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Oral-examination:

Development and application of a high throughput cell based assay to identify novel modulators of ERK1/2 activation and,
Functional characterisation of the candidate Radial spokehead like (Rshl1)

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To my Grandfather

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Summary

The aim of my project was to identify and functionally characterise novel human proteins that influence cancer relevant cellular processes like cell proliferation, signalling, and apoptosis upon over-expression. The focus of my work was 1) The establishment of a high throughput cell based assay to screen for proteins involved in the modulation of cell signalling pathways, specifically the activation of the ERK1/2 pathway, 2) to apply this assay in a screen of previously uncharacterised proteins, and 3) to characterise one candidate protein from this assay and to validate its association with the ERK1/2 pathway.

The principle of the assay is based on the detection of phosphorylated ERK1/2 in cells over-expressing N- and C-terminal YFP tagged proteins. Data acquisition was done using a flow cytometer with an integrated 96-well plate reader. A total of 200 proteins were screened, out of which eleven novel cancer relevant modulators of ERK1/2 activation were identified.

One of the candidates, the Radial spoke head like -1 (Rshl1), which was identified as an inhibitor of ERK1/2 activation was followed up, and shown to be down regulated in kidney cancer. The protein was identified as an inhibitor of proliferation in another cell based assay. The corresponding gene is located on chromosome 19q13.3 at the primary ciliary dyskinesia locus, and the encoded protein contains a radial spoke domain. However, the biological role of this protein was not described. I found that Rshl1 indeed localizes to primary cilia but also to the cytoplasm and nucleus of human kidney cells. Further, I found that its localisation is cell cycle phase dependent. Rshl1 co-localised with MEK1, ERK1/2 and CDK2 and interacts with MEK1, CDK2 and ERK3. Its role as an inhibitor of proliferation was elucidated by the finding that over-expression of Rshl1 caused a G0/G1 phase arrest in human kidney cells via an up-regulation of $p57^{KIP2}$ expression and stabilization of ERK3. Rshl1 thus regulates the cell cycle by inhibiting the ERK1/2 kinase. It interacts with critical signalling proteins in the cell and maintains homeostasis by arresting cells in the G0/G1 phase.

In conclusion, I screened 200 novel proteins for their influence on ERK1/2 activation and identified eleven novel modulators of ERK1/2 pathway. Detailed functional analysis of Rshl1, which was an inhibitor of ERK1/2 activation, identifies this protein as a novel player in the MAPK pathway, and shed light on its role in homeostasis and tumorigenesis.

Zusamenfassung

Das Ziel dieses Projektes war die Identifizierung und funktionelle Charakterisierung unbekannter Proteine die, nach Überexpression, Krebs-relevante zelluläre Prozesse wie z.B. Proliferation, Signaltransduktion und Apoptose beeinflussen. Der Fokus meiner Arbeit lag in der Etablierung eines zellbasierten Hochdurchsatz-Assays zur Untersuchung von Proteinen auf die Modulation von Zell-Signalwegen, im Speziellen der Aktivierung des ERK1/2-Signalweges. Das Prinzip des Assays basiert auf der Detektion der phosphorylierten Form von ERK1/2 in Zellen, die Fusionsproteine mit N- und C-terminalem YFP überexprimieren. Die Datenaufnahme wurde mit einem Durchflußzytometer mit integriertem 96-Well-Platten Lesegerät durchgeführt. Insgesamt wurden 200 Proteine untersucht, von denen schließlich sieben als Krebs-relevante ERK1/2-Modulatoren identifiziert wurden. Einer der Kandidaten, das Radial Spoke Head Like-1 (Rshl1) Protein, welches als Inhibitor der ERK1/2 Kinase identifiziert wurde, habe ich im Rahmen meiner Arbeit funktionell charakterisiert. In vorherigen Studien wurde gezeigt, dass Rshl1 in Nierenkrebs herunter reguliert ist und es wurde als Inhibitor der Proliferation beschrieben. Das Rshl1-Gen ist auf Chromosom 19q13.3 im Primary Ciliary Dykinesia Lokus lokalisiert und das Protein enthält eine Radial-Spoke-Domäne, jedoch ist die biologische Funktion bisher nicht bekannt.

In der vorliegenden Studie habe ich die Lokalisation des Rshl1 Proteins in primären Cilien, im Cytoplasma und im Kern von Nierenzellen nachgewiesen und konnte eine Zellzyklus-abhängige Lokalisation feststellen. Ich habe gezeigt, dass Rshl1 mit den Proteinen MEK1, ERK1/2 und CDK2 co-lokalisiert und habe seine direkte Interaktion mit MEK-1, CDK2 und ERK3 nachgewiesen. Seine Rolle als Inhibitor der Proliferation wurde durch die Blockade von Nierenzellen mit Rshl1-Überexpression in der G0/G1-Phase des Zellzyklus, sowie durch die verstärkte Expression des Zellzyklus-Repressors $p57^{KIP2}$ und die Stabilisierung von ERK3 erläutert. Diese Studie zeigt somit zum ersten Mal, dass Rshl1 den Zellzyklus durch die Inhibierung der ERK1/2-Kinase reguliert. Es interagiert mit Schlüsselproteinen der Signaltransduktion und erhält das Gleichgewicht während der G0/G1-Phase des Zellzyklus. Zusammengefasst habe ich 200 Proteine auf ihren Einfluss auf die ERK1/2-Aktivierung untersucht und sieben neue Modulatoren des ERK1/2-Signalweges identifiziert. Die Ergebnisse aus der detaillierten funktionellen Analyse des Proteins Rshl1, für das eine Inhibierung des ERK1/2-Siganlweges nachgewiesen wurde, bestätigt die Stärke und Effizienz dieses Ansatzes und hebt die Bedeutung einer solchen Untersuchung im Rahmen der funktionellen Genomanalyse hervor.

1 Introduction

Eukaryotic cells respond to a variety of extra-cellular stimuli by transducing extra-cellular signals mostly via cell-surface receptors to cytoplasmic and nuclear molecules. Key processes like cell division, growth and differentiation, and cell death mechanisms are regulated through so called signal transduction pathways. There, the transmission of extra-cellular signals to their intracellular targets is mediated by a network of interacting proteins. Among the intracellular signalling pathways that have been identified to date, growth factor stimulation of intracellular events is of particular interest because altered regulation of the processes regulated by these factors often leads to cellular transformation or altered proliferation.

Growth factor signals are transmitted via their transmembrane receptors, which upon stimulation can activate several signalling pathways leading to an array of responses. The stimuli can be rather diverse in nature, comprising several distinct classes of biological molecules that include hormones, growth factors, cytokines, osmotic stimuli and UV light. The response generated by these stimuli is often overlapping and is modulated by regulatory mechanisms operating within the cell. However, the pathways not only operate within their modules but also interact and affect other pathways, thus forming networks with cross talk between different pathways. Perturbations in these pathways, for example mutations, can cause abnormal functioning of more than one cellular process and in consequence may lead to diseases, including cancer.

1.1 The Mitogen Activated Protein Kinase pathway

The Mitogen Activated Protein Kinase (MAPK) pathway is one of the central pathways that is highly conserved from primitive to higher eukaryotic organisms [2]. MAPK modules in general consist of three distinct kinases that are arranged in a linear cascade, and the generic arrangement is similar in lower eukaryotes like yeasts and in mammals (Fig 1.2). The nomenclature and homology between these kinases however differs from species to species.

