such as EGF, insulin or LDC-3. The data was collected by the Software Duolink® Ligation Assay Tool and analysed by Graph Pad Prism 6 (GraphPad Software©, La Jolla, CA, USA) and the Mann-Whitney-U-Test. Additionally, immunostaining was used to investigate the semi-quantification of the PTPIP51 interactome with the respective interacting partners and measurements of the gab took place.

Here, the scratch was applied slowly, in order to partly leave the monolayer uninjured and to avoid the cell demolition of the wound rims. Compared to unscratched cells, there was no significant impact described in this study on cells surrounding the scratch, yet the migrating cells displayed a significant increase of the PTPIP51 interactome for FAK, Rac-1 and β-actin (see Fig 17 B).

Measurements of the width of the gap detected a cell count loss (detached and a few migrated cells within the wound bed were partly lost) after the fixation process. In the eighth hour, the wound rims were almost closed so that preparation were started for the DPLA and immunochemistry experiment.

The measurements of the gap width are demonstrated in Figure 15 B.

A



B

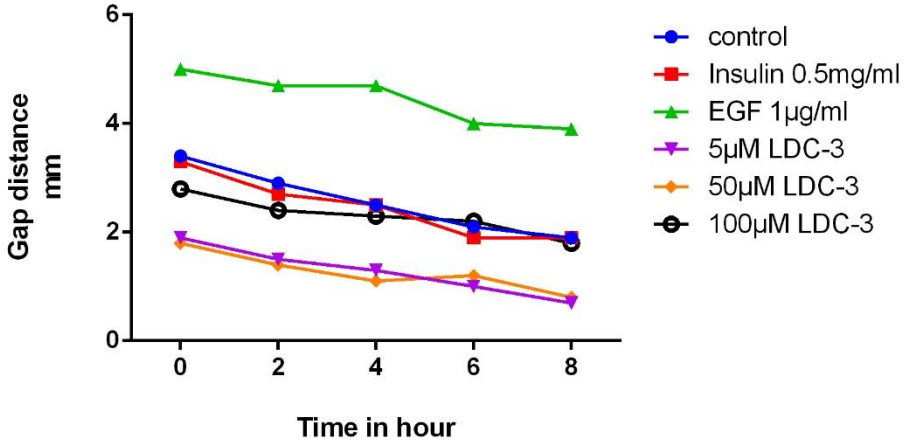


Figure 15 A: Example photo of the scratched HaCaT monolayer by inverted reflected-light microscopy.

15 B: Measurements of the scratch gap taken every two hours under treatment with LDC-3 (5µM, 50µM, 100µM concentration), insulin and EGF and under controlled conditions. After these measurements, cells were fixated before the DPLA or immunostaining experiments started.

C

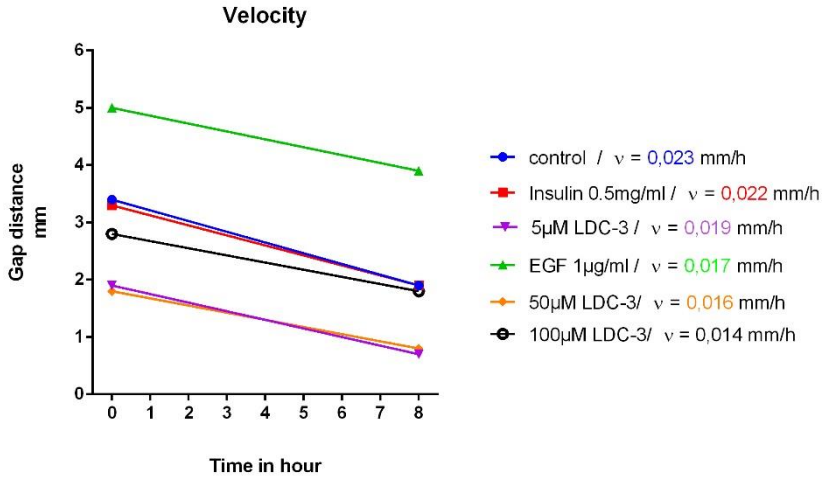
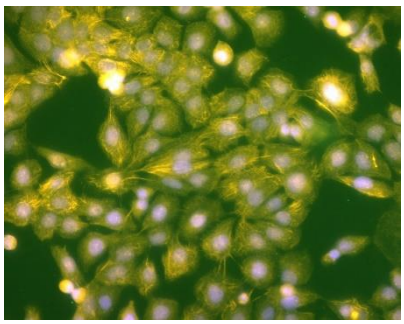


Figure 15 C: Mathematical velocity of migrating HaCaT cells. Collected data of gap width measurements with metric scale (see Figure 15 A) by inverted reflected-light microscopy.

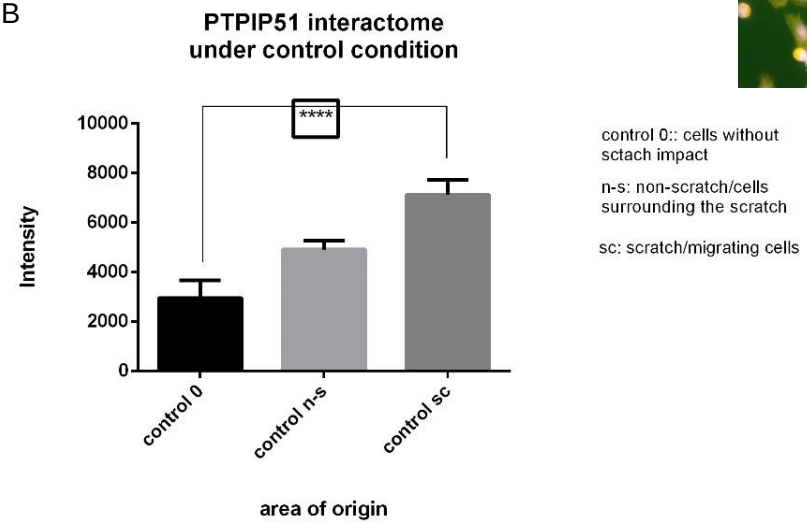
The highest velocity of cell migration was here demonstrated with 0.023 mm/h in the control group. Application of the compound insulin led almost to the same speed with 0.022 mm/h. Interestingly, by submitting 100µM LDC-3 to the migrating cells, the velocity decreased to 0.014 mm/h.

Semi-quantification of the PTPIP51 interactome with the respective binding proteins during migration demonstrated a significant intensity increase (Figure 16 A/ B).

A



B



control 0:: cells without sctach impact

n-s: non-scratch/cells surrounding the scratch

sc: scratch/migrating cells

Figure 16 A: Example of FAK/ PTPIP51 immunostaining of a scratched region.
B: Example of the semi-quantification of the PTPIP51/Rac-1 interactome during migration. Zero (0) describes the cell groups without any manipulation by scratching; non-scratch (n-s) describes the cells surrounding the scratch; scratched (sc) describes the cells within the migration area. Significances are represented with **** of a p value <0.0001.

Therefore, the PTPIP51 interactome has shown, on the one hand, a high level (and upregulation) of its presence in migrating cells. On the other hand, it could be demonstrated so far that the above-described supplements impact cell migration behaviour through the PTPIP51 interactome by reducing the velocity.

3.1 The influence of the scratch on the PTPIP51 interactome in control cells

Mechanical manipulation of cells affected cell signalling and in subsequent resulted in proliferation and migration [60]. Scratching HaCaT cells changed the PTPIP51 interactome. Figure 17 A gives examples of the PTPIP51 interactome in untreated cells. The PTPIP51 interactome with the proteins Rac-1, β-actin and the FAK was investigated. The numerical data is given in Figure 17B.

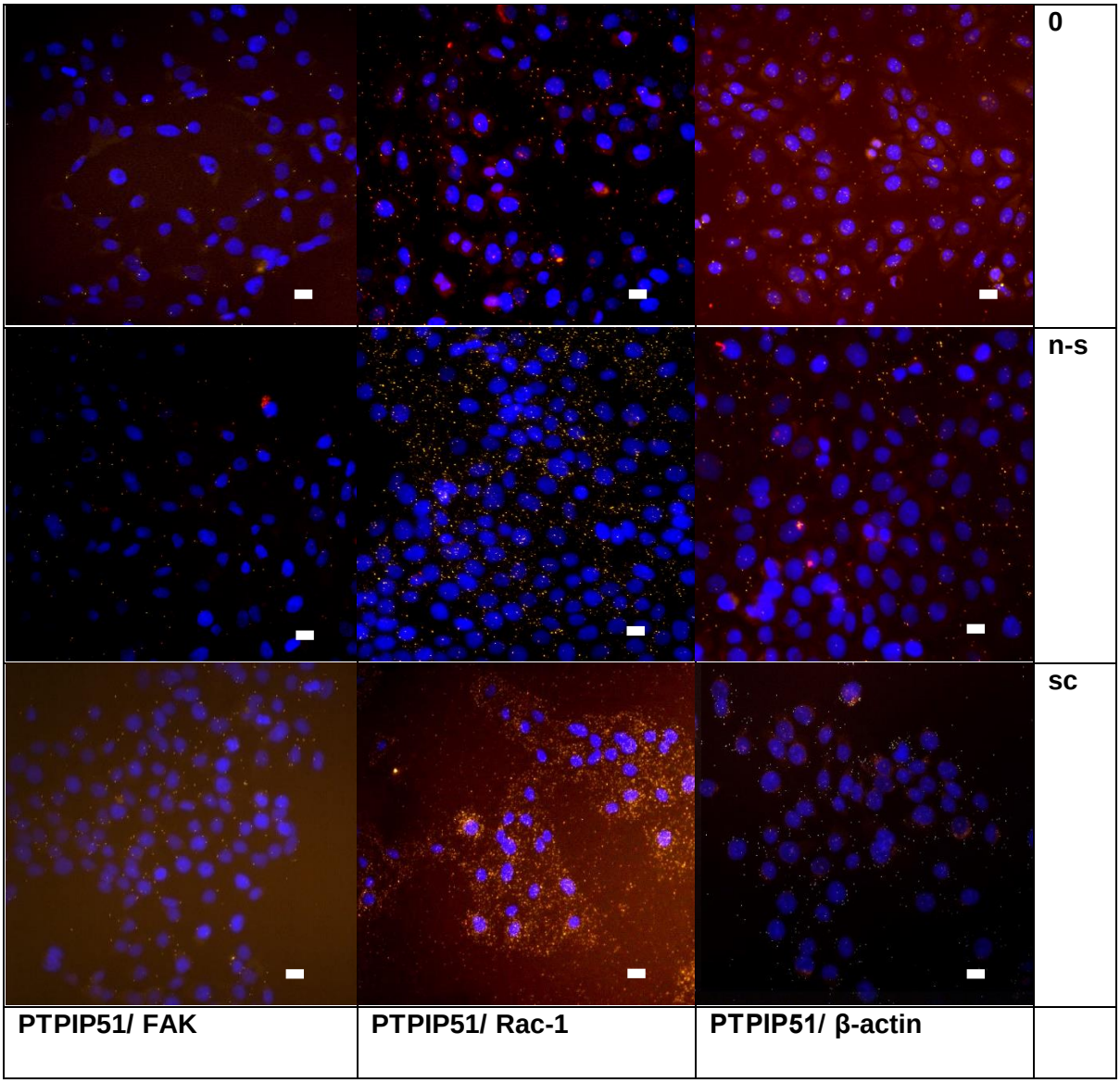


Figure 17 A: DPLA of the PTPIP51 for β-actin, Rac-1 and FAK in **untreated** HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with the respective interaction partner. Zero (0) describes the cells without any manipulation by scratching; non-scratch (n-s) describes the cells surrounding the scratch; scratched (sc) describes the cells within the migration area. Nuclei are stained with DAPI (blue colour). *n* = 3. Scale bar 20µm.

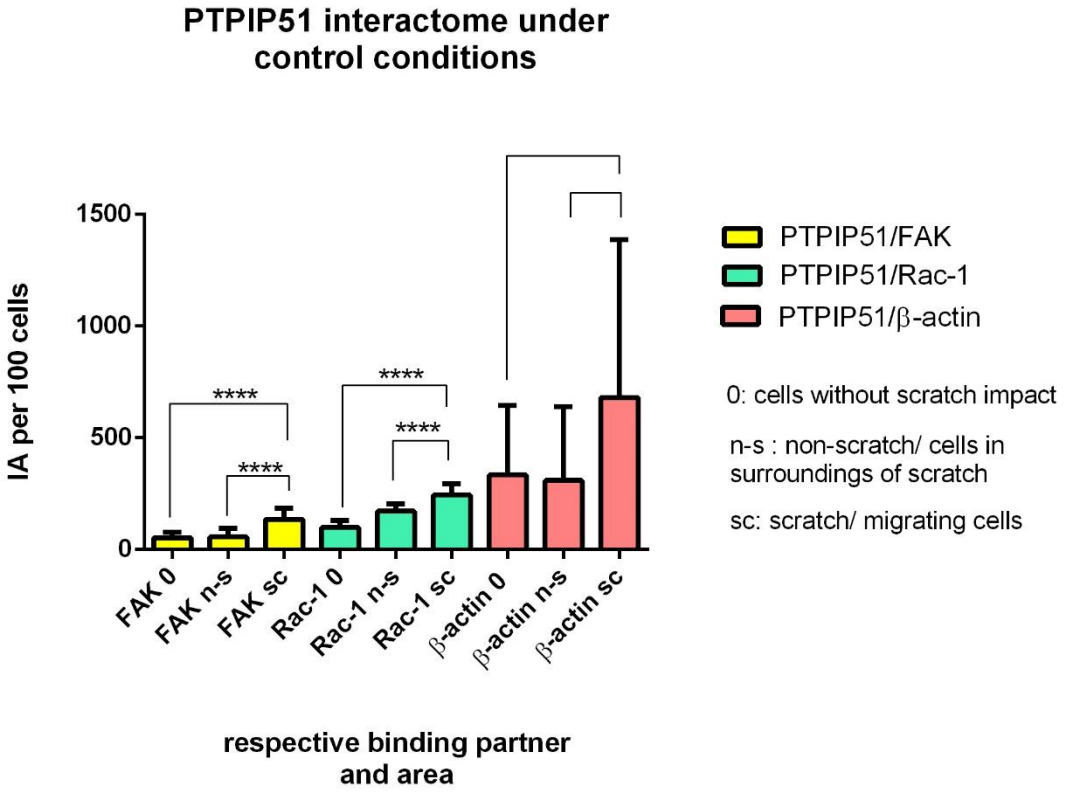


Figure 17 B Interaction profile of PTPIP51 with FAK, Rac-1, β-actin respectively during migration in control cells. Yellow bars: PTPIP51/FAK interactions, turquoise bars: PTPIP51/Rac-1 interactions and pink bars: PTPIP51/β-actin interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with **** of a p value <0.0001.

Cells exposed to scratching have shown an upregulation in the number of interactions of PTPIP51 independent from the investigated interaction partner in comparison to the non-scratched HaCaT cells and zero-scratched HaCaT cells, respectively. As seen in the figure 17 B all interactions relevant for migration were upregulated. The lowest level of interactions was seen for the PTPIP51/FAK group and the highest for the PTPIP51/β-actin group (FAK 0 53 IAs, FAK n-s 57 IAs, FAK sc 136 IAs; Rac-1 0 100 IAs, Rac-1 n-s 172 IAs, Rac-1 sc 244 IAs, β-actin 0 334 IAs, β-actin n-s 310 IAs, β-actin 681 IAs).
Compared to unscratched cells the PTPIP51 interactions with FAK and Rac-1 in migrating cells were significantly increased by scratching (**** p <0.0001).
No significant changes were seen in the interaction of PTPIP51/β-actin neither by scratching, nor in the surrounding of the scratch compared to the zero group. Nevertheless, this group showed the highest number of interactions within the migratory zone.

3.2 The influence of the scratch on the PTPIP51 interactome in EGF treated cells

As established by DPLA experiments with EGF-treated HaCaT cells in a scratched area, the migrating HaCaT cells displayed a changed PTPIP51 interactome for FAK, Rac-1 and β-actin. This can be seen in Figure 18 A of the DPLA of EGF-treated (1µg/ml) cell repair of the scratch/during migration and in unscratched HaCaT cells. The numerical evaluation is given in Fig. 18 B, 19, 20.

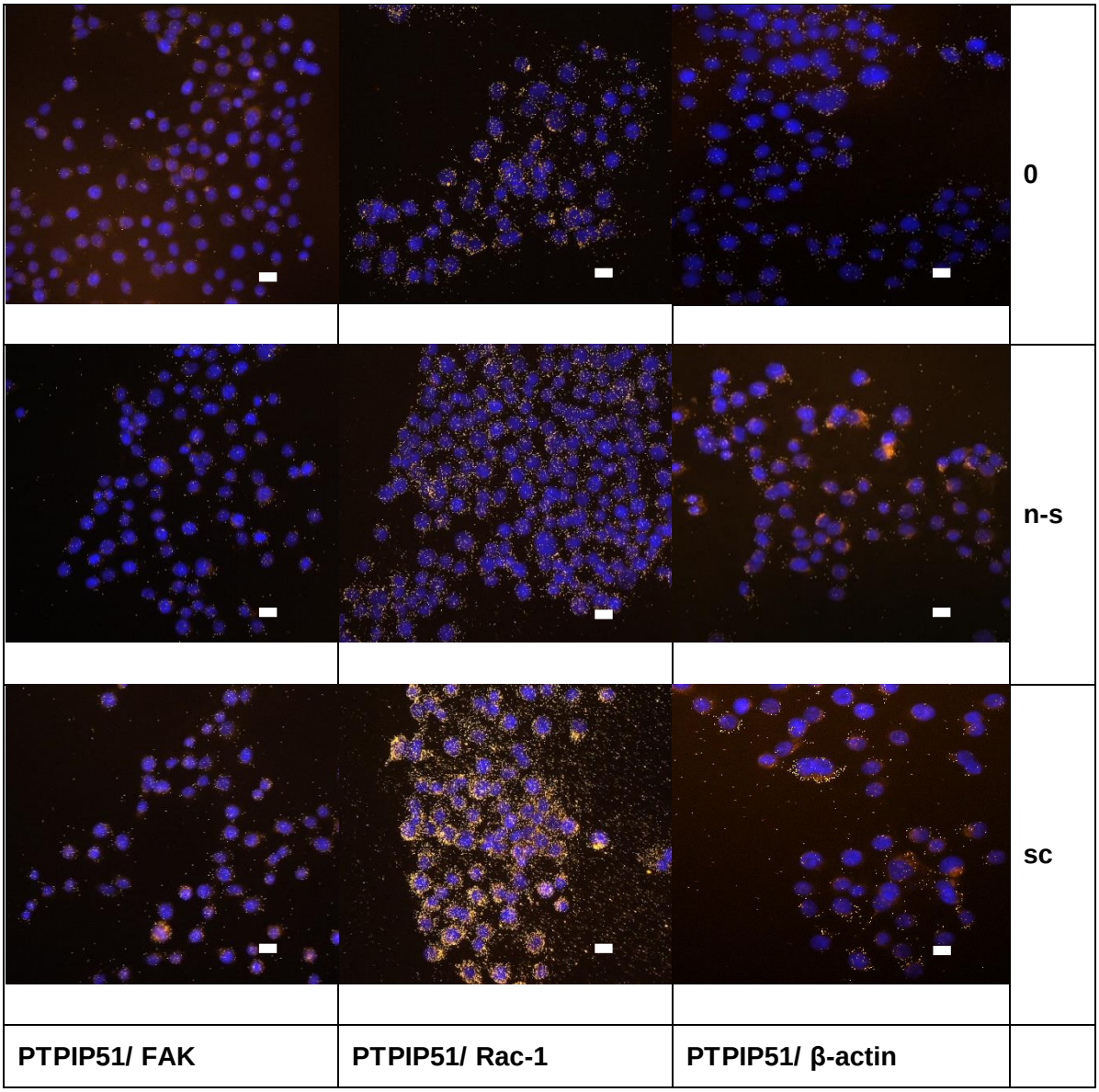


Figure 18 A: DPLA of the PTPIP51 for β-actin, Rac-1 and FAK in EGF-treated (1µg/ml) HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with the respective interaction partner. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Nuclei are stained with DAPI (blue colour). *n* = 3. Scale bar 20µm.

3.2.1 PTPIP51/ β-actin interaction in migrating EGF-treated HaCaT cells

Interestingly, the DPLA scratch experiments in migrating EGF-treated (1µg/ml) HaCaT cells displayed no difference in PTPIP51/ β-actin interactions despite their relation to the scratch (0, n-s, sc). EGF treatment increased the level of PTPIP51/ β-actin interactions in all investigated conditions (0, n-s, sc) to the same level as seen in untreated "migrating" cells. The induced upregulation could not be stimulated further (Fig. 18 A and Fig. 18 B).

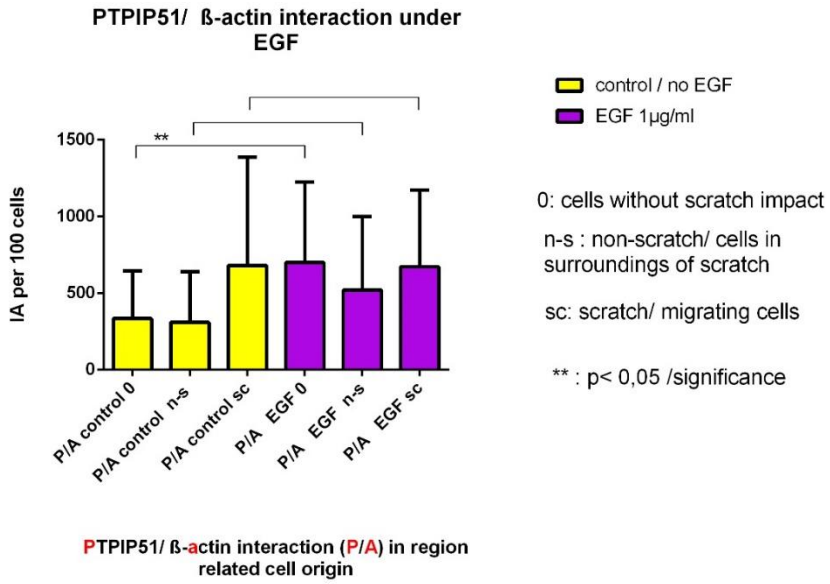


Figure 18 B: PTPIP51/ β-actin interactome in HaCaT control cells and in EGF-treated HaCaT cells (1µg/ml). Yellow bars: HaCaT cell controls, without EGF treatment. Violet bars: EGF-treated HaCaT cells. P/A: PTPIP51/ β-actin interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with ** p< 0.05 calculated by the Mann-Whitney-U-Test.

EGF treatment of HaCaT cells resulted in a 110% upregulation of PTPIP51/ β-actin (P/A) interaction (Fig. 18 B) in both, the control zero cell group compared to the EGF-treated zero cells (p< 0.05 (**); 334 P/A control 0 vs 701 P/A EGF 0)). Scratching did not result in a further increase in the number of interactions in the EGF group. Migrating cells of both experimental set-ups (control vs. EGF treatment) displayed no significant differences in the number of interactions.

.

3.2.2 PTPIP51/ Rac-1 interaction in migrating EGF-treated HaCaT cells

The identical experimental setup was used to analyse the interactome of PTPIP51 and Rac-1. EGF treatment increased the level of PTPIP51/Rac-1 interactions rapidly and significantly within the groups (0, n-s, sc).

Figure 18 A (as seen before) represented an example of a DPLA on the PTPIP51/Rac-1 interactome in EGF-treated (1µg/ml) HaCaT cells during migration and in unscratched cells. The numerical data are seen in Figure 19 further below. EGF-treated cells revealed a significant ($p<0.0001$) upregulation of the PTPIP51/Rac-1 interaction, even in the absence of a scratch, in comparison to the control group (P/R control 0 vs P/R EGF 0). Thus, there was an additive effect of scratching and EGF.

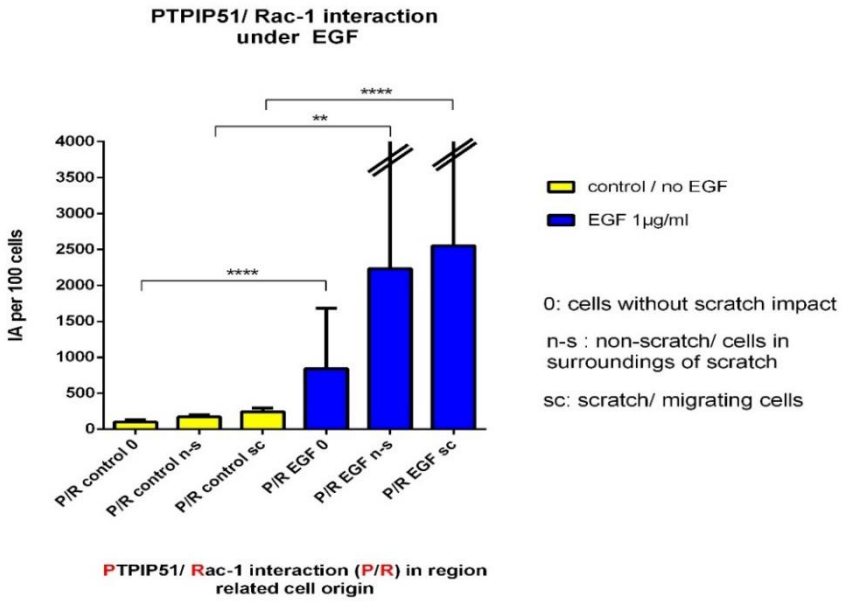


Figure 19: PTPIP51 interactome for Rac-1 in HaCaT control cells and under EGF treatment (1µg/ml). Yellow bars represent the control HaCaT cells and blue bars represent the HaCaT cells under EGF treatment. P/R represents PTPIP51/ Rac-1 interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with **$p<0.0043$, ****$p<0.0001$

EGF treatment augmented the number of PTPIP51/Rac-1 interactions to 845 (745%) interactions/100cells in the non-manipulated group (P/R EGF 0). In the surroundings of the scratch (n-s) EGF treatment increased the PTPIP51/Rac-1 interactions to a number of 2236 (1200%) with a significance value of $p< 0.0043$ (P/R EGF n-s). Moreover, migrating cells with EGF treatment show a strong significant ($p<0.0001$) increase in PTPIP51/Rac-1 interactions to a number of 2553 (946%) interactions/100cells (P/R EGF sc).

3.2.3 PTPIP51/FAK interaction in migrating EGF-treated HaCaT cells

EGF treatment increased the level of PTPIP51/FAK interactions but only in the groups of the manipulated cells (n-s, sc). The application of EGF did not result in a significant PTPIP51/FAK interaction upregulation in the non-manipulated group (0).
Figure 18 A (as seen before) represented an example of the DPLA with EGF-treated (1µg/ml) HaCaT cells during migration and in unscratched cells with the PTPIP51 interactome for FAK. The numerical data of these experiments are seen in Figure 20.

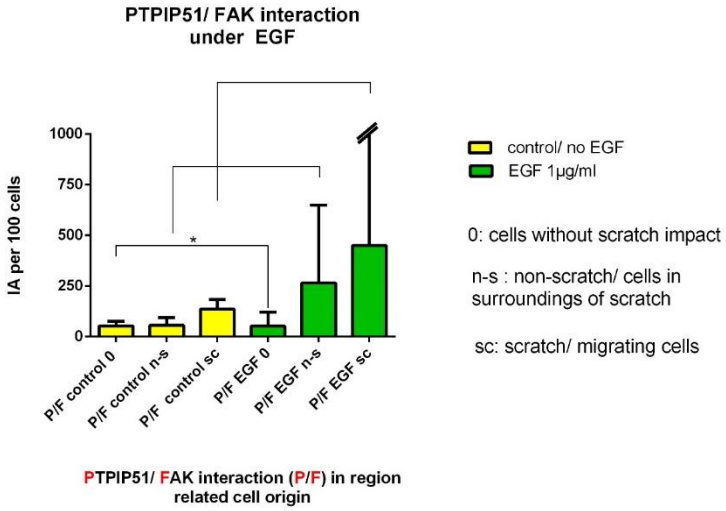


Figure 20: PTPIP51 interactome for FAK in HaCaT control cells and under EGF treatment (1µg/ml). Yellow bars represent the control HaCaT cells and green bars represent the HaCaT cells under EGF treatment. P/F represents PTPIP51/ FAK interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with *p< 0.04.

Submitting EGF to scratched cells revealed ambiguous data. Firstly, EGF treatment produced no significant increase in the non-manipulated groups (54 IAs control 0 vs 53 IAs EGF 0). Secondly, the cells surrounding the scratch augmented their PTPIP51/FAK interactions by submitting EGF by 364% and migrating cells upregulated their PTPIP51/FAK interactions by submitting EGF by 231%. Both upregulations didn't show significances in the Mann-Whitney-U-testing. Although the numbers of PTPIP51/FAK interactions demonstrated a further increase by submitting EGF to migrating cells. (53 IAs EGF 0 vs 265 IAs EGF n-s vs 451 IAs EGF sc in comparison to 54 IAs control 0 vs 57 IAs control n-s vs 136 IAs control sc). The most significant differences were due to scratching, not by EGF treatment.

3.3 The PTPIP51 interactome in migrating insulin-treated HaCaT cells

As established by DPLA experiments with insulin-treated HaCaT cells in a scratched area, the migrating HaCaT cells displayed a changed PTPIP51 interactome for FAK, Rac-1 and β-actin. This can be seen in Figure 21 A: the DPLA of insulin-treated (0.5mg/ml) cells repair of the scratch/during migration and in unscratched HaCaT cells. The numerical evaluation is given in Fig. 21 B, 22, 23.