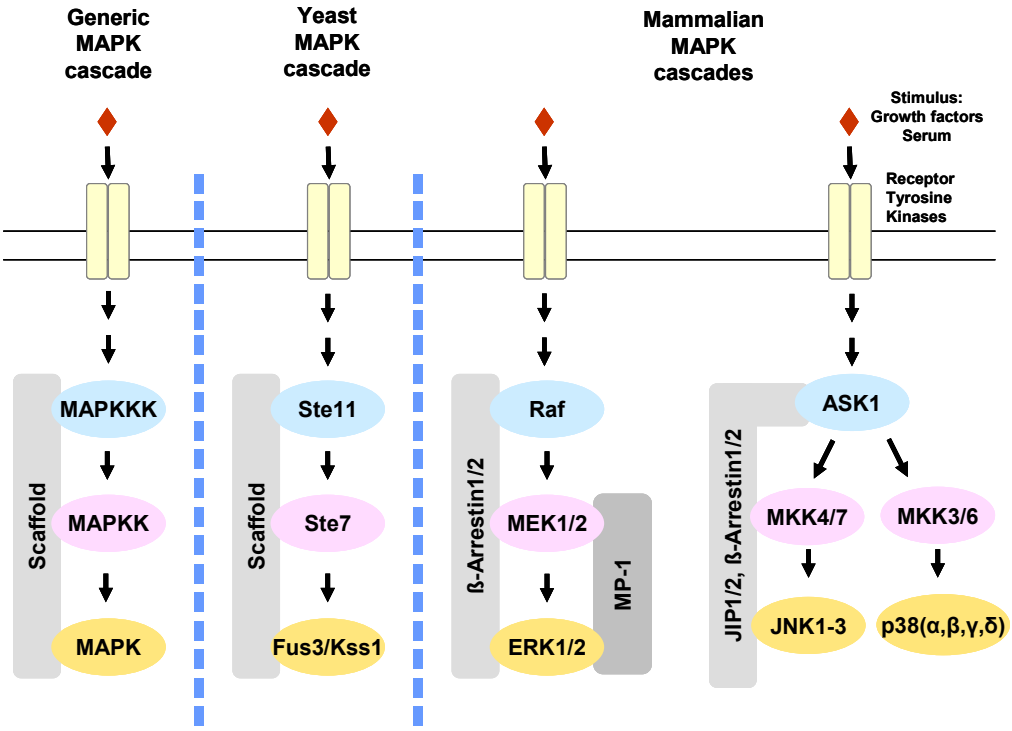


Figure 1.2: The MAPK cascade. The mitogen activated protein kinase pathway is highly conserved and plays an important role in a variety of cellular processes from proliferation to apoptosis. The pathway is made up of a cascade of three protein kinases – the MAPK Kinase Kinase (MAPKKK), the MAPK Kinase (MAPKK or MEK) and the MAPK. The mammalian MAP Kinases can be divided into three distinct classes; a) Extra-cellular signal regulated MAPK (ERK) – which responds to extra-cellular stimuli like growth factors or mitogens. b) Jun-associated protein kinase or Stress associated protein kinases (SAPK) and c) the p38/HOG MAP Kinase (Figure adapted from [1]).

The mammalian MAPK kinases can be distinguished into 3 major categories, a) The extra-cellular signal regulated MAP Kinases or ERKs and b) the Stress associated protein kinases or SAPKs, also called the c-Jun associated protein Kinase (JNK) and c) the p38 MAPK. The ERKs are involved in signalling mechanisms that lead to cell growth and survival [3]. They are involved in proliferation, differentiation and development. The SAPK and the p38 MAPK are involved in response to stress and they play a role in the induction of apoptosis and influence development and other cellular processes [4].

1.1.1 The Extra-cellular signal regulated Kinase (ERK) pathway and its mediators

The Extra-cellular signal regulated kinases are a group of MAPKs that are activated in response to extra-cellular stimuli. Several isoforms of ERK (1-8) have been reported [5]. However, ERK1 and ERK2 are the most studied due to their ubiquitous expression and to their indispensable role in a variety of cellular processes. ERK1/2 and the mediators of the

pathway have been the focus of numerous studies especially because of their involvement in various cancer relevant processes for e.g. adhesion, differentiation, proliferation and apoptosis.

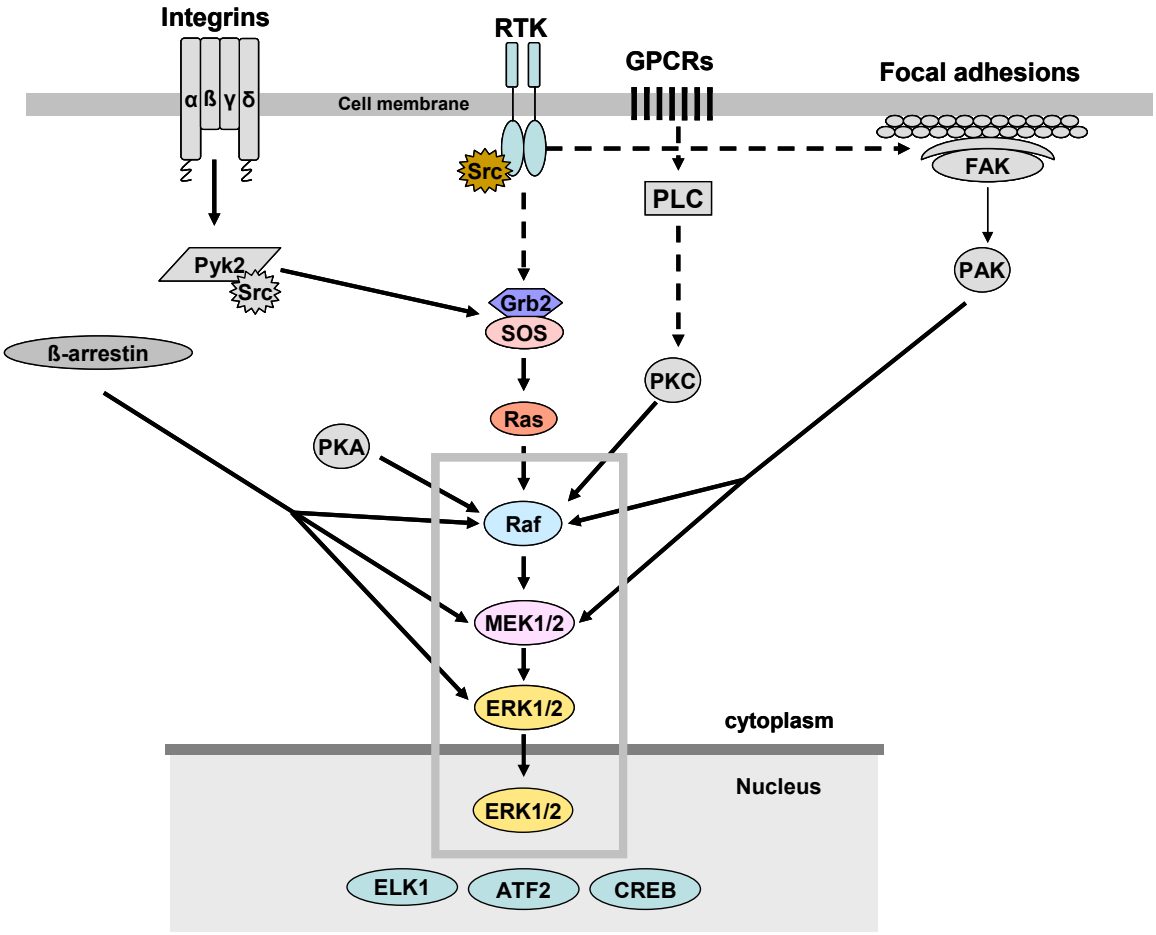


Figure 1.3: Schematic representation of the ERK1/2 pathway. The ERK pathway responds to extra-cellular stimuli like growth factors and mitogens. These signals are received at the cell surface with the help of receptor tyrosine kinases or GPCRs and then transferred into the cell, there recruiting Ras and then the ERK1/2 cascade. Raf is also activated by a variety of proteins like PAK, PKC and PKA. Raf then specifically activates MEK which in turn phosphorylates ERK1/2. Phosphorylation activates ERK1/2 and induces nuclear translocation followed by activation of transcription factors, e.g. ELK-1, that induce expression of genes necessary for growth and cell cycle progression.