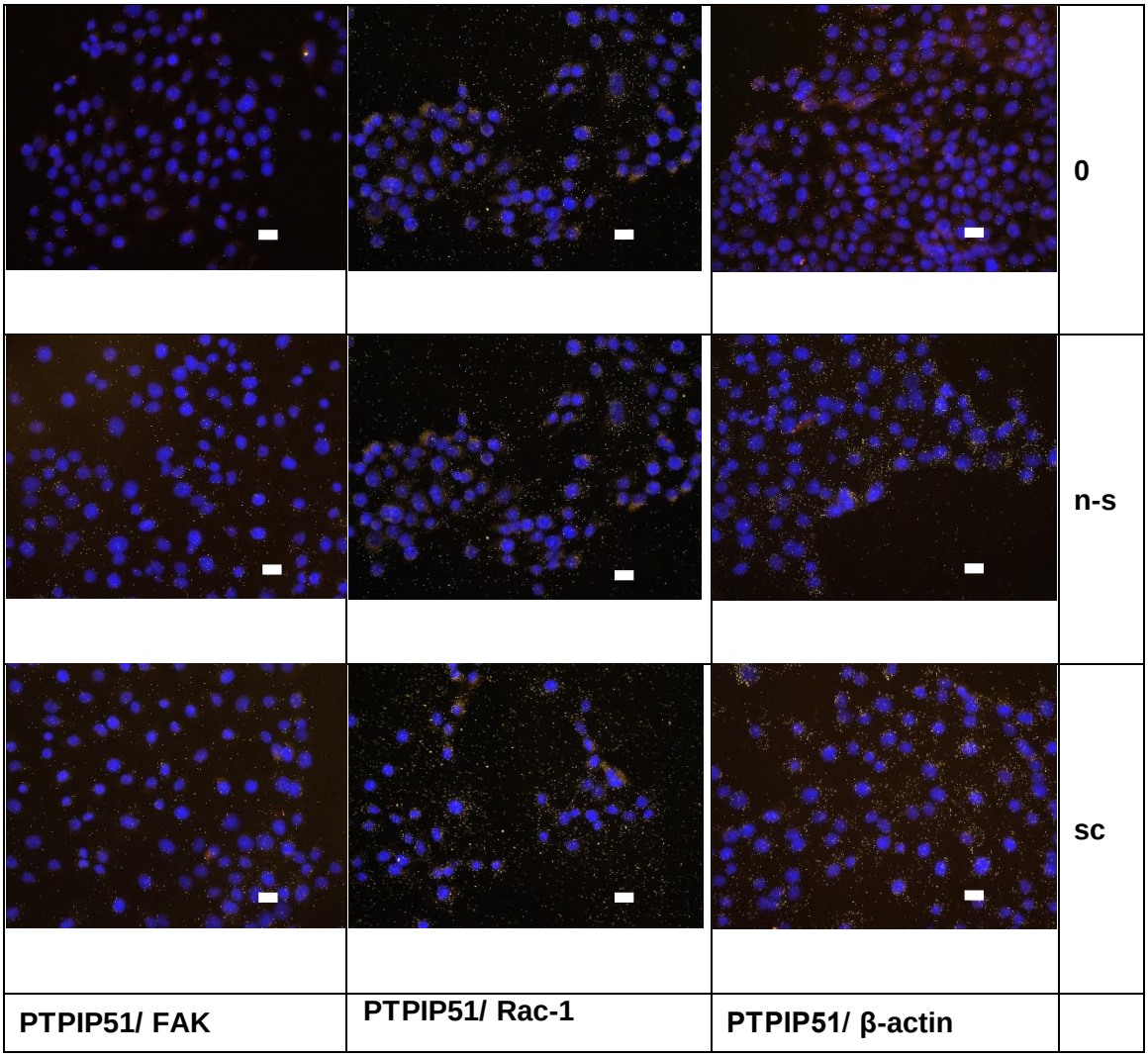


Figure 21 A: DPLA of the PTPIP51 for β-actin, Rac-1 and FAK in insulin (0.5mg/ml) HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with the respective partner. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Nuclei are stained with DAPI. *n* = 3. Scale bar 20µm.

3.3.1 PTPIP51/ β-actin interaction in migrating insulin-treated HaCaT cells

The DPLA scratch experiments in migrating insulin-treated (0.5mg/ml) HaCaT cells displayed an augmentation of PTPIP51/ β -actin interactions with significant differences, which were demonstrated in the relation to the scratch respectively (0, n-s, sc).
The upregulation of IAs in the insulin-treated 0 group is by 80%, by 364% in the n-s group and 92% in the migrating (sc) group.

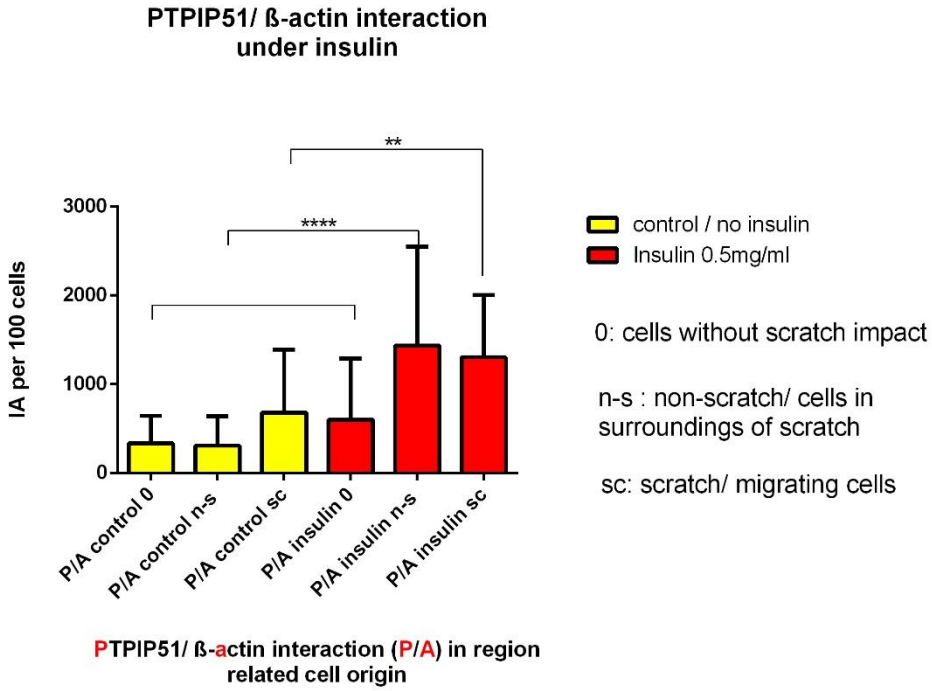


Figure 21 B: PTPIP51 interactome for β-actin in HaCaT control cells under insulin treatment (0.5mg/ml). Yellow bars represent the control HaCaT cells and red bars represent the HaCaT cells while insulin was applied. P/A represents the PTPIP51/β-actin interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with **p<0.005, ***p<0.0002.

The non-manipulated control cells displayed a number of 334 PTPIP51/β-actin interactions/100 cells (P/A control 0). Insulin treatment augmented the number of PTPIP51/β-actin interactions to 602 interaction/100cells (P/A insulin 0), without a significant difference. Insulin treatment upregulated the PTPIP51/β-actin interactions in the cells near the scratch to a number of 1435 IAs significantly ($p < 0.0001$). Additionally, the migrating insulin-treated cells displayed a significant ($p < 0.005$) increase of PTPIP51/β-actin interactions to an amount of 1303 IAs.

3.3.2 PTPIP51/ Rac-1 interaction in migrating insulin-treated HaCaT cells

The identical experimental setup was used to analyse the interactome of PTPIP51 and Rac-1. Insulin treatment increased the level of PTPIP51/Rac-1 interactions rapidly and significantly within the groups (0, n-s, sc). Figure 21 A (as seen above) represented an example of the DPLA with insulin-treated (0.5mg/ml) HaCaT cells during migration and in unscratched cells with the PTPIP51 interactome for Rac-1. The numerical data of these experiments is seen in Figure 22. Insulin-treated cells revealed a significant ($p<0.0001$) upregulation of the PTPIP51/Rac-1 interaction, even in the absence of a scratch, in comparison to the control group (P/R control 0 vs P/R insulin 0).

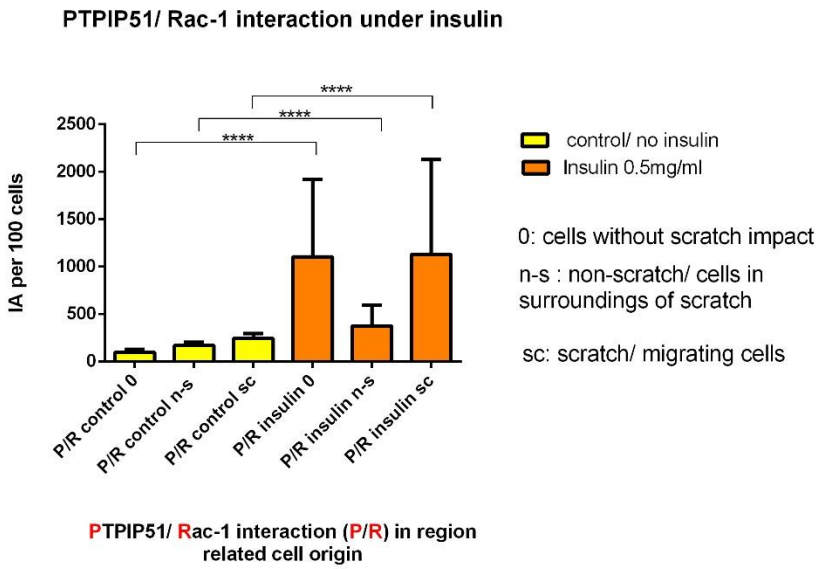


Figure 22: PTPIP51 interactome for Rac-1 in HaCaT control cells and under insulin treatment (0.5mg/ml). Yellow bars represent the control HaCaT cells and orange bars represent the HaCaT cells under insulin treatment. P/R represents PTPIP51/ Rac-1 interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with ****$p<0.0001$.

The non-manipulated control cells have shown a number of 100 PTPIP51/Rac-1 interactions. Due to insulin treatment, these cells augmented the interactions by 1006% to a number of 1106 IAs (P/R control 0 vs P/R insulin 0). Moreover, the migrating cells also displayed a high upregulation by 363% to a number of 1130 PTPIP51/Rac-1 IAs (P/R control sc vs P/R insulin sc). The cells in the surrounding area of the scratch showed a drop in numbers of PTPIP51/ Rac-1 interactions in insulin-treated cells (172 IAs P/R control n-s vs 376 IAs P/R insulin n-s). This still corresponded to an increase by 119%.

3.3.3 PTPIP51/ FAK interaction in migrating insulin-treated HaCaT cells

As established by DPLA experiments, the PTPIP51/FAK interactome in migrating insulin-treated HaCaT cells revealed an increase by 80% compared to the control migrating group (244 IAs P/F insulin sc vs 136 IAs P/F control sc). The insulin-treated cells near the scratch boost their PTPIP51/FAK interactome by 54% compared to the adjacent control HaCaT cells (Fig. 23). Thus, there was an additive effect of scratching and insulin. Figure 21 A (as seen before) represented an example of the DPLA with insulin-treated (0.5mg/ml) HaCaT cells during migration and in unscratched cells with the PTPIP51 interactome for FAK. The numerical data of these experiments can be seen in Figure 23.

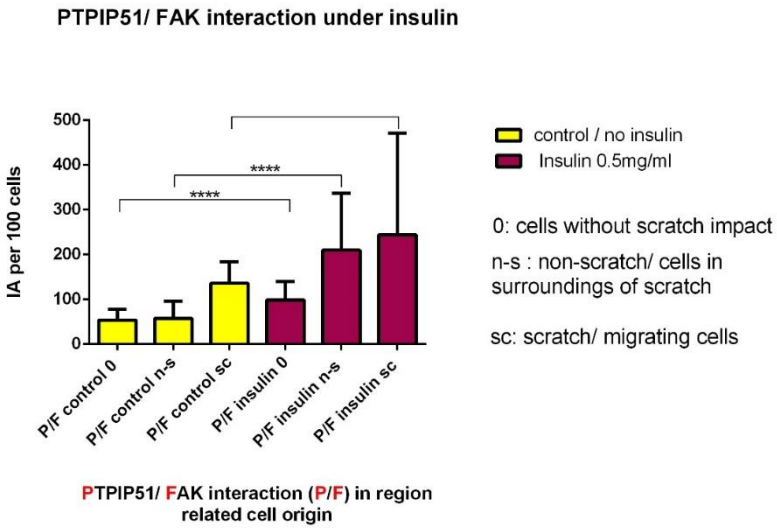


Figure 23: PTPIP51 interactome for FAK in HaCaT control cells and under insulin treatment (0.5mg/ml). Yellow bars represent the control HaCaT cells and magenta bars represent the HaCaT cells under insulin treatment. P/F represents PTPIP51/ FAK interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with ****p<0.0001.

Non-manipulated control cells displayed a number of 54 PTPIP51/FAK interactions/100 cells, followed by an increase of 57 interactions in cells in the scratch surroundings and 136 interactions in migrating cells. The non-manipulated insulin-treated cells demonstrated an increased number of 99 PTPIP51/FAK interactions followed by a further increase of 210 PTPIP51/FAK interactions in the cells near the scratch, with a peak of 244 PTPIP51/FAK interactions in migrating cells with insulin treatment. Additionally, insulin treatment increased significantly ($p < 0.0001$) the PTPIP51 interactome for FAK in the zero 0 and n-s groups. Migrating insulin-treated cells have shown no significant difference, even with the markable upregulation of PTPIP51/FAK interactions.

Summary of the PTPIP51 interactome in control cells and in insulin, EGF-treated scratched and unscratched HaCaT cells

Scratching increased the PTPIP51 interactome for Rac-1, FAK and β-actin. EGF and insulin treatment impacted the PTPIP51 interactome for Rac-1, FAK and β-actin differently. Additive effects were evident for the PTPIP51/Rac-1 interactome with EGF treatment compared to the untreated scratched, n-s and 0 groups. EGF treatment did not show a significant PTPIP51/FAK interactome upregulation for all groups (0, n-s, sc). For the PTPIP51/ β-actin interactome, EGF treatment increased the interactions but no further increase could be demonstrated due to scratching. Additive effects of scratching and insulin treatment were demonstrated by the PTPIP51 interactome for FAK and β-actin also, but not for the PTPIP51/ Rac-1 interactome (see figure 24).

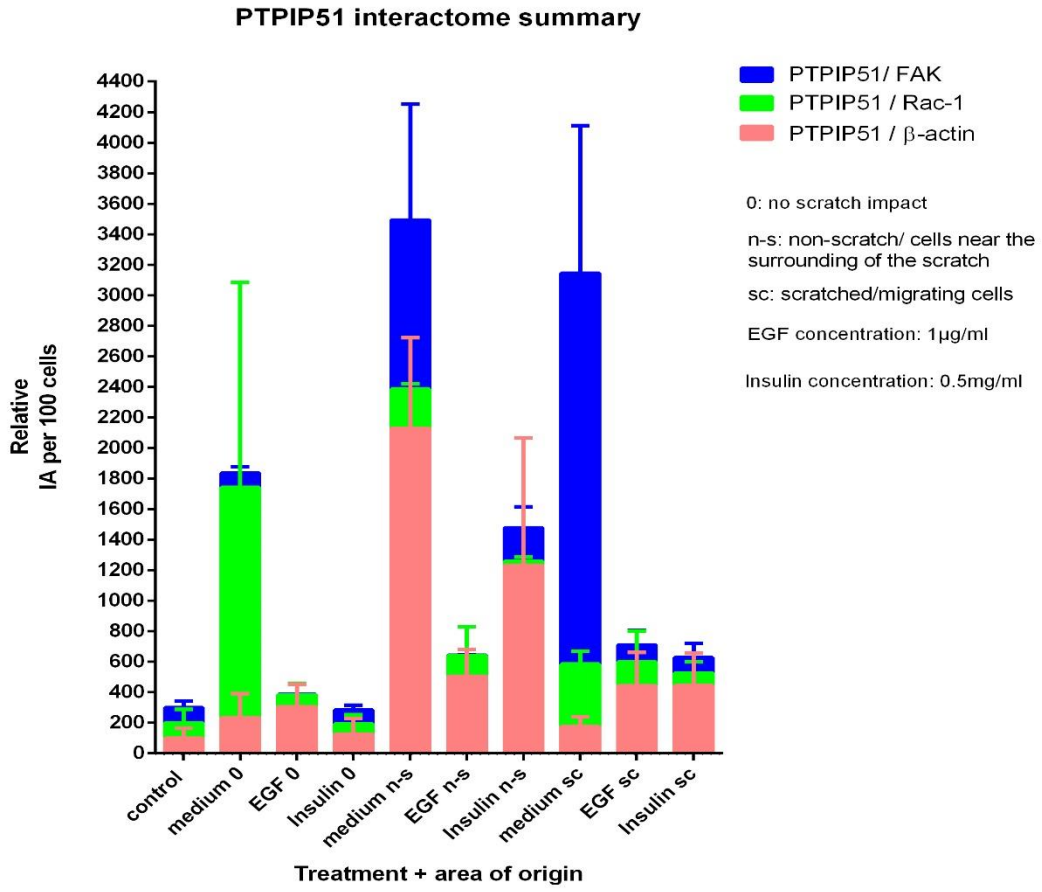


Figure 24: Cellular protein complex shift of PTPIP51 within three protein–protein interactions. Quantitative analysis of the DPLA of PTPIP51 with three different interaction partners in untreated controls and in the control area (0), in the surrounding of the scratch (n-s) and in migrating (sc) HaCaT cells (n = 3). The control values of three different PTPIP51 protein interactions were equalized to 100% (first stacked column) and the following columns show the standardized interaction values related to the controls (equaling 100%) for sc/ns/0 and for the insulin treatment and EGF treatment.

3.4 The PTPIP51 interactome in migrating LDC-3-treated HaCaT cells

To analyse the effect of the small molecule LDC-3 on the PTPIP51 interactome, HaCaT cells were treated with three different concentrations (5, 50, 100µM) of LDC-3. Submitting LDC-3 to HaCaT cells during migration, the PTPIP51 interactome partners, such as FAK, Rac-1 and β-actin were investigated. Interestingly, the migrating HaCaT cells displayed a changed PTPIP51 interactome for FAK, Rac-1 and β-actin.
Data was analysed by quantifying the number of interactions in each experiment and subjecting them to the Mann-Whitney-U test.

3.4.1 PTPIP51/β-actin interaction in migrating LDC-3-treated HaCaT cells with increasing concentrations

As established by DPLA experiments with LDC-3-treated HaCaT cells, dose dependent PTPIP51/β-actin interactions in migrating were demonstrated. The application of 5µM LDC-3 increased the PTPIP51/ β-actin significantly in the zero (non-manipulation) cells. Elevated PTPIP51/β-actin interaction levels were also seen in the cells near the surrounding of the scratch and in the migrating cells, but not significantly. Treatment with 50µM LDC-3 increased the interactions as well, but interestingly, cells with 100µM LDC-3 concentration showed a decline of PTPIP51/β-actin interactions.
Figure 25 represents an example of the DPLA with LDC-3-treated (5, 50, 100µM) HaCaT cells during migration and in unscratched cells with the PTPIP51 interactome for β-actin. The numerical data of these experiments are seen in Figure 26.

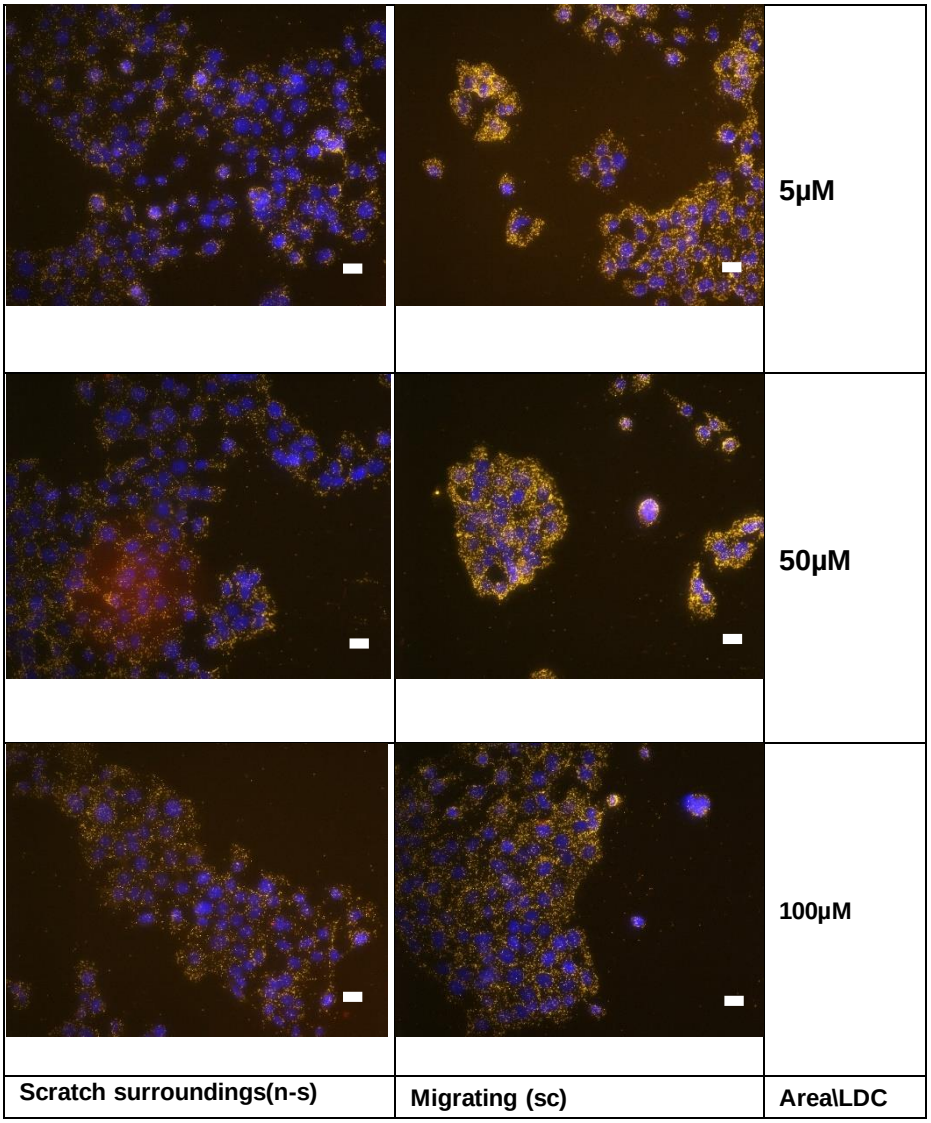


Figure 25: DPLA of the PTPIP51 for β-actin LDC-3 (5, 50, 100µM) treated HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with β-actin; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Nuclei are stained with DAPI *n* = 3. Scale bar 20µm.

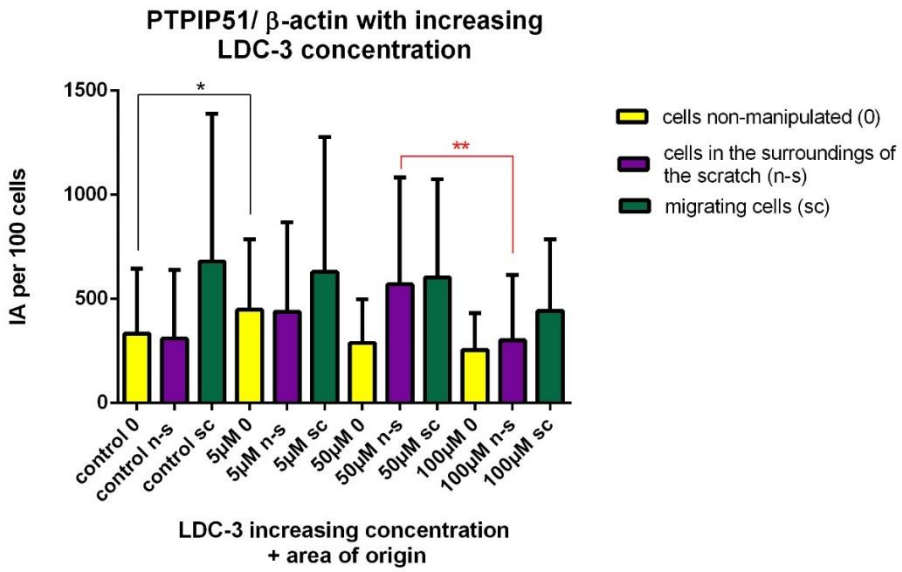


Figure 26: PTPIP51 interactome for β-actin in HaCaT control cells and with LDC-3 treatment (various concentrations: 5, 50, and 100µM). Yellow bars represent the zero (non-manipulated by scratch) groups of HaCaT cells. Purple bars represent the LDC-3-treated HaCaT cells in the surrounding area of the scratch and green bars represent the LDC-3-treated migrating HaCaT cells. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with **p<0.005 and *p<0.05 are described.

Scratching the control cells led to an increase of PTPIP51/β- actin interactions by 104%. Submitting 5µM LDC-3 significantly (p<0.05/*) upregulated the interactions by 34%, but there was no additive effect with 5µM LDC-3 in the migrating cells. By submitting 50µM LDC-3, HaCaT cells even showed a slightly decrease of PTPIP51/ β-actin interactions, which was significant (p< 0.005) in the cells near the scratch.

Interestingly, migrating cells demonstrated a further reduction of interactions by 54% with 100µM LDC-3.

However, application of LDC-3 did not result in a significant further augmentation of PTPIP51/β-actin interactions during migration; there was even a reduction evident in the group with 100µM LDC-3.

3.4.2 PTPIP51/Rac-1 interaction in migrating LDC-3-treated HaCaT cells with increasing concentrations

As assessed by the DPLA in scratch experiments, LDC-3 also had a profound impact on the PTPIP51 interactome for Rac-1. Migrating LDC-3-treated cells have shown an average increase of PTPIP51/Rac-1 interactions by 34%, but in the 100µM treated group, a decline of PTPIP51/Rac-1 interactions was demonstrated.

Figure 27 represents an example of the DPLA with LDC-3-treated (5, 50, 100µM) HaCaT cells during migration and in unscratched cells with the PTPIP51 interactome for Rac-1. The blue staining represents DAPI staining of nuclei. Each of the small fluorescent dots represent a single interaction of PTPIP51 with Rac-1. Figure 28 shows numerical data.

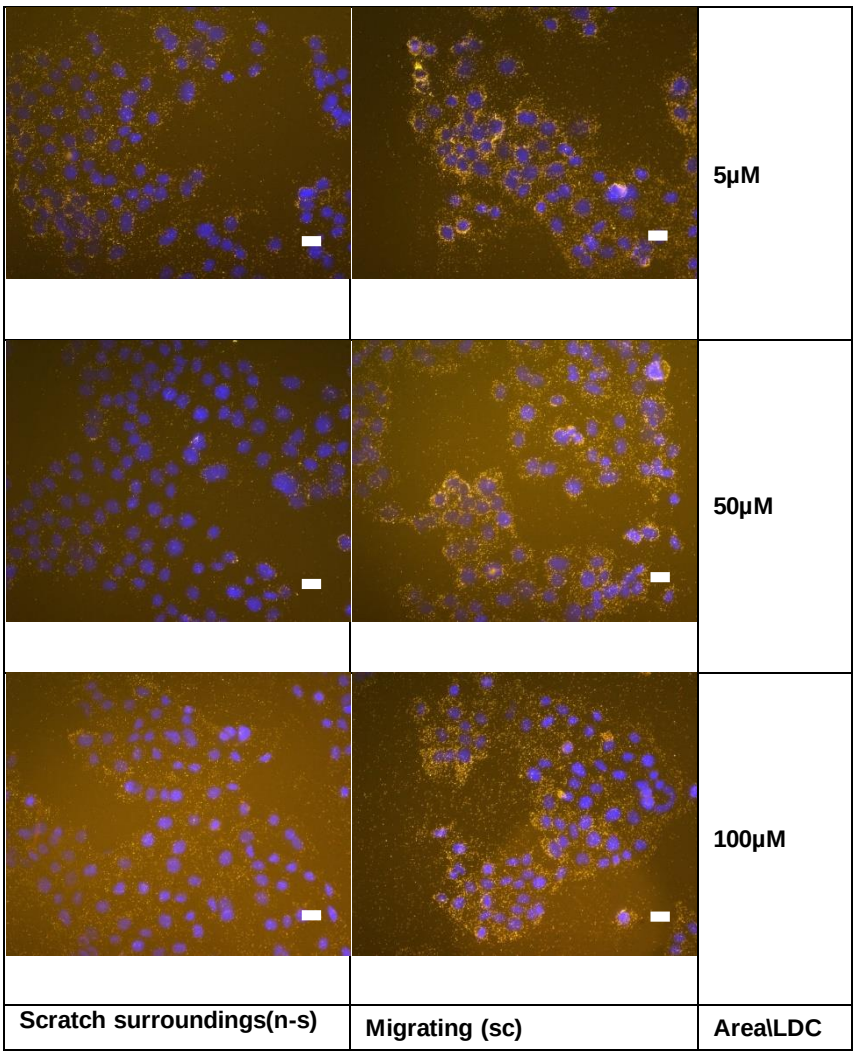


Figure 27: DPLA of the PTPIP51 for Rac-1 LDC-3-treated (5, 50, 100µM) HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with Rac-1.; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Nuclei are stained with DAPI. *n* = 3. Scale bar 20µm.

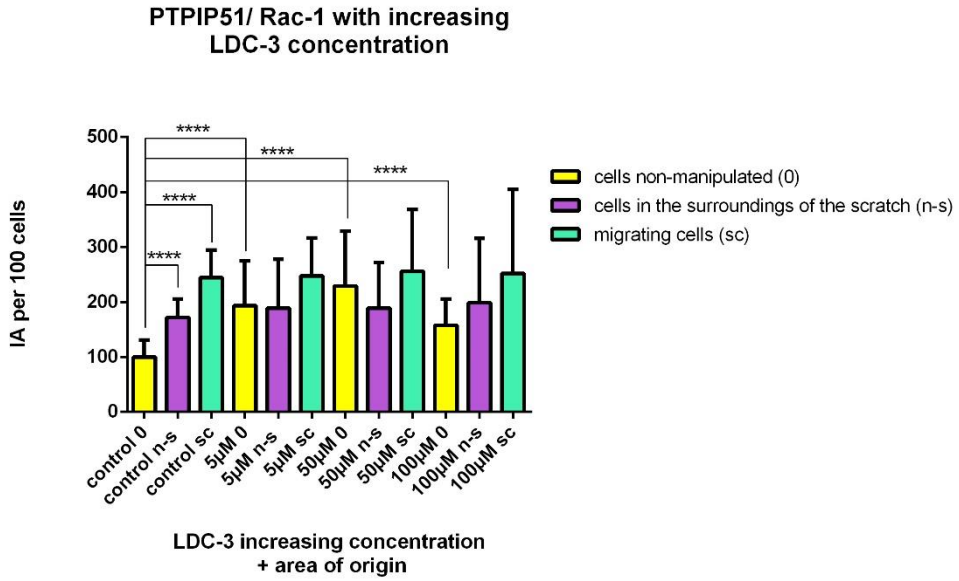


Figure 28: PTPIP51 interactome for Rac-1 in HaCaT control cells and with LDC-3 treatment (various concentrations: 5, 50, and 100µM). Yellow bars represent the zero (non-manipulated by scratch) groups of HaCaT cells. Purple bars represent the LDC-3-treated HaCaT cells in the surrounding area of the scratch and green bars represent the LDC-3-treated migrating HaCaT cells. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances with ****$p<0.0001$ are described.