The mammalian ERK1/2 module, also termed as the classical mitogen activated protein kinase cascade, responds primarily to growth factors and stimulates transcriptional responses in the nucleus. Growth factors, through receptor tyrosine kinases (RTKs, eg: EGFR, erbB2 etc.), G-protein coupled receptors (GPCRs), or other cell surface proteins like Integrins, recruit a large network of signalling proteins to execute their cellular programs. These receptor proteins then recruit an array of proteins that include adaptor proteins, like GRB2. The GRB2 binds to SOS, a nucleotide exchange factor which stabilizes the GTP bound form of Ras thus maintaining it active. Ras is a 21kDa protein with GTPase activity.

Activation mutations in Ras isoforms are present in almost one-third of all cancers. After becoming activated through Ras, Raf then moves away from the membrane into the cytoplasm where it phosphorylates MEK1/2 specifically. MEK1/2 are dual specificity protein kinases that can phosphorylate Ser/Thr and Tyrosine residues targeting a Thr-X-Tyr motif on ERK1/2. While the activation of MEK1/2 is specifically via Raf alone, the activation of Raf can occur via proteins other than Ras. Proteins kinases like PKA, PKC and PAK are also capable of activating Raf (Fig: 1.3). Activation of MEKs and ERKs is further modulated by scaffolding proteins, like ß-arrestin by bringing the components of the module together and thereby increasing the efficiency of signal transduction.

1.1.2 Cytosolic substrates

ERK1/2, once activated, target a variety of proteins both in the cytoplasm and in the nucleus leading to the activation of proteins involved in different cellular processes. Cytosolic substrates for ERK include several pathway components (Fig: 1.4). Multiple residues on SOS are phosphorylated by ERK following growth factor stimulation. MAPK-interacting kinase 1 (MNK1) and MNK2 are cytosolic Ser/Thr protein kinases initially discovered in two-hybrid screens for ERK-interacting proteins [6]. MNKs are known to be activated by both ERK and p38. Active MNKs regulate eukaryotic initiation factor-4E (eIF-4E) that binds to 7-methylguanosine cap structures, directing ribosomes to the 5' ends of mRNAs and enhancing translation efficiency [7]. ERK1/2 regulate transcription indirectly by phosphorylating the 90kDa ribosomal protein S6 kinases (RSKs), a family of broadly expressed Ser/Thr kinases activated in response to mitogenic stimuli, including growth factors and tumor-promoting phorbol esters [8, 9]. RSKs are solely phosphorylated by ERK1/2 [10]. Active RSKs appear to play a major role in transcriptional regulation, translocating to the nucleus and phosphorylating such factors as the product of proto-oncogene c-*fos*, serum response factor (SRF), and cyclic AMP response element-binding protein (CREB) [11, 12]. ERK2 is also known to phosphorylate MAPK phosphatase 3 (MKP-3) [13, 14]. Cytoskeletal proteins like paxillin, FAK, MLCK and Calpain are also targets of ERK1/2 [15-18].

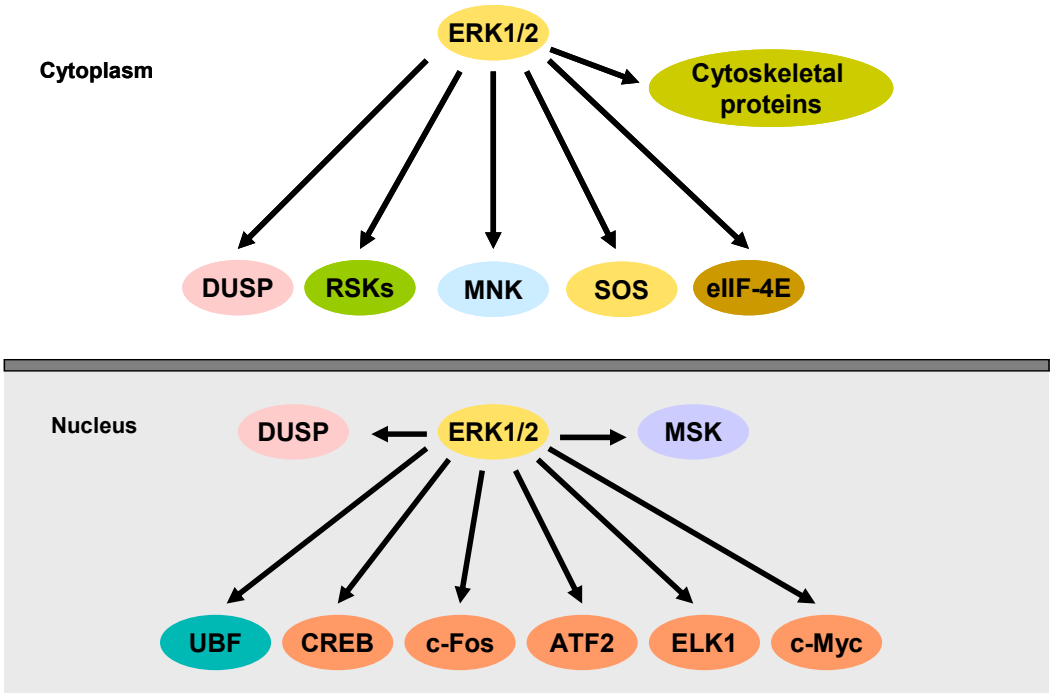


Figure 1.4: ERK1/2 substrates. ERK1 and ERK2 are kinases that phosphorylate their substrates rendering them either active or inactive. Certain substrates need to be phosphorylated in order to perform their cellular function and other become inactive. Substrates of ERK are distributed in the cytoplasm and the nucleus. Cytoplasmic targets include SOS, RSK, phosphatases (DUSP/MKP), MNK etc. RSK and MNK are proteins that are activated by ERK phosphorylation and activate their down stream targets. MKPs on the other hand are stabilized by ERK phosphorylation and further inhibit ERKs by dephosphorylating them both in the nucleus.

1.1.3 Nuclear Targets of ERK1/2

Upon phosphorylation, ERK1 and ERK2 translocate into the nucleus. Nuclear translocation of ERK1 and ERK2 is critical for both gene expression and DNA replication induced by growth factors [19]. In the nucleus, ERK phosphorylates an array of targets, including transcription factors and a family of RSK-related kinases, the mitogen- and stress-activated protein kinases (MSKs) [20] (Fig: 1.3). MSK1 and MSK2, activated by both ERK and p38, share the same tandem kinase structure as the RSKs, and also appear to be activated by sequential phosphorylation following MAPK docking. MSKs phosphorylate and activate the AP-1 component ATF1 and may be more important *in vivo* than RSKs in CREB phosphorylation. [21, 22].

The best-characterised transcription factor substrates of ERKs are ternary complex factors (TCFs), including Elk-1, which is directly phosphorylated by ERK1 and ERK2 at multiple sites [23]. Upon complex formation with serum response factor (SRF), phosphorylated TCFs transcriptionally activate numerous mitogen-inducible genes regulated

by serum response elements (SREs) [24]. The ERK pathway has been reported to directly link growth factor signalling to ribosome biogenesis. Following serum induction, ERK phosphorylates the BRF1 subunit of RNA polymerase (pol) III-specific transcription factor TFIIIB, both *in vitro* and *in vivo*, at an unknown site [25]. Phosphorylation of this pol III subunit enhances translational efficiency, inducing tRNA and 5s rRNA synthesis.

1.2 Regulation of ERK1/2 pathway

The ERK1/2 pathway is a cascade with at least three levels, MAPKKK (Raf), MAPKK (MEK) and the MAPK (ERK1/2) (Fig: 1.2). It is susceptible to regulatory inputs at multiple levels within the cascade as well as via multiple mechanisms. Important and well known mechanisms of regulation are described below.

1.2.1 Regulation via stimulus intensity and duration

The timing and duration of a stimulus has a direct effect on the type of specific response that cells make to a particular signal. A sustained or transient activation of ERK would determine whether a cell undergoes differentiation or proliferation. For example, in PC12 cells, epidermal growth factor (EGF) transiently stimulates ERK1/2 leading to cellular proliferation. In contrast, nerve growth factor (NGF) stimulation leads to the sustained activation of ERK1/2 and subsequently leads to neuronal differentiation. It has been shown that both the magnitude and longevity of MAPK activation governs the nature of the cellular response [26].

1.2.2 Regulation by Raf specificity

Since the biological outcome is determined by the strength and duration of the activation of this pathway, it is tightly regulated with the most intricate controls operating at the level of Raf [27]. Raf activation is a consequence of its binding to Ras and subsequent complex changes in phosphorylation. Although all three Raf isoforms can interact with Ras, there are important differences. For instance, Ras binding alone is sufficient to activate B-Raf, but not Raf-1 or A-Raf, both of which require secondary signals [28]. Rap1, a Ras-related G protein, has been reported to activate B-Raf but to inhibit Raf-1 [29]. These examples indicate that the ERK1/2 pathway has a complex but effective regulation mechanism at the level of Raf.

1.2.3 Regulation by cellular localisation

ERK activity has also been reported to be regulated by its localisation. ERK1/2 usually are bound to MEK1/2 and present in the cytoplasm, sequestered from the nuclear targets thus preventing unnecessary activation and cell proliferation. Upon activation, the ERK quickly diffuse into the nucleus and are retained there by anchoring proteins [30]. Retention of ERK within the nucleus sequesters ERK from active MEK that has a strong nuclear export signal and hence is present in the cytoplasm. Membrane bound, cytoplasmic and ERK present in the form of complexes with scaffolding proteins, can all be activated by MEK. However, the amount of activation and the accessibility is highly dependant on its localisation. For e.g.; the cytoplasmic ERK is more readily activated when compared to the membrane bound [31], but the ERK in complexes with scaffolding proteins has a stoichiometric advantage for activation or inhibition depending on the nature of the scaffolding protein [2].

1.2.4 Regulation by scaffolding proteins

Several isoforms of Raf, MEK, and ERK exist. This suggests that a combination of different isoforms of these proteins could determine the specific biological response to a given extracellular stimulus. Cells may express more than one isoform of each signalling component in parallel, therefore mechanisms that coordinate the assembly and localisation of specific active signalling complexes must exist. The identification of scaffold proteins which help to assemble MAPK pathway components into a localized signalling complexes explains how this coordination could be achieved [32]. ERK activation is a chain reaction that results in the formation of large multimeric signalling complexes. The scaffolding protein MP1 (MEK partner 1) specifically binds MEK1 and ERK1, thus favouring activation of ERK1 but not ERK2 [33]. Another scaffolding protein, the Kinase suppressor of Ras (KSR), also binds Raf, MEK and ERK forming an active signalling complex in stimulated cells [34]. These findings clearly indicate the role of scaffolding proteins and their importance in the regulation of the ERK1/2 pathway.

1.2.5 Regulation by phosphatases

Phosphatases also play another key role in the regulation of ERK activity. ERKs are activated by phosphorylation and hence are inactivated by de-phosphorylation via single and dual specificity protein phosphatases. These dual specificity phosphatases can dephosphorylate Serine/Threonine as well as Tyrosine in contrast to the single specific

phosphatases that either dephosphorylate Serine/Threonine or Tyrosine residues. MAPK phosphatase-1 (MKP-1) and MAPK phosphatase-2 (MKP-2) are known to de-phosphorylate all MAPKs, however, MKP-1 is found only in the nucleus and MKP-2 is distributed both in the nucleus and the cytoplasm. MKP-3 inactivates ERK2 with a greater affinity and is found predominantly in the cytoplasm [35].

Activation of ERK1/2 also leads to the nuclear accumulation of ERK1/2, apart from cytoplasmic redistribution, where ERK1/2 are de-phosphorylated. Some phosphatases also act as nuclear anchors for ERK1/2 and retain them in the nucleus thus sequestering them from MEKs in the cytoplasm and in consequence terminating the signalling event in the nucleus [36]. The ERKs also phosphorylate phosphatases thereby stabilizing them and delaying their degradation by the proteasome pathway.

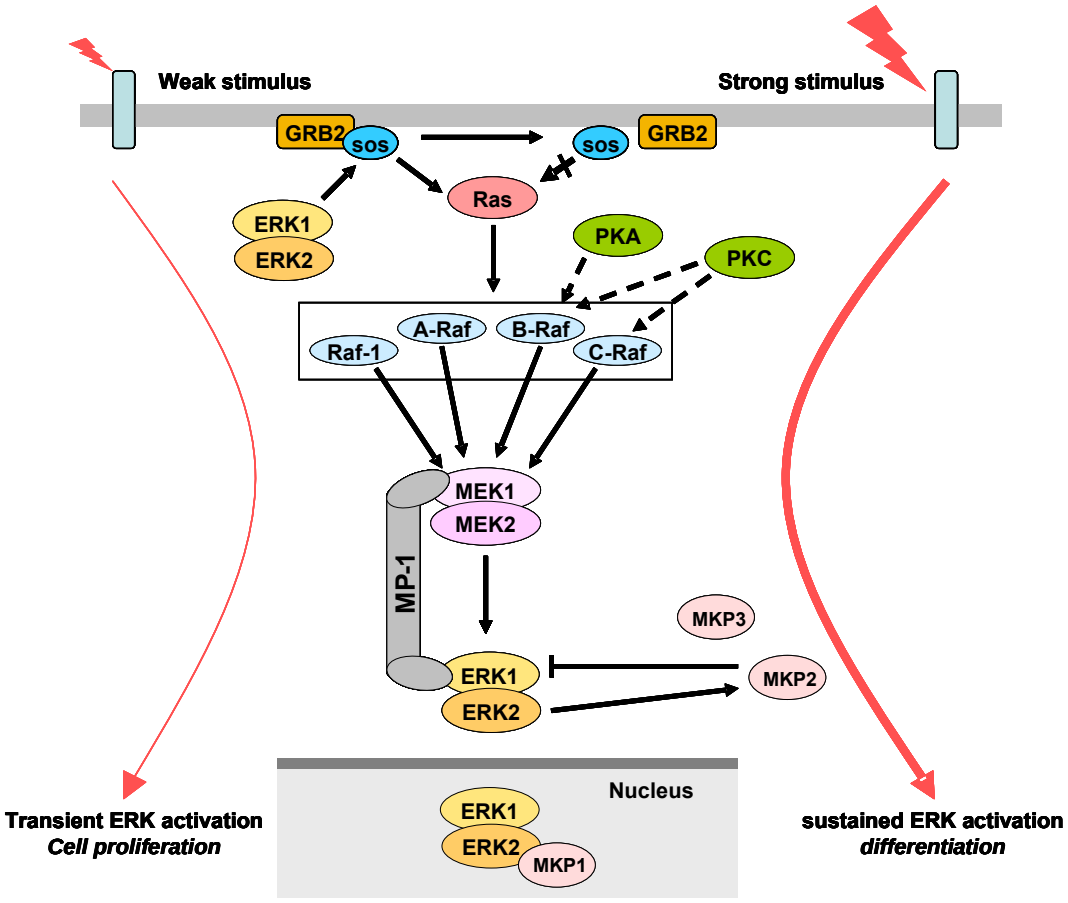


Figure 1.5: Regulation of the ERK1/2 pathway. ERK1/2 pathway is regulated by multiple mechanisms at different levels of the cascade. At the level of Raf, regulation is brought about by differential specificity of Ras and other kinases like PKA and PKC towards the different isoforms. The outcome of the signal thus depends on the isoform of Raf that is activated. Signal intensity and duration also influence ERK1/2 activation and in turn decide the fate of the cells. Scaffolds also play an role in regulation. MP1, for e.g. binds Mek1 and ERK1 and favors activation of ERK1 to ERK2. ERK1/2 phosphorylate DUSP, which is stabilized due to the phosphorylation and then inactivates ERK1/2. This is an example of the feed back mechanisms that are also involved in the regulation of ERK1/2 pathway.