Migrating control cells upregulated the PTPIP51/Rac-1 interaction by scratch manipulation (as mentioned in figure 15B). Submitting LDC-3 to HaCaT cells significantly ($p<0.0001$) led to a further dose dependent increase of interactions, but the 100µM groups show a decline. The lowest number of 100 PTPIP51/Rac-1 interactions was seen for control zero cells (control 0, yellow bar). In comparison, the highest numbers were represented in the 50µM groups with a peak of 256 PTPIP51/Rac-1 interactions in migrating cells. 50µM LDC-3-treated cells, which were not manipulated, summed up to 229 PTPIP51/Rac-1 interactions (50µM 0). The 5µM LDC-3-treated cells have shown a number of 194 interactions (without the scratch impact) and 158 interactions with 100µM LDC-3 (5µM 0, 100µM 0, yellow bars). The migrating cells with 100µM LDC-3 treatment have also shown a decline of PTPIP51/Rac-1 interactions.

Therefore, LDC-3 treatment upregulated the PTPIP51/Rac-1 interactions up to a concentration with 50µM LDC-3 and downregulated the interactions with 100µM treatment. Additionally, 100µM LDC-3 treatment downregulated the migrating cells. In spite of that, a significant downregulation of the interactions was not represented and neither was an additive effect evident.

3.4.3 PTPIP51/FAK interaction in migrating LDC-3 treated HaCaT cells with increasing concentrations

Interestingly, LDC-3 had an impressive impact on the interaction profile of the PTPIP51 interactome for FAK, seen in the DPLA investigations. With the application of LDC-3, the number of PTPIP51/FAK interactions increased almost thrice. Furthermore, in migrating LDC-3-treated HaCaT cell monolayers, an increase of PTPIP51/FAK interactions between 4-5 times was evident. Figure 29 shows an example of the DPLA for HaCaT cells treated with LDC-3 (5, 50, 100µM concentrations), during migration and in unscratched cells with the PTPIP51 interactome for FAK. Each of the small fluorescent dots represent a single interaction of PTPIP51 with FAK. Figure 30 shows numerical data.

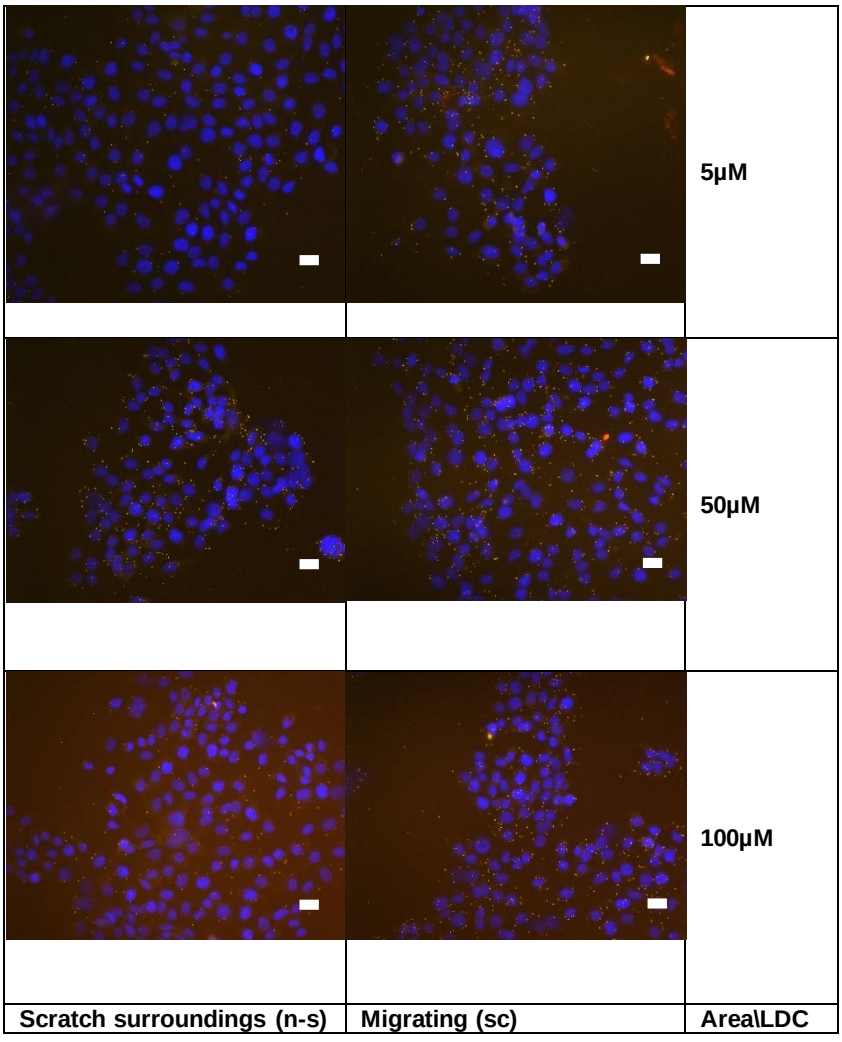


Figure 29: DPLA of the PTPIP51 for FAK LDC-3-treated (5, 50, 100µM) HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with FAK; non-scratch (n-s) describes the cells surrounding the scratch; scratched (sc) describe the cells within the migration area. Nuclei are stained with DAPI. *n* = 3. Scale bar 20µm.

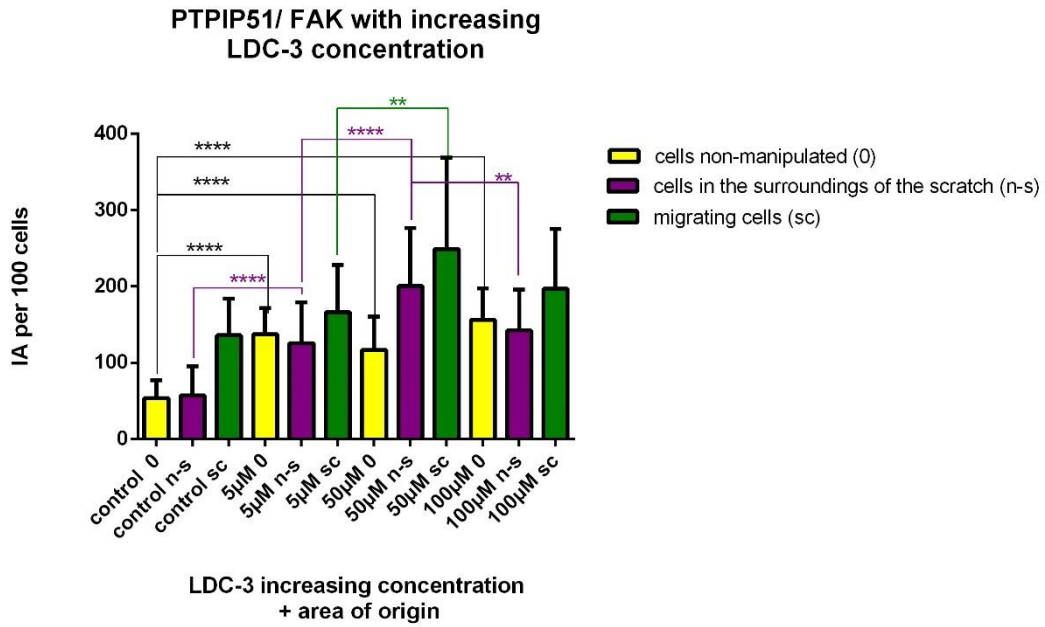


Figure 30: PTPIP51 interactome for FAK HaCaT control cells and with LDC-3 treatment (various concentrations: 5, 50, and 100µM). Yellow bars represent the zero (non-manipulated by scratch) groups of HaCaT cells. Purple bars represent the LDC-3-treated HaCaT cells in the surrounding area of the scratch and green bars represent the LDC-3-treated migrating HaCaT cells. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with **p<0.005, ** p< 0.002 and ****p<0.0001.

The augmentation of PTPIP51/FAK interactions in control migrating cells, with the significant difference of p<0.0001 (****), was described in figure 15 B before. LDC-3 treatment increased the PTPIP51/FAK interactions significantly (p<0.0001) in the zero cells with the highest number of 156 PTPIP51/FAK interactions in the 100µM zero group. The LDC-3-treated manipulated cells also increased their PTPIP51/FAK interactions with a significance of p<0.0001, especially in the cells surrounding the scratch with a concentration of 50µM. The 100µM treated cells near the scratch displayed a significantly (p< 0.002) decline of interactions. Interestingly, the migrating cells displayed a significantly (p<0.005) upregulation of the PTPIP51 interactome for FAK with the highest peak of 249 interactions in the migrating 50µM LDC-3-treated cells (Green bar 50µM sc). The 100µM LDC-3-treated migrating cells decreased the PTPIP51/FAK interactions to a number of 197. To sum up, in migrating cells the PTPIP51 interactome for FAK revealed an additive effect due to LDC-3 treatment with dose dependency and a decrease in the 100µM treated groups.

Conclusion data set of the PTPIP51 interactome in LCD-3-treated HaCaT cells

The PTPIP51 interactome with all three respective binding partners during LDC-3 treatment and the impact of the scratch is summed up in the following figure.

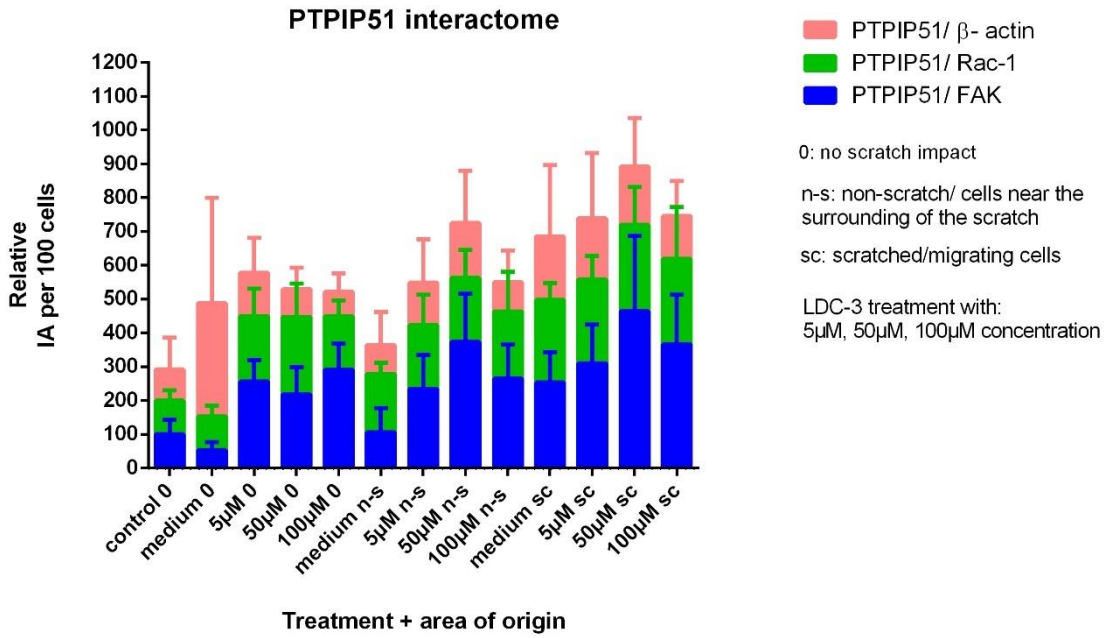


Figure 31: Cellular protein complex shift of PTPIP51 within three protein–protein interactions. Quantitative analysis of the DPLA of PTPIP51 with three different interaction partners in untreated controls and in the control area (0), in the surrounding of the scratch (n-s) and in migrating (sc) HaCaT cells ($n = 3$). The control values of three different PTPIP51 protein interactions were equalized to 100% (first stacked column) and the following columns show the standardized interaction values related to the controls (equaling 100%) for sc/ns/0 and for the LDC-3 treatment with the concentration of 5µM, 50µM and 100µM.

Submitting LDC-3 with increasing concentrations upregulated the PTPIP51 interactome. There is a slightly decrease in the 100µM LDC-3-treated cells for the PTPIP51 interactions with all three interacting partners (Rac-1, FAK and β-actin). The highest relative increase is seen in 50µM LDC-3-treated HaCaT cells in the surroundings of the scratch and in the migrating area for PTPIP51/ FAK and PTPIP51/ Rac-1 interactions.

4 Discussion

4.1 Wound healing and the PTPIP51 interactome

Migration of single cells has been investigated in depth in the past. Migration is required for wound healing, growth renewal, inflammatory responses and tumourigenesis. Cell migration is a complex process which requires controlling the cytoskeletal structure and adhesion processes [83]. Processes in collective migration, a different method of cell movement, is less understood. Additionally, PTPIP51 is known to regulate different cellular processes by regulating signal transduction via interactions with involved proteins. The biomolecular aspects of PTPIP51 during cell migration function is still an open question. Thus, this study aimed to identify the interactome of PTPIP51 and key proteins in migration, such as Rac-1, FAK and β-actin.

The migration of cells in a monolayer can be induced either on demand, such as in wound healing after activation by EGF, reactive oxygen species (ROS), vascular endothelia growth factor (VEGF), platelet-derived growth factor (PDGF), fribroblast growth factor and TGF-β) [22, 84], or self-induced across an intact basal cell layer, therefore, members of the cadherins groups are also involved (cadherin members, non-cadherin members and integrin members) [22, 82]. During wound repair, collective cell migration of keratinocytes leads to epidermal wound closure of the provisionally wound bed [20]. Initially, keratinocytes form simple monolayers which undergo a multi-layered stratification after hours or even days, and receive signals from integrin-mediated binding extracellular ligands [22, 70, 82] which are connected to cytoskeletal adaptor proteins, such as talin, praxilin, vinculin and contractin. Connecting integrins to the actin cytoskeleton and the growth factors (see above) [22, 70, 82] generate intracellular mitogen-activated protein kinase (MAPK) signalling. Activation of the MAPK pathway propagates from cell to cell within the monolayer in a rearward direction and promotes transcription and, therefore, proliferation and differentiation via the Erk family, JNK family and p38 kinase family signalling [30, 85]. To generate a multicellular sheet, the keratinocytes create mechanically robust cell-cell connections which are mediated by adherens junction proteins, integrins, cadherins and other immunoglobulin superfamily members. All of these are connected directly or indirectly to the actin filament (or intermediate filament) cytoskeleton and thereby provide dynamic but robust coupling [22, 82]. By closing the wound, the keratinocyte layer provides coverage and protection of the underlying tissue [20]. This study analysed the relation of PTPIP51 activation after an induced injury to HaCaT monolayers. A common method of analysing the regulation of signal transduction in wound healing during cell proliferation and migration is by scratching the monolayers of a special type of cell [18] [45] [64]. By scratching, the

proliferation and migration are upregulated in HaCaT cells. Furthermore, several metabolites, which are released after scratching, as well as the velocity by which the tip creating the scratch is moved, influence the cellular behaviour and the downstream pathways within the cell in the wound sides [53] [55]. Here, the scratch was applied slowly, in order to partly leave the monolayers uninjured and to avoid cell demolition of the wound rims.