1.2.6 Regulation by feed back inhibition

ERK1/2 pathway is also regulated by feed back inhibition mechanisms. For example, ERK1/2 phosphorylates SOS which in turn destabilizes the SOS-Grb2 complex. This eliminates SOS recruitment to the plasma membrane and interferes with Ras activation of the ERK pathway. ERKs are also part of a negative feed-back loop as they phosphorylate MKPs, thus reducing degradation of these phosphatases through the ubiquitin-directed proteasome complex and stimulating their own inactivation [14].

1.2.7 Cross talk between signalling pathways

Specificity of the ERK signalling pathway is the highest at the level of MEK where MEK1/2 specifically phosphorylate ERK1/2 [37]. However, upstream to MEK, members of different pathways can activate Raf. For example, it has been shown that GPCR mediated activation of PKC and PKA lead to phosphorylation of Raf isoforms independent of Ras (Fig: 1.3) [29]. In the same way, ATF-2 is activated both by p38 and ERK [38]. It has also been described that ERKs can activate the JAK/STAT pathway [39].

1.3 Physiological roles of ERK1/2 cascade

1.3.1 Proliferation and Cell cycle

There is a correlation between extra-cellular agents that lead to cell proliferation and stimulation of components of the MAPK cascade. Numerous publications describe the activation of Raf-1, MEKK, MAPKK, and MAPK in response to various mitogenic signals. Direct evidence using mutants of the various components has also been used to link the cascade to cellular proliferation [3, 40-43]. It has been reported that an inactive mutant of Raf interferes with cell proliferation, and a constitutively-activated Raf-l has an accelerated effect on cell proliferation. Raf-1 is both sufficient and necessary to activate a subset of early and late growth response genes [44]. Similarly, mutations in the regulatory domain of MEK and ERK have been used to show a direct linkage of these enzymes to proliferation. Over-expression of non-activatable forms of MEK-1 in NIH-3T3 cells significantly reduced their rate of proliferation, which was correlated to a similar reduction in MAPK activity. Constitutively activated MEK-1 raised the basal ERK activity and caused accelerated proliferation. It was also demonstrated that a dominant negative form of ERK-1 and its anti-

sense cDNA, caused a reduction in the number of cells and reduced their ability to proliferate [42]. Though all the data suggest the importance of ERK in proliferation, activation does not always lead to proliferation. ERK activation has also been associated to cell differentiation [45], which is often accompanied by growth arrest. A biphasic activation of two ERKs during the cell cycle in CHO cells has been reported [46]. In these cells ERK-1 and ERK-2 showed enhanced activities in the G1 through S and G2/M phases and were activated bi-phasically in the G1 phase and around the M phase (Fig: 1.6).

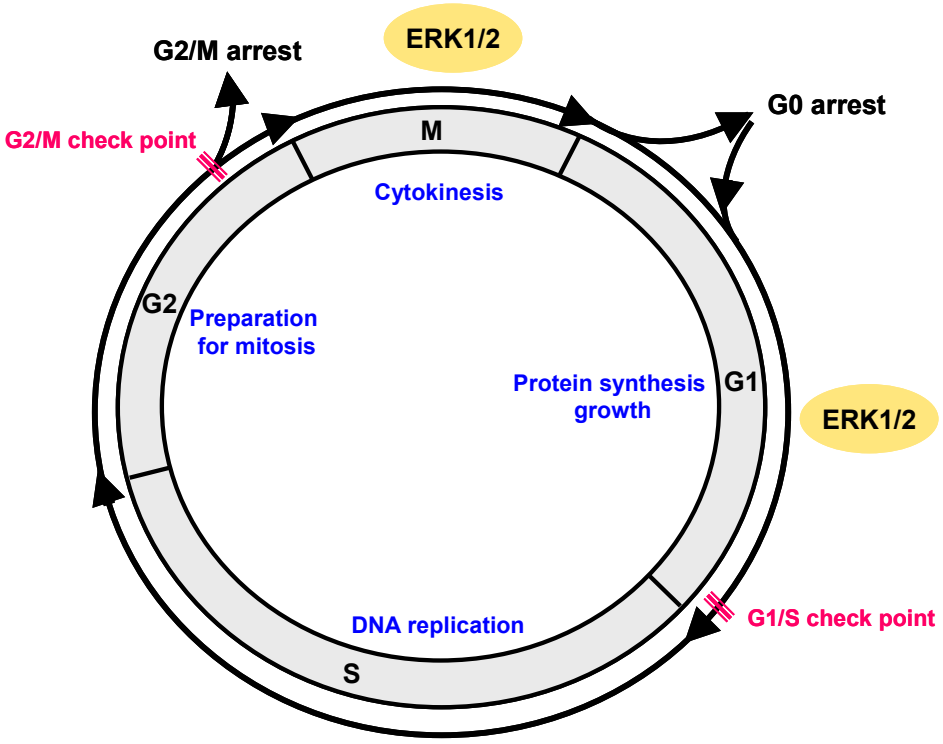


Figure 1.6: ERK1/2 in cell cycle and cell proliferation. ERK1/2 activation is required for cells to pass through the G1/S phase. ERK activation during the G1 phase induces expression of genes essential for cells to pass through the S phase. ERK activation is also important during mitotic phase of the cell cycle, where it associates with MTOC and aids in chromatid alignment and separation.

Another role for ERKs in the cell cycle may be the regulation of the microtubule organizing center (MTOC) [47], as ERKs seems to be activated in metaphase and to associate with MTOCs. This provides a potential structural basis for the cell cycle-dependent change in MTOC activity. However, in most systems examined, such as fibroblasts, clam oocytes, or *Xenopus* oocytes, activation of ERK is a onetime event that regulates the G0/G1 transition of the cell cycle. Another role of ERK in the cell cycle is demonstrated in studies on the *Xenopus* meiotic oocyte [48], where ERK is active during the natural arrest of unfertilized vertebrate oocytes in the second meiotic metaphase. Upon fertilization, the ERKs are inactivated.

1.3.2 Differentiation

Another physiological response that is regulated through the ERK signalling pathway is cellular differentiation. Different members of the MAPK cascade have been implicated in processes such as monocytic differentiation, neuronal outgrowth of PC12 cells [49], T cell maturation [45], and mast cell development [50]. Because ERKs are activated in somatic cells in response to many extra-cellular stimuli; ERK is also involved in developmental processes requiring proliferation of a new group of cells when new organs develop in the growing organisms [51].

1.3.3 Apoptosis

ERK1/2 pathway has multiple functions in a variety of cell types and is important for anti-apoptotic signals. Several experiments with knockout mice showed that knock out of MEK1 and other components of the ERK pathway sensitised cells to apoptosis or caused embryonic lethality [52]. However, in certain cell types sustained activation of ERKs can induce apoptosis [53]. It has been reported that ERK1/2 activation induces cell death in neurons [54]. It has been reported that apoptosis increases in Raf knockout mice even after activation of ERK1/2 via other pathways, indicating the role of MEK and Ras also in other functions related to apoptosis.

1.3.4 Cell Adhesion and Migration

ERK1/2 have also been reported to be involved in cell adhesion and migration [55]. Integrins that are cell surface adherence molecules also mediate ERK1/2 activation and nuclear translocation [56].

1.4 ERK pathway and disease

1.4.1 Cancer

Mutations, deletions or over expression of the ERK pathway members are linked to the majority of cancers [3, 41, 57-62]. Over-expression of the EGF receptor [59] and point mutation in Ras have been reported to cause transformation in several cell types. Approximately 30% of known cancers are associated to mutated forms of Ras isoforms [63].

Raf, the upstream activator of the MEK, is a protein with known oncogenic potential [64]. It has been demonstrated that mutations in the regulatory phosphorylated serines of MEK-1 enables it to induce cellular transformation [65].

1.4.2 ERK pathway and cardiovascular diseases

It is known that the ERK/MEK pathway has an impact on cardiac diseases. Various upstream events lead to the activation of ERK1/2 pathway and thus bring about remodelling of heart tissue in cardiac hypertrophy [66, 67]. It has been shown that ERK activation induces cyto-protection in cardiac ischemia and oxidative stress [68, 69].

1.4.3 Neuronal disorders

ERK1/2 have been linked to neuronal and synaptic plasticity in a variety of species. In mammals, several experiments have proved the role of ERK1/2 in central nervous system development at embryonic stages to memory and behaviour in adults [70]. Recent studies have also indicated that ERK activity is responsible for biochemical changes that occur in neurons of patients with Alzheimer's disease [71, 72]. Further, sustained ERK activation has been linked to different forms of neuronal death and neurodegenerative disorders [73].

1.5 Overview of the project

As the human genome has been mostly sequenced on genomic level [74] [75], the identification, isolation and functional characterisation of the human genes and proteins especially in view of their role in disease processes, thus remains the next challenge. The German cDNA Consortium has generated and sequenced a large number novel human cDNAs and protein coding regions (Open reading frames, ORFs) in the recent years [76]. The availability of full-length cDNAs is essential for the process of correct gene identification, and also constitutes the ideal physical clone resource for functional genomic approaches. Characterisation of the biological role of these proteins, also with relation to disease, would be the next step towards functional genomics. All novel ORFs generated within the consortium were therefore cloned into Gateway [77] compatible cloning vectors that allow for the further sub-cloning into a different prokaryotic and eukaryotic expression vectors. Considering the number of proteins that remain to be analysed functionally, establishment of

a high throughput screening platform to systematically identify the cellular role of proteins that are involved in major cellular processes is essential.

Recently, there have been many advances in the field of high throughput screening in terms of technology and data handling. Most of these advances focus on reporter system based assays, cell based assays, or *in vitro* assay systems which often do not represent the "true" biological context as within the cells. Cell based assays are the ideal choice for such screening approaches. First of all, these assays allow to characterise, analyse and screen for potential candidates in-situ. Secondly, they also take advantage of the fact that cells by themselves serve as convenient providers of assay components.

The biological phenomenon chosen to screen for, should be an indicator of normal cell activity and changes or differences in this phenomenon must bring about a clear impact on cancer relevant cellular processes. Cellular processes are tightly regulated by an array of signalling pathways. The complexity, diversity, and regulation of signalling networks and their effects on cellular processes are not yet completely understood. Unravelling novel players and the mechanism of their involvement in these complex networks are essential to ultimately understand disease mechanisms and to design new strategies for treatment. Therefore, we selected cell signalling as a biological parameter for the screening. Knowing the key role of the ERK1/2 signalling pathway in disease and cancer relevant cellular processes, we chose to detect the perturbations in ERK1/2 activation after over-expression of the novel uncharacterised proteins. With the novel ORFs cloned in suitable mammalian expression vectors, we over-expressed these proteins in well defined mammalian cell systems aiming at picking out potential candidates that show an effect on the activation of ERK1/2 pathway . These candidates would be further validated and a selected for detailed functional analysis. The scheme below illustrates the overview of the project (Fig: 1.9).

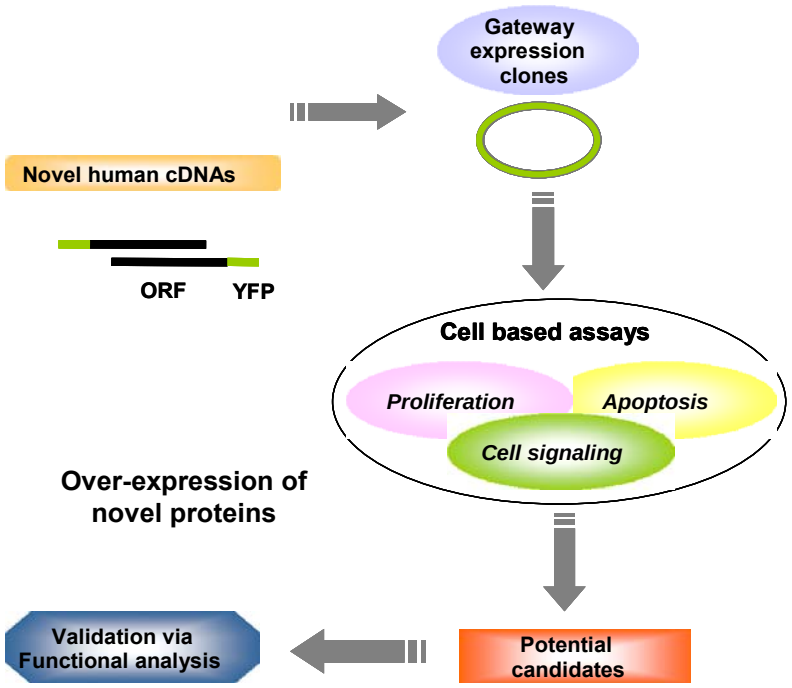


Figure 1.9: Overview of the project. Several novel ORFs have been identified in the German cDNA consortium in the recent years. In order to functionally analyze these proteins, the ORFs were cloned into Gateway compatible expression vectors. The proteins coded by these ORFs would be over-expressed in well characterised cell systems. The effect of over-expression on three cellular processes, proliferation, cell signaling and apoptosis would then be measured. The proteins that show an effect on these processes would then be validated and then selected for detailed functional analysis. The screen would therefore unravel novel players involved in important cellular processes.

1.5.1 Detection of perturbations in ERK1/2 activity

Activation of ERK1/2 occurs when they are phosphorylated by the immediate upstream dual specificity kinases, MEK1/2. Once the signal is received, ERK activation occurs rapidly and activated ERK translocates into the nucleus [30, 36]. ERK1/2 are accumulated in the nucleus with time. The accumulation of ERK1/2 in the nucleus can be measured using antibodies specific for ERK1/2 (Fig:1.4). With the help of Immunofluorescence and fluorescence microscopy, one can measure the amount of ERK in the nucleus at a defined time point after stimulation. If the over-expression of a particular protein affects ERK activation (increase/decrease) at a defined time point, the amount of ERK translocating into the nucleus would also vary accordingly and thus could be detected by fluorescence microscopy. Using automated microscopy and integrated software for signal quantification and image analysis one can adapt and use this method for the high throughput detection of ERK1/2 activation.