Furthermore, in a second set of experiments the effects of compounds such as EGF, insulin and the small molecule LDC-3 on the PTPIP51 interactome with key proteins in migration, such as Rac-1, FAK and β-actin were tested. This study shows the profound impact of the PTPIP51 protein interactome.

Interestingly, during the migration of HaCaT cells in monolayers, the PTPIP51 protein seemed to increase its interactions with important cytoskeletal binding partners. Additionally, the respective compounds, such as epidermal growth factor, insulin or the small effective molecule LDC-3, which strongly enhances the affinity of the PTPIP51 to MAPK-binding, present here an interesting outcome of the PTPIP51 interactome during migration. The impact of the PTPIP51 interactome, in terms of the speed of cell migration and the effectiveness in closing the wound bed, is of greatest interest and is described in more detail below.

The basic scratch experiments set on HaCaT cells without any treatment have demonstrated a significant increase in the PTPIP51/Rac-1 interactome by a factor of 2.44 and with FAK by a factor of 2.56. Migrating HaCaT cells insignificantly increased the PTPIP51 interactome for β-actin by a factor of 2.04.

The β-actin protein is responsible for polymerization of the G-actin within the assembly part of the cytoskeleton reorganisation. Rac-1 activates through the WAVE protein the actin remodelling and FAK stabilizes focal adhesion and regulates downstream pathways in cell spreading. Consequently, the PTPIP51 protein stabilizes preferably FAK complex binding, therefore, cell adhesion, and regulates by Rac-1 binding the actin filament, but also takes part of the G-actin polymerization.

This adaptation of the PTPIP51 interactome in scratched cells will probably influence (by adding effectors like insulin) the repair of the wound bed paralleled by increasing closure speed.

4.2 Framing the PTPIP51 in the migration process under EGF treatment

The PTPIP51 interactome with Rac-1, FAK- and β-actin differed in migrating cells after EGF treatment. First, EGF treatment upregulated the PTPIP51/ β-actin interactome only in cells without any influence of an injury, by a factor of 2.1. Interestingly, EGF treatment did not affect the PTPIP51/ β-actin interactome, neither in migrating cells, nor in the cells near the scratch.

With EGF treatment, migrating cells were expected to upregulate the G-actin polymerization at the leading edge and also at the lamellipodia to form robust F-actin filaments during migration [54] and, therefore, to upregulate the PTPIP51 protein interaction with β-actin. Surprisingly, this study revealed no further augmentation of PTPIP51/ β-actin interactome.

When EGF binds to its receptor, the actin fibre/EGFR association directly induces the organization of G-actin and, thus, G-actin (β-actin) directly forms F-filaments. The presence of ATP alone, which is upregulated by EGF/EGFR activation, is already polymerizing G-actin (β-actin) [12][72]. Consequently, there is a direct connection between the EGFR and the F-actin, whereas the PTPIP51 protein complex binding with G-actin could be a detour.

Second, EGF treatment significantly increased the PTPIP51/Rac-1 interactome impressively. Upregulation of the PTPIP51/Rac-1 interactome by a factor of 8.5 in cells without any induction by injury was seen. Additionally, EGF treatment in migrating cells showed an increase of the PTPIP51/Rac-1 interactome by a factor of 10.5 and in cells near the scratch, an increase of the PTPIP51/Rac-1 interactome by a factor of 13. Considering the increase of the PTPIP51/Rac-1 interactome due to the injury in the control group (factor 2.44) increased by EGF treatment (factor 8.5, 10.5 and 13), an additive effect is seen.

Rac-1 is known to play a key role during proliferation and migration near the leading lamellipodia [16] [44]. There are several Rac-1 pathways activated if the cell migrates but up to now, not all have been well understood. One pathway is that Rac-1 induces through the WAVE protein the α3β1 integrin to form protrusions and stabilizes the leading lamellipodia [16] [70] [27], where the PTPIP51 protein, whilst binding to Rac-1, might take part. Another additional pathway is known of Rac-1 inducing filament growth through the IRsp53 (insulin receptor Tyr-kinase substrate p53) and the formin actin nucleator mammalian diaphanous 2 (DIA2), which link Rac to the actin nucleators WAVE2 and Ena/VASP-like protein [22], where the PTPIP51 protein also might play a key role here.

Consequently, a parallel induced pathway due to the injury and, furthermore, to EGF treatment could cause the additive effect on the PTPIP51/Rac-1 interactome upregulation under EGF treatment (see figure 32).

Interestingly, EGF treatment displayed an ambiguous effect on the PTPIP51/FAK interactome in migrating cells. The cells without any injury influence did not upregulate PTPIP51/FAK interactome by EGF treatment. The cells near the scratch upregulated the PTPIP/FAK interactions by a factor of 4.6 and the migrating cells by a factor of 3.3 due to EGF treatment. The FAK promotes migration as important linking protein between growth factor receptors and integrin signalling [63]. As complex with the Scr (Tyrosine kinase Scr) FAK also activates through GRB2/SOS the MAPK pathway [77]. The ambiguous results on the PTPIP51/FAK interactions with EGF-treated migrating cells could be the result of preventing an overshooting MAPK signalling. On the one hand, EGF treatment results in a higher phosphorylation of the Tyr176 residue, which inhibits the MAPK pathway [10][19].

On the other hand, if cells get stressed by the injury, where FAK gets activated, or the EGF/EGFR activation induces the FAK, the PTPIP51/FAK complex could focus on cell survival and proliferation [32] necessary for the needs of the cell, therefore, the highest increase of the PTPIP51/FAK interactome was detected in the cells near the scratch.

De Vos and colleagues reported the regulations characteristic of the PTPIP51 protein on the level of MAMs (mitochondria-associated membranes), connecting ER and mitochondria, thus regulating the intracellular Ca^{2+} levels [76]. Furthermore, it is reported that high Ca^{2+} in the rear (Figure 35 A) of the migrating cell facilitates the turnover of stable focal adhesion complexes [73]. Consequently, during wound healing, the interaction of PTPIP51 and FAK seems to focus on cell proliferation, regulating Ca^{2+} homeostasis, stabilizing focal adhesion and MAPK signalling is finely tuned to control the migration of cells [12].

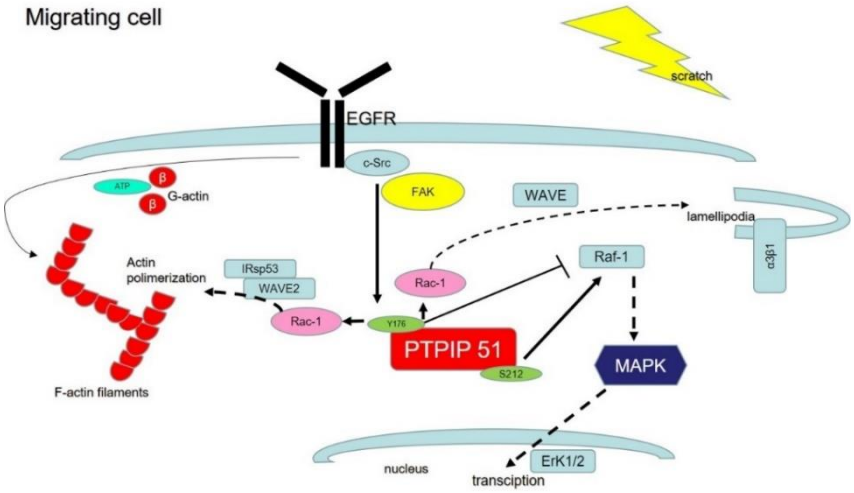


Figure 32: The cell gets activated by the scratch and the EGF (epidermal-growth-factor) treatment activates the EGFR (epidermal-growth-factor-receptor). The G-actin gets direct activated to form the F-actin filaments. Additionally, the PTPIP51 Tyr176 is upregulated which finely tunes the MAPK (mitogen-activated-protein-kinase) pathway, preventing an overshoot. Therefore, the PTPIP51/FAK interactions focus on cell survival and proliferation, where the PTPIP51/Rac-1 interactions stimulate two pathways. One is where PTPIP51/Rac-1 interactome induce through WAVE the α3β1 integrin and the other is additionally activation WAVE2/ WASF2 followed by the activation of the IRsp53 (insulin-receptor-substrate 53) for actin remodelling.

4.3 Framing the PTPIP51 in the migration process under insulin treatment

Insulin treatment results display differing adaptations of the PTPIP51 interactome either with Rac-1, FAK and β-actin.

The migrating insulin-treated cells displayed upregulation of PTPIP51/β-actin by a factor of 1.9 and the cells near the scratch display an even higher PTPIP51/ β-actin interactome upregulation by a factor of 4.6. The PTPIP51/Rac-1 interactome was upregulated in the cells without any influence of the injury due to insulin treatment by a factor of 11.6 and the cells near the injury showed a lower PTPIP51/ Rac-1 interactome upregulation by a factor of 2.2. The migrating cells, closing the wound bed, displayed an increased PTPIP51/Rac-1 interactome by the factor 4.6. The highest PTPIP51/FAK interactome upregulation was seen in migrating insulin-treated cells with a factor of 3.7, but the upregulation of the PTPIP51/FAK interactome in the cells near the scratch was significantly ($p < 0.0001$) with a factor of 3.6.

Consequently, the insulin-treated cells which were near the injury (expected proliferation zone) showed the highest PTPIP51/β-actin interactome upregulation, where the PTPIP51/FAK interactome was the most upregulated during migration but still high in the

proliferating cells. The insulin treatment induced the overall highest PTPIP51 interactome with Rac-1 in cells without any injury influence.

As mentioned before, the FAK protein regulates cell survival and adhesion through the stabilizing effect on integrins [32, 63]. Additionally, receptor bound insulin activates the autophosphorylation of downstream molecules [4] with activation of the MAPK pathway and results in the phosphorylation by ERK1/2, of various nuclear, cytosolic and cytoskeletal proteins [43]. The MAPK pathway is also activated by the activated FAK due to the integrin adhesion to ECM molecules [43]. Furthermore, the structure of FAK can be modified within its FERM domain by the small ubiquitin-related modifier (SUMO), which causes a nuclear enrichment of FAK [47]. However, insulin treatment upregulates the Tyr176 -phosphorylation status of PTPIP51, which reduces the interactions with 14-3-3 beta and Raf-1. Therefore the MAPK pathway is suppressed, and the formation of the spindle apparatus is increased by CGI-99 and Nuf-2 [3].

In consequence, PTPIP51 may, when activated by insulin through the IR, activates or stabilizes FAK on the adhesion-integrin level and/or on the SUMOylated nuclear level, whilst activated FAK induces cell motility and survival in migrating cells.

In addition, PTPIP51 could use the bypass (CGI-99 and Nuf-2) to the MAPK pathway and also prevent an overshoot of the MAPK pathway.

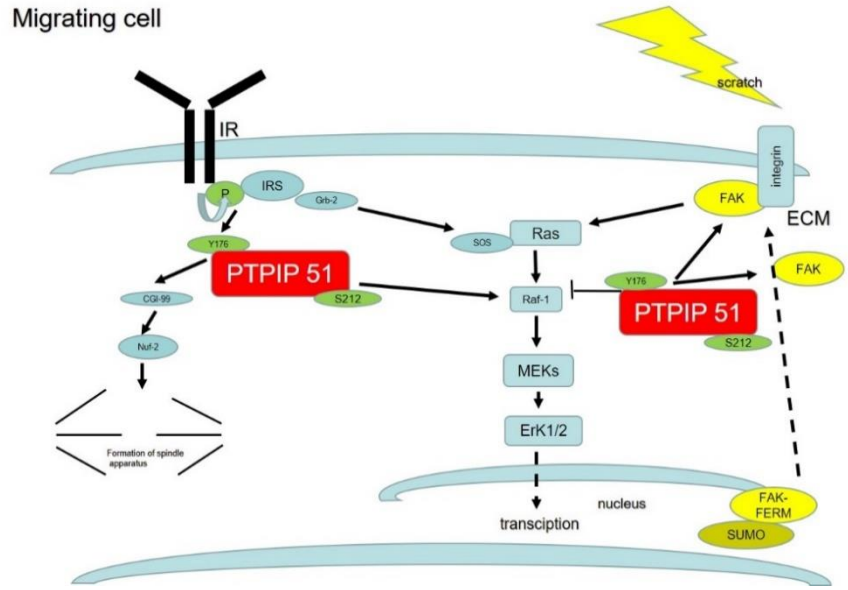


Figure 33: Insulin Receptor (IR) activity induces through PTPIP51 (176y) the formation of the spindle apparatus (= activation of CGI-99, followed by Nuf-2). PTPIP51 stabilizes FAK on integrin level and/or SUMOylated nuclear level, if phosphorylated on Tyr176 preventing overshoot of the MAPK pathway.

Yet, cells, representing the proliferation zone, displayed a drop of PTPIP51/Rac-1 interactome under insulin treatment and a peak of the PTPIP51/β-actin interactome. Both results suggest that the PTPIP51 interactome with Rac-1 and β-actin (G-actin) respectively plays a different role in these cells.

Insulin receptor (IR) activation leads amongst others in the PI3K activation, which in turn results into Rac-1 activation, which cause PAK1 (p21-activated kinase) activation and this plays a crucial role in the actin remodelling pathway [74]. Moreover, activated Rac drives the SCAR/WAVE complex to nucleate F-actin assembly at the leading edge, increasing the adhesion [55] and plays a crucial role in the PIP3-dependent feedback loop forming the leading edge in chemotaxis [65].

The high PTPIP51/Rac-1 interaction level in the migrating cells in this study, plus less upregulation of the PTPIP51/ β-actin interaction, puts PTPIP51 in the formation and assembly part on the leading edge. Consequently, PTPIP51 interacts with β-actin, preferably on the leading edge in proliferating cells, to form F-actin to provide the leading edge. During migration, the PTPIP51/Rac- 1 interactions are elevated, probably stabilizing the filament remodelling.

Additionally, the IR activity is also determined by the PTP1B phosphatase, which is known as a crucial protein for the dephosphorylation of the PTPIP51 [4, 19, 71]. PTP1B is able to dephosphorylate PTPIP51 at the Tyrosine 176 mark, which consequently restores PTPIP51/Raf-1 binding and, therefore, induces the MAPK pathway [10], which seems to maintain a level of insulin sensitivity [19].

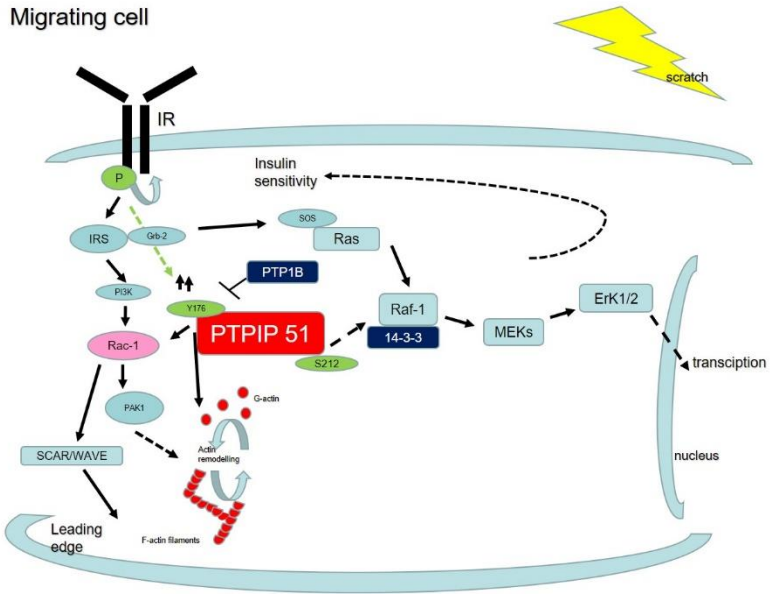


Figure 34: IR pathway. By autophosphorylation, PTPIP51 Tyr 176 gets upregulated (green dart). The PTPIP51/ Rac-1 interactome activates actin remodelling in migrating cells and the PTPIP51/β-actin interactome is highly elevated during proliferation to provide F-actin filaments on the leading edge. MAPK activity keeps insulin sensitivity and PTP1B might prevent the overshoot of F-actin polymerisation.

4.4 Framing PTPIP51 in the migration process under LDC-3 treatment

LDC-3 treatment profoundly changed the PTPIP51 interactome in migrating cells. The PTPIP51 interactome with all three investigated interacting partners (Rac-1, FAK and β-actin) displayed an augmentation with increasing LDC-3 concentrations (5µM, 50µM), but there was a slightly decrease with 100µM. Migrating and proliferating 50µM LDC-3-treated cells displayed high PTPIP51/ FAK- and PTPIP51/ Rac-1 interaction levels. Remarkably, the PTPIP51/ β-actin interactome differed in the outcome.

Increasing concentrations of LDC-3 reduced the PTPIP51/ β-actin interactions levels in non-manipulated cells (except under 5µM concentration) and in migrating cells in a dose dependent manner.

Dietel and Brobeil (et al.) [9, 21] have previously described the small aminothiazole LDC-3 as an effective small molecular inhibitor of PTPIP51. It is known to enhance the phosphorylation of the Tyr176 residue of PTPIP51 and, despite the high Tyr176 phosphorylation, PTPIP51 is forced into the PTPIP51/Raf-1/14-3-3β complex by LDC-3. Furthermore, LDC-3 blocks the Akt signalling and, by stabilizing the RelA/ IκB/ PTPIP51 interactome, LDC-3 is able to abolish the effects caused by TNFα stimulation [18].

Interestingly, the present study revealed that LDC-3-treated migrating cells displayed the lowest velocity in wound closure. Considering this new fact, the LDC-3 modulated PTPIP51 might support wound healing or reduce metastatic invasion.

When the PTPIP51/ β-actin interactome is reduced by LDC-3, the essential actin remodeling at the leading edge and, therefore, the F-filament growth and a forward locomotion are suppressed (here also seen by the reduced velocity).

Furthermore, this reduced migration was probably conveyed by the augmentation of the PTPIP51/ FAK interactome. If bound to FAK, PTPIP51 can stabilize the integrin signaling and form focal adhesion for cell survival. The PTPIP51/ FAK interactions increase by a factor of 4-5 in migrating cells. However, the PTPIP51/Rac-1 interactome upregulation during migration contradicts this hypothesis. When the actin remodelling is activated through the PTPIP51/Rac-1 interactome, followed by WAVE2 and the IRsp53 proteins, the forward locomotion on the leading edge can be forced too. These opposite theses are possibly explained by the time dependency. The longer LDC-3 incubates on the cells, the more the inhibitory effect of LDC-3 acts on the cells.

LDC-3 is a direct modulator of PTPIP51 interactome [21] in a dose - and time-dependent manner. PTPIP51 was forced to bind with FAK and Rac-1 during the migration process, but with progressing incubation time, migration was slowed down. It is well known [21] that PTPIP51 gets forced into the MAPK pathway due to longer incubation times, even if PTPIP51 shows a high phosphorylation status.

The regulation of the PTPIP51 interactome is modulated by its tyrosine and serin phosphorylation status. PTPIP51 normally displayed a reduced interaction when phosphorylated at Tyr176, and displayed under LDC-3 treatment an enhanced interaction with the Raf-1, despite its high Tyr176 residue phosphorylation status.

Therefore, regardless of its regulatory phosphorylation status, LDC-3 treatment led to a strongly enhanced MAPK binding affinity of PTPIP51 in a dose dependent manner [9]. Yet, a concentration of 100µM LDC-3 resulted in a decrease of PTPIP51 interactions with all respective binding partners (β-actin, FAK and Rac-1). Probably, the PTPIP51 interactome increased and decreased during the migration process according to the need of the cells. Lower LDC-3 concentrations activate PTPIP51/G-actin, as a consequence, F-filament growth is induced. This takes place in parallel to Rac-1 interaction when actin remodelling takes place and FAK activation stabilizes focal adhesion at the beginning in the migration process. Further on, cells get slower with time, and the PTPIP51 interactome is switched to the MAKP pathway to prevent an overshooting reaction.

This aspect, additional to the reduced velocity, demands further investigation of PTPIP51 under the influence of LDC-3 treatment during migration in tumourigenic and metastatic cell lines.

4.5 The PTPIP51 interactome within a migrating cell

This study reveals that the Protein-Tyrosin-Phoshophase-Interacting Protein 51 has a profound impact during proliferation and migration of the HaCaT cell line monolayers while they heal from injury. By the formation of the asymmetric structure, forming the leading edge [75] polarizing [44] the cell via calcium [73] shifts and stabilizes at the rear and front of the cell, creating filaments and preventing an MAPK overshoot, PTPIP51 takes a crucial regulating part by interacting with FAK, Rac-1 and β-actin. Figure 35 A illustrates a migrating cell and figure 35 B shows the PTPIP51 interactome with Rac-1, β-actin and FAK in more detail and under the treatment with EGF, insulin and LDC-3.

A

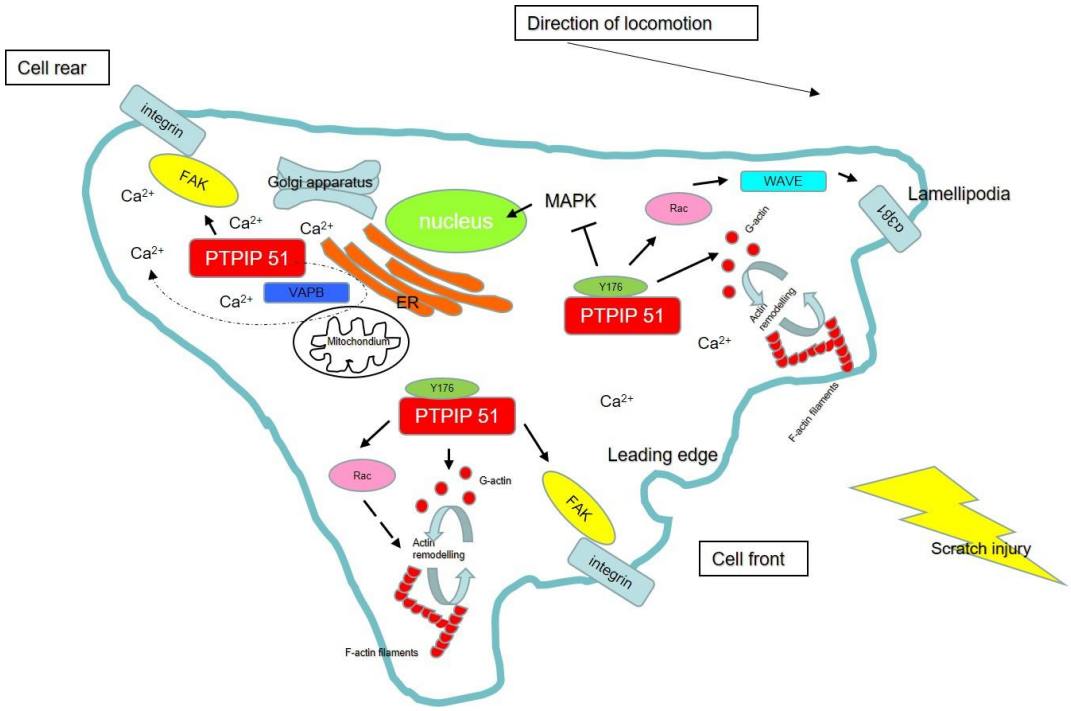


Figure 35: A: Schematic illustration of the migrating cell during wound healing. The cell front creates membrane extension and the cell forms an asymmetric structure: Furthermore, the cell polarizes and the leading edge and the lamellipodia create the adhesive expansion region. Meanwhile, the back of the cell stabilizes and the restructuring and the reorganisation of the cell can start. The PTPIP51 interacts with Rac-1 to create the actin remodelling and it interacts with β-actin (G-actin) for actual filament growth at the cell front. The PTPIP51 interacts at the back with VAPB and creates within the cell a Ca^{2+} concentration gradient. Additionally, the PTPIP5/ FAK interactions form according to the need of the cell, the adhesion complex with interacting with integrins for (re)attachments of the cell.

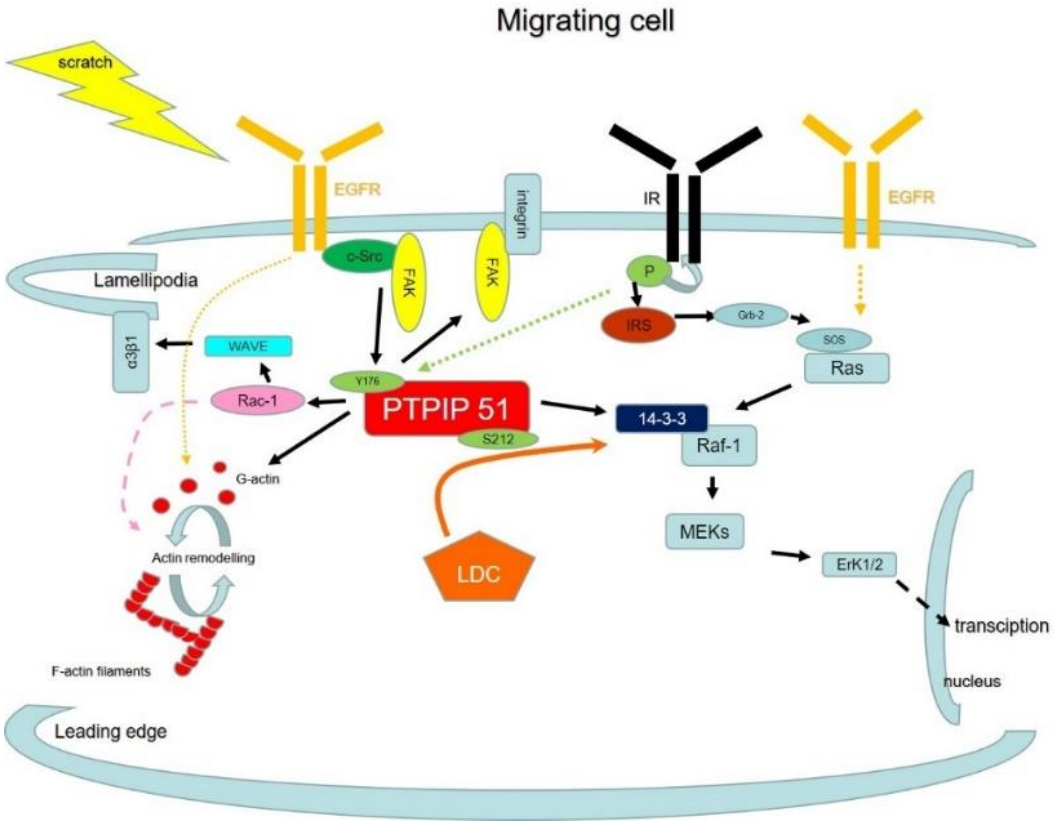


Figure 35 B: PTPIP51 interactome during the migration process: PTPIP51 with its tyrosin 176 residue and serin 212 residue (serin 46 resdidue not illustrated). After the scratch, cells start to migrate into the wound bed. The MAPK pathway, also known as the Ras-Raf-MEK-ErK pathway, is activated. Treatment with EGF through the EGFR leads to activation actin remodelling. Additionally, the PTPIP51 Tyr 176 interactome with FAK, Rac-1 and G-actin (also β-actin) is upregulated. Actin remodelling at the leading edge and the stabilizing effect through PTPIP51 probably takes place at the lamellipodia and integrin levels. Through autophosphorylation of the IR by insulin, the PTPIP51 interactome with Rac-1, FAK and β-actin is upregulated as well. The small molecule LDC-3 enhances PTPIP51 affinity to MAPK binding. The cell velocity decreases with LDC-3 treatment in time and dose dependency.

The importance of Rac and FAK [47] during wound healing [62], as well as the intergins and actin rearrangements [14], were discovered beforehand. The interesting findings that these cytoskeletal proteins actually interact with the scaffold protein PTPIP51 in HaCaT cells can also be an interesting part for future studies in carcinoma cell lines. For instance, Nowak (et al.) [20] published data about colon adenocarcinoma cells, where β-actin expression, actin cytoskeletal organization and the state of actin polymerization showed a distinct correlation to a metastatic capacity. Especially the fact that with LDC-

3 treatment, the locomotion of the HaCaT cells can be influenced through the PTPIP51 is of utmost interest concerning metastasis.

Other studies have shown the importance of macrophage migration, which can lead to heart failure when infected with SARS-Cov-2 [72]. Therefore, it is of utmost importance to study possible effectors reducing cellular migration. Here, investigations of the PTPIP51 interactome and the effector LDC3 in immune-responding cell lines are crucial as well.

5 Summary

The migration of cells in the human body is an important mechanism to support healing or to avoid inflammatory progresses. Therefore, the cells have to adapt and reorganise the cytoskeletal structure to actually create locomotion. The reorganisation and restructuring of the cytoskeleton filaments are regulated by the activation of specific actin binding proteins, of signal transductions pathways of membrane receptors and of kinases and phosphatases proteins. The protein tyrosine phosphates interacting protein (PTPIP51) regulates and facilitates with important signalling pathways, such as MAPK, calcium homeostasis, nuclear factor κB signalling and Akt signalling. PTPIP51 acts as a scaffold protein for signalling proteins, such as Her2 (human epidermal growth factor receptor 2), the EGFR (epidermal growth factor receptor), the IR (insulin receptor) and other scaffold proteins, e.g. 14.3-3-β. This review illuminates the PTPIP51 interactome in migrating HaCaT cells. The cells were grown to monolayers which were injured with a tip to induce the migration process. After the treatment with EGF (epidermal growth factor), insulin and LDC-3, the wound bed width was measured and the PTPIP51 interactome was detected by the Duolink proximity Assay and immunohistochemistry.

PTPIP51 seem to stabilize and induce crucial cytoskeletal proteins such as the FAK (focal adhesion kinase), β-actin (also known as G-actin) and Rac-1 (Ras-related C3 botulinum toxin substrate 1).