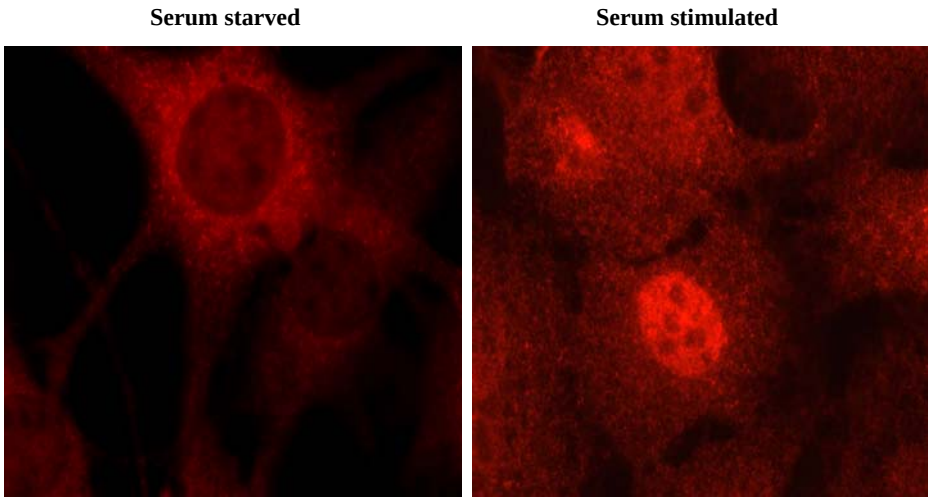


Figure 1.7: ERK activation and nuclear accumulation. ERK1 and ERK2 are activated by Mek1/2 respectively upon receiving a specific signal (serum, EGF etc). Activation of ERK1/2 leads to the nuclear translocation of ERK1/2, where it phosphorylates many of its targets. It is also dephosphorylated in the nucleus and is retained in the nucleus by anchoring proteins. Thus, inactive ERK accumulates within the nucleus with time. This accumulation can be measured using antibodies specific for ERK1/2 by fluorescence microscopy. Increase or decrease in the amount of nuclear ERK1/2 can be measured using integrated software and automated image analysis systems.

Cells are exposed to a large number of signals in vivo. In order to differentiate between “real” signal and noise, cells have developed a mechanism of regulation where, upon initial contact with MEK, ERK is phosphorylated only at one of the two residues. When the signal is strong enough or persistent, then the second phosphorylation occurs, enhancing ERK affinity for its substrates by a factor of 1000. Hence, under normal conditions cells posses a pool of mono-phosphorylated ERK, which upon further stimulation, becomes phosphorylated at the second site and thus become fully active. This shift from mono-phosphorylated to double-phosphorylated ERK, however, occurs rapidly ranging from a few minutes to an hour, depending on the cell system. This shift can be monitored or measured using antibodies that specifically identify the phosphorylated ERK1/2. When a protein interferes with the activation of ERK1/2, by speeding up or slowing down the activation, this perturbation can be measured and thus determine the effect of the protein to be either activating or inhibiting. Phospho-specific antibodies are commercially available and can be used to detect the level of phosphorylated ERK efficiently by using different techniques like flow cytometry (Fig: 1.8) or plate reader based methods.

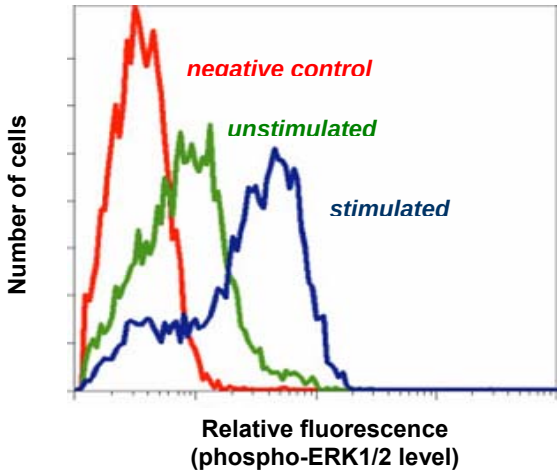


Figure 1.8: Detection of ERK1/2 activation by flow cytometry. Cells treated with a specific stimulating agent of ERK1/2 or left untreated are shown here in the histogram. Untreated cells have a low relative fluorescence when stained with phospho-ERK1/2 antibody as compared to cells treated with stimulant.

1.7 High throughput cell based assay for identification of novel modulators in MAPK signalling

My work focuses on establishment of a high throughput cell based assay for the identification of proteins that affect the MAPK signalling upon over-expression. From the multitude of signalling pathways and networks operating within cells, we chose the mitogen activated protein kinase pathway, specifically, the Extra-cellular signal Regulated protein Kinase (ERK) pathway for the screen. Involvement of ERK in physiological processes and malfunction in major disease conditions and cancer makes it an appropriate choice for the screening. The ERK pathway is regulated at different levels by several complex mechanisms which are not yet completely understood. Identification of novel proteins involved directly or indirectly in the regulation of the pathway will shed light on the still unknown biological mechanisms operating in cells and be a very significant improvement in understanding disease mechanisms.

The specific aims of my work are

a) To establish a High throughput ERK1/2 activation assay based on single cell detection
b) To automate the assay
c) Screening novel human proteins for potential candidates
d) Confirmation of the effects of candidates that come out of the screen
e) To perform an in-depth functional analysis of a selected candidate protein.

2 Materials and Methods

2.1 Materials

2.1.1 Instruments and Equipment

37°C Incubator	Binder GmbH
Agarose gel casting chambers	Renner GmbH
Autoradiography cassettes, IEC 60406	Rego
Cell culture incubators	Heraeus
Centrifuge, RC5C	Sorvall, Langenselbold
DNA- (SmartSpec3000)	Biorad
DNA electrophoresis Apparatus	Renner GmbH
Electroblotting Apparatus, Transblot SD	Biorad
Electroporater, Gene Pulser II	Biorad
Flow Cytometer, FACS Calibur	Becton-Dickenson
Fluorescence Microscope, Axiovert 25	Zeiss
Laminar flow hood, HeraSafe	Heraeus
Laser Scanning Microscope	Zeiss
Light microscope	Hund Wetzler
Magnetic Stirrers, Ikamag RTC	IKA Labortechnik
PCR Machine, GeneAmp	Applied Biosystems
pH-meter	HANNA, Kehl
Pipetboy, acu	Integra Biosciences
Protein Electrophoresis apparatus, MiniProtean II	Biorad
Rocker Platform, PolyMax 1040	NeoLab
Rotator	NeoLab
Spectrophotometer, SpectraMax190	Molecular Devices
Table centrifuge, Biofuge Fresco	Heraeus
Thermocycler, Thermomixer Comfort	Eppendorf
Vortex Mixer	NeoLab
Waterbath	Julabo, Seelbach

2.1.2 Plastics and Glassware

24 well plates	Falcon
6 well plates	Nunc, Falcon
96 well plates	
- *clear bottomed*	*Falcon*
- *v-bottomed*	*Nunc*
- *black plates*	*Packard biosciences*
- *white plates*	*Packardbiosciences*
- *u-bottomed , polypropylene*	*Falcon*
bottle top filters	Millipore
cell culture flasks	Greiner
cell scrapers	Roth
cell cuture petridishes	Greiner
chamber slides	Nunc, Nalgene
cover slips	Menzel glaeser
cryotubes	Nunc
cuvettes	Biorad
Eppendorf tubes	Eppendorf
Falcon tubes 15ml, 30ml	Falcon
glass slides	R.Langenbrick
Glassware	Fisherbrand
magnets for stirring	Neolab
Pasteur pipettes	Brand
petridishes for bacteria	Greiner
pipette tips	Starlab, Steinbrenner

2.1.3 Chemicals, Reagents and Media

1kb DNA Ladder	Invitrogen
2-Mercaptoethanol	Sigma
3MM Filterpapier	Whatman, Göttingen
Acrylamid	Sigma, Steinheim
Agarose	Gibco BRL, Karlsruhe
Albumin Standard	Pierce biotechnology

Ammonium peroxo disulphate (APS)	Roth
Bacto-Agar	Difco
Bacto-Tryptone	BD Bioscience, Heidelberg
Blotting equipment	Biorad
Blotting Paper	Sartorius
Bromophenolblue	AppliChem, Darmstadt
BSA	BioLabs, New England
Cell culture media	Gibco, PAN biosciences
Dimethylsulfoxide (DMSO)	Sigma
Dipotassium hydrogen phosphate (K_2HPO_4)	Vertriebs GmbH
Ethanol	Applichem
Ethidiumbromide	Sigma
FBS	Gibco
Glycerol	Roth, Karlsruhe
Glycine	AppliChem
Hybond-P, PVDF Membrane	Amersham Biosciences
Hydrochloric Acid	Riedel-de Haen
Isopropanol	Sigma-Aldrich
L-Glutamine	Sigma
Magnesium chloride ($MgCl_2$)	BioLabs
Methanol	Riedel-de Haen
Milk-Powder	Roth
n-Butanol	Sigma
PBS	Gibco
Pellet Paint	Novagen
Penicillin	Invitrogen
Ponceau-S Solution	Serva
Potassium chloride (KCl)	Sigma
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Darmstadt
Protein Loading Buffer (4X)	Roth
Protogel (Acrylamide/Bisacrylamide)	National Diagnostics
Rainbow Marker	Amersham Pharmacia
Restriction enzymes and buffers	New england biolabs
S.O.C Medium	Invitrogen

SDS	Gerbu
Sodium Acetate	Fischer
Sodium acetate (NaAc, (3M solution)	Novagen, Wisconsin
Sodium chloride (NaCl)	J.T.Baker, Griesheim
Sodium hydroxide (NaOH)	Gerbu
Sodium meta vanadate (NaVO3)	Sigma
TEMED	Roth
Topoisomerase	Invitrogen
Tris-Base	Sigma
Tris-Sodiumcitrate	Fluka, Seelze
Triton X-100	Sigma
Trypsin	Sigma
Tween 20	Gerbu
WST-1 reagent	Roche
X-ray film	Amersham Biosciences

2.1.4 Kits

APC-BrdU Flow Kit	BD biosciences
ECL Western Blotting detection reagents	Amersham biosciences
Effectene Transfection Reagent	Qiagen
Gateway BP-Clonase Enzyme Mix	Invitrogen
Gateway LR-Clonase Enzyme Mix	Invitrogen
Lipofectamine 2000	Invitrogen
Micro BCA Protein Assay Reagent Kit	Pierce biotechnology
Plasmid DNA MaxiPrep-Kit	Qiagen
Plasmid DNA MiniPrep-Kit	Qiagen

2.1.5 Antibodies

Primary antibodies

Antibody	Company	Catalog no.	Species	WB	IP	IF/ICC
Acetylated tubulin (clone 6-11B-1)	sigma	T6793	mouse	NA	NA	1 :4000
Actin (20-33)	sigma	A 5060	rabbit	1:500	NA	1:200
Caspase-7 mono	BD	551239	Mouse	1:2000	4µg/200µg lysate	NA
CDK-2 (Ab-1)	Oncogene	PC44	rabbit	1:500	1:500	NA
CDK-2 mono	BD	610146	mouse	1:2500	NA	1:100
Cyclin A	upstate	06-138	rabbit	1:500	NA	NA
Cyclin D	upstate	06-137	rabbit	1:1000	NA	NA
Cyclin E (mono)	BD	554182	mouse	1:1000	NA	NA
ERK1/2-CT	upstate	06-182	rabbit	1:5000	NA	1:500
ERK3	BD	E16320	mouse	1:250	NA	1:100
GFP (mono)	Roche	1 814 460	mouse	1:1000	1:200	NA
MEK1 mono	BD	610121	mouse	1:1000	1:200	1:50
p16 (Ab-1) (mono)	Oncogene	NA29	mouse	1:100	NA	NA
p21 Waf1/Cip1 (DCS60) (mono)	CST	2946	mouse	1:2000	1:100	1:100
p27 Kip-1	CST	2552	rabbit	1:1000	1:100	1:50
p44/42(ERK1/2)	CST	9102	rabbit	1:1000	1 :100	NA
p57 Kip-2	CST	2557	rabbit	1:1000	NA	NA
p70	BD	611260	mouse	1:1000	NA	1:50
pan-ERK	BD	610123	Mouse	1:1000	NA	NA
Phospo-ERK1/2	CST	9101	rabbit	1:1000	NA	1:100

Secondary antibodies

Antibody	Company	Catalog no.	Species	IF/ICC	FACS	WB
HRP labeled anti-rabbit IgG	Sigma	A6154	Goat	-	-	1:10000
HRP labeled anti-mouse IgG	dianova	115-035-003	Goat	-	-	1:10000
APC labeled anti-rabbit IgG	Invitrogen	A-10931	Goat	-	1:250	-
APC labeled anti-mouse IgG	Invitrogen	A-865	Goat	.	1:250	-
Alexa488 labeled anti-rabbit IgG	Invitrogen	A-11034	Goat	1:600	1:250	-
Alexa488 labeled anti-mouse IgG	Invitrogen	A-11029	Goat	1:500	1:250	-
Alexa568 labeled anti-rabbit IgG	Invitrogen	A-11036	Goat	1:500	-	-
Alexa568 labeled anti-mouse IgG	Invitrogen	A-11031	Goat	1:500	-	-
Alexa647 labeled anti-rabbit IgG	Invitrogen	A-21245	Goat	1:400	1:250	-
Alexa647 labeled anti-mouse IgG	Invitrogen	A-21236	Goat	1:400	1:250	-

2.1.6 Peptides used for antibody generation

i) 15i5-386-405 5´- AHGEEEGEEDEEKAVDIVPK - 3´

ii) 15i5-361-380 5´- EFREGEEEAEEEEVEEMTEG – 3´

2.1.7 Buffers and Media

PBS (pH 7.4) :

NaCl	140 mM
KCl	2.7 mM
KH2PO	41.5 mM
Na2 HPO	4 8.1 mM

TE-Buffer:

Tris-HCl (pH 7.5)	10 mM
EDTA	1 mM

TAE (10x):

Tris-base	48.46 g/L (400 mM)
EDTA-Na_2-salt	3.72 g/L (10 mM)
Acetic acid	12.01 g/L (200 mM)

Make up volume to 1 litre with millipore water.

TBS:

Trizma base	6.1 g/L
NaCl	9.0 g/L

pH 7.6

Buffers for SDS-PAGE

a) 4x Running gel buffer

Tris-HCl	1.5 M, pH 8.8

b) 4x Stacking gel buffer

Trsi-HCl	0.5 M, pH 6.8

c) 10 x Tank/Running buffer

Tris	0.25M
Glycine	1.92 M

pH 8.3

Diluted 1:10 in distilled water and SDS was added to a final concentration

Buffers for Western blotting

a) Semi-dry transfer buffers

Anode solution I

Tris base	300 mM
Methanol	20%(v/v)

Anode solution II

Tris base	25 mM
Methanol	20%(v/v)

Cathode solution

6-Amin-n-caproic acid	40mM
Methanol	20%(v/v)

b) Wash buffer for western blots

TBST: TBS with 0.1% Tween20

c) Blocking buffer

5% milk in TBST

d) Blocking buffer for Immunofluorescence

3% BSA in PBS

LB-Medium

Trypton	10 g/L
Yeast-Extract	5 g/L
NaCl	10 g/L

pH 7.0

LB-Agar: 3% Agar dissolved in LB-Medium

Buffers for the ß-galactosidase assay

a) Lysis buffer: 0.1% triton X-100/0.1 M Tris-HCl (pH 8.0)

b) 100 X Mg solution:

Magnesium Chloride	0.1 M
2-mercaptoethanol	4.5 M

store at 4°C

c) 0.1 M sodium phosphate buffer (pH 7.5)

Disodium hydrogen phosphate	0.2 M
sodium dihydrogen Phosphate	0.2 M
Distilled water	50 ml

4mg/ml ONPG in 0.1 M sodium phosphate (pH 7.5) containing