This study has shown that PTPIP51 is a very important interacting partner in migrating cells. When the cells were treated with the EGF, the PTPIP51/ Rac-1 interactions might induce the actin remodelling and also stabilize the lamellipodia itself through the integrins. Additionally, the PTPIP51/ G-actin interactome takes actual place of the F-actin filament growth on the leading edge. By treatment with insulin, the migration process gets induced through the PTPIP51/Rac-1 interactome, forming the F-actin filament through the PTPIP51/G-actin interactome on the leading edge, whereas the PTPIP51 stabilize the FAK on the integrin level plus prevents an overshoot of the MAPK pathway. The velocity of the migrating cells can be reduced by the dose and time dependency of LDC-3 treatment on the above-illustrated PTPIP51 interactome, plus prevents an overshoot of the MAPK activity.

Future studies will show the importance of the PTPIP51 interactome with the interacting partners Rac-1, FAK and β-actin in carcinoma cell lines.

Summary (German Version)

Der Migrationsprozess von Zellen im menschlichen Körper ist ein sehr wichtiger Mechanismus für die Wundheilung oder um Entzündungsprozesse zu verhindern. Die Zellen müssen sich dazu anpassen und erst durch die Umstrukturierung des Zytoskeletts wird die Vorwärtsbewegung erst möglich. Die Re-Organisation und die Umstrukturierung des Zytoskeletts passiert durch die Aktivierung spezifischer Actin Proteine, membranständige Rezeptoren, Kinasen und Phosphatasen und der darauffolgenden Signalkaskaden.

Das PTPIP51 reguliert und erleichtert wichtige Signalwege, wie den MAP-Kinase Weg, Akt-Signalweg, den nukleären κB-Weg und die Calcium Homöostase. Es dient als Gerüst und Überträger für verschiedene Signalproteine, wie den Her2, den EGFR, den IR und andere Signalproteine wie 14.3-3-β. Diese Dissertation illustriert nun die Bedeutung des PTPIPs51 in migrierenden HaCaT Zellen. Diese Zellen wurden zunächst zu einer nahezu konfluenten Einzel-Reihe- Zellschicht gezüchtet. Diese Schicht wurde mit einer Pipettenspitze verletzt, sodass die Migration dieser Zellen induziert wurde. Es folgte die Behandlung mit jeweils EGF, Insulin und LDC-3 im Inkubator. Die Breite des Kratzers wurde im 2-stündlichen Abständen gemessen. Bei meist vollständigem Verschluss des Wundbetts erfolgte das Abstoppen und durch DPLA und immunhistochemische Versuche wurde das PTPIP51-Interaktom mit den Bindungspartners, wie Rac-1, β-actin und FAK, die für die Migration wichtig sind, detektiert. Unter der Behandlung mit EGF scheint das PTPIP51/Rac-1 Interaktom einen Anteil am Actin-Umbauprozess und einen stabilisierenden Effekt an dem Lamellipodia zu haben. Zusätzlich scheint das PTPIP51/ β-actin Interaktom sich am führenden Ende der Zelle am F-Filament Wachstum zu beteiligen. Unter der Behandlung mit Insulin wies die PTPIP51 Interaktion mit FAK eher einen stabilisierenden Effekt auf Integrin-Ebene auf und zeigte die Regulation des MAPK Signalwegs, zusätzlich zum Aufbau des F-Filaments durch das PTPIP51/Rac-1 Interaktom.

Es konnte interessanterweise auch durch die Behandlung mit LDC-3 über das PTPIP51 Interaktom Einfluss auf die Geschwindigkeit der migrierenden Zellen gezeigt werden. In Zeit- und Konzentrationsabhängigkeit konnte über das PTPIP51 Interaktome die Geschwindigkeit der migrierenden Zellen reduziert werden und auch hier wurde eine potenzielle überschießende Signalkaskade des MAPK mithilfe von PTPIP51 reguliert. Diese Aspekte des PTPIP51 Interaktom mit Rac-1, β-actin und FAK stellen Ansatzpunkte für zukünftige Untersuchungen in Carcinom Zellen, vor allem in metastasierenden Zellen dar.

6 Table of Abbreviation

ATP: Adenosine triphosphate

DAPI: 4',6-Diamidin-2'-phenylindol-dihydrochlorid

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

FCS: Fetal calf serum

GSK3β: Glycogen synthase kinase 3 β

HaCaT: Spontaneously immortalized human keratinocyte cells

IR: Insulin receptor

IRS: Insulin receptor substrate

MAPK: Mitogen-activated protein kinases/ also ERK: extracellular signal-regulated kinases (ErK1/2), also MEK: Mitogen-activated protein kinase kinase

NFκB: Nuclear factor ´kappa-light-chain-enhancer´ of activated B-cells

PBS: Phosphate buffered saline (Buffer solution)

Raf: Rapidly accelerated fibrosarcoma (protein kinase family) belongs to MAPK pathway

Rac-1: Ras-related C3 botulinum toxin substrate 1

Ser 46: Serine site 46 of PTPIP51

Ser 212: Serine site 212 of PTPIP51

Src, also c-Src: Tyrosine kinase named Src

Tyr176: Tyrosine site 176 of PTPIP51

WASP: Wiskott-Aldrich Syndrome Protein also WAVE

7 Figures:

15 B: Measurements of the scratch gap taken every two hours under treatment with LDC-3 (5µM, 50µM, 100µM concentration), insulin and EGF and under controlled conditions. After these measurements, cells were fixated before the DPLA or immunostaining experiments started.

Figure 15 C: Mathematical velocity of migrating HaCaT cells. Collected data of gap width measurements with metric scale (see Figure 15 A) by inverted reflected-light microscopy.
Figure 16 A: Example of FAK/PTPIP51 immunostaining of a scratched region.
B: Example of the semi-quantification of the PTPIP51/Rac-1 interactome during migration. Zero (0) describes the cell groups without any manipulation by scratching; non-scratch (n-s) describes the cells surrounding the scratch; scratched (sc) describes the cells within the migration area. Significances are represented with **** of a p value <0.0001.

Figure 17 A: DPLA of the PTPIP51 for β-actin, Rac-1 and FAK in **untreated** HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with the respective interaction partner. Zero (0) describes the cells without any manipulation by scratching; non-scratch (n-s) describes the cells surrounding the scratch; scratched (sc) describes the cells within the migration area. Nuclei are stained with DAPI (blue colour). *n* = 3. Scale bar 20µm.

Figure 17 B Interaction profile of PTPIP51 with FAK, Rac-1, β-actin respectively during migration in control cells. Yellow bars: PTPIP51/FAK interactions, turquoise bars: PTPIP51/Rac-1 interactions and pink bars: PTPIP51/β-actin interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with **** of a p value <0.0001.

Figure 18 A: DPLA of the PTPIP51 for β-actin, Rac-1 and FAK in EGF-treated (1µg/ml) HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with the respective interaction partner. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Nuclei are stained with DAPI (blue colour). *n* = 3. Scale bar 20µm.

Figure 18 B: PTPIP51/ β-actin interactome in HaCaT control cells and in EGF-treated HaCaT cells (1µg/ml). Yellow bars: HaCaT cell controls, without EGF treatment. Violet bars: EGF-treated HaCaT cells. P/A: PTPIP51/ β-actin interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with ** p< 0.05 calculated by the Mann-Whitney-U-Test.

Figure 19: PTPIP51 interactome for Rac-1 in HaCaT control cells and under EGF treatment (1µg/ml). Yellow bars represent the control HaCaT cells and blue bars represent the HaCaT cells under EGF treatment. P/R represents PTPIP51/ Rac-1 interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with **p<0.0043, ****p<0.0001

Figure 20: PTPIP51 interactome for FAK in HaCaT control cells and under EGF treatment (1µg/ml). Yellow bars represent the control HaCaT cells and green bars represent the HaCaT cells under EGF treatment. P/F represents PTPIP51/ FAK interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with *p< 0.04.

Figure 21 A: DPLA of the PTPIP51 for β-actin, Rac-1 and FAK in insulin (0.5mg/ml) HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with the respective partner. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Nuclei are stained with DAPI. *n* = 3. Scale bar 20µm

Figure 21 B: PTPIP51 interactome for β-actin in HaCaT control cells under insulin treatment (0.5mg/ml). Yellow bars represent the control HaCaT cells and red bars represent the HaCaT cells while insulin was applied. P/A represents the PTPIP51/β-actin interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with **p<0.005, ***p<0.0002.

Figure 22: PTPIP51 interactome for Rac-1 in HaCaT control cells and under insulin treatment (0.5mg/ml). Yellow bars represent the control HaCaT cells and orange bars represent the HaCaT cells under insulin treatment. P/R represents PTPIP51/ Rac-1 interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with ****p<0.0001.

Figure 23: PTPIP51 interactome for FAK in HaCaT control cells and under insulin treatment (0.5mg/ml). Yellow bars represent the control HaCaT cells and magenta bars represent the HaCaT cells under insulin treatment. P/F represents PTPIP51/ FAK interactions. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with ****p<0.0001.

Figure 24: Cellular protein complex shift of PTPIP51 within three protein–protein interactions. Quantitative analysis of the DPLA of PTPIP51 with three different interaction partners in untreated controls and in the control area (0), in the surrounding of the scratch (n-s) and in migrating (sc) HaCaT cells (n = 3). The control values of three different PTPIP51 protein interactions were equalized to 100% (first stacked column) and the following columns show the standardized interaction values related to the controls (equaling 100%) for sc/ns/0 and for the insulin treatment and EGF treatment.

Figure 25: DPLA of the PTPIP51 for β-actin LDC-3 (5, 50, 100µM) treated HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with β-actin; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Nuclei are stained with DAPI n = 3. Scale bar 20µm

Figure 26: PTPIP51 interactome for β-actin in HaCaT control cells and with LDC-3 treatment (various concentrations: 5, 50, and 100µM). Yellow bars represent the zero (non-manipulated by scratch) groups of HaCaT cells. Purple bars represent the LDC-3-treated HaCaT cells in the surrounding area of the scratch and green bars represent the LDC-3-treated migrating HaCaT cells. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with **p<0.005 and *p<0.05 are described.

Figure 27: DPLA of the PTPIP51 for Rac-1 LDC-3-treated (5, 50, 100µM) HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with Rac-1.; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Nuclei are stained with DAPI. *n* = 3. Scale bar 20µm

Figure 28: PTPIP51 interactome for Rac-1 in HaCaT control cells and with LDC-3 treatment (various concentrations: 5, 50, and 100µM). Yellow bars represent the zero (non-manipulated by scratch) groups of HaCaT cells. Purple bars represent the LDC-3-treated HaCaT cells in the surrounding area of the scratch and green bars represent the LDC-3-treated migrating HaCaT cells. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances with ****p<0.0001 are described.

Figure 29: DPLA of the PTPIP51 for FAK LDC-3-treated (5, 50, 100µM) HaCaT cells. Each dot corresponds to a single interaction of PTPIP51 with FAK; non-scratch (n-s) describes the cells surrounding the scratch; scratched (sc) describe the cells within the migration area. Nuclei are stained with DAPI. *n* = 3. Scale bar 20µm.

Figure 30: PTPIP51 interactome for FAK HaCaT control cells and with LDC-3 treatment (various concentrations: 5, 50, and 100µM). Yellow bars represent the zero (non-manipulated by scratch) groups of HaCaT cells. Purple bars represent the LDC-3-treated HaCaT cells in the surrounding area of the scratch and green bars represent the LDC-3-treated migrating HaCaT cells. Zero (0): non-manipulated cells; non-scratched cells (n-s): cells surrounding the scratch; scratched cells (sc): migrating cells. Significances are represented with **p<0.005, ** p< 0.002 and ****p<0.0001.

Figure 31: Cellular protein complex shift of PTPIP51 within three protein–protein interactions. Quantitative analysis of the DPLA of PTPIP51 with three different interaction partners in untreated controls and in the control area (0), in the surrounding of the scratch (n-s) and in migrating (sc) HaCaT cells (*n* = 3). The control values of three different PTPIP51 protein interactions were equalized to 100% (first stacked column) and the following columns show the standardized interaction values related to the controls (equaling 100%) for sc/ns/0 and for the LDC-3 treatment with the concentration of 5µM, 50µM and 100µM.

Figure 32: The cell gets activated by the scratch and the EGF (epidermal-growth-factor) treatment activates the EGFR (epidermal-growth-factor-receptor). The G-actin gets direct activated to form the F-actin filaments. Additionally, the PTPIP51 Tyr176 is upregulated which finely tunes the MAPK (mitogen-activated-protein-kinase) pathway, preventing an overshoot. Therefore, the PTPIP51/FAK interactions focus on cell survival and proliferation, where the PTPIP51/Rac-1 interactions stimulate two pathways. One is where PTPIP51/Rac-1 interactome induce through WAVE the α3β1 integrin and the other is additionally activation WAVE2/ WASF2 followed by the activation of the IRsp53 (insulin-receptor-substrate 53) for actin remodelling.

Figure 33: Insulin Receptor (IR) activity induces through PTPIP51 (176y) the formation of the spindle apparatus (= activation of CGI-99, followed by Nuf-2). PTPIP51 stabilizes FAK on integrin level and/or SUMOylated nuclear level, if phosphorylated on Tyr176 preventing overshoot of the MAPK pathway.

Figure 34: IR pathway. By autophosphorylation, PTPIP51 Tyr 176 gets upregulated (green dart). The PTPIP51/ Rac-1 interactome activates actin remodelling in migrating cells and the PTPIP51/β-actin interactome is highly elevated during proliferation to provide F-actin filaments on the leading edge. MAPK activity keeps insulin sensitivity and PTP1B might prevent the overshoot of F-actin polymerisation

Figure 35: A: Schematic illustration of the migrating cell during wound healing. The cell front creates membrane extension and the cell forms an asymmetric structure: Furthermore, the cell polarizes and the leading edge and the lamellipodia create the adhesive expansion region. Meanwhile, the back of the cell stabilizes and the restructuring and the reorganisation of the cell can start. The PTPIP51 interacts with Rac-1 to create the actin remodelling and it interacts with β-actin (G-actin) for actual filament growth at the cell front. The PTPIP51 interacts at the back with VAPB and creates within the cell a Ca^{2+} concentration gradient. Additionally, the PTPIP5/ FAK interactions form according to the need of the cell, the adhesion complex with interacting with integrins for (re)attachments of the cell.

Figure 35 B: PTPIP51 interactome during the migration process: PTPIP51 with its tyrosin 176 residue and serin 212 residue (serin 46 resdidue not illustrated). After the scratch, cells start to migrate into the wound bed. The MAPK pathway, also known as the Ras-Raf-MEK-ErK pathway, is activated. Treatment with EGF through the EGFR leads to activation actin remodelling. Additionally, the PTPIP51 Tyr 176 interactome with FAK, Rac-1 and G-actin (also β-actin) is upregulated. Actin remodelling at the leading edge and the stabilizing effect through PTPIP51 probably takes place at the lamellipodia and integrin levels. Through autophosphorylation of the IR by insulin, the PTPIP51 interactome with Rac-1, FAK and β-actin is upregulated as well. The small molecule LDC-3 enhances PTPIP51 affinity to MAPK binding. The cell velocity decreases with LDC-3 treatment in time and dose dependency.

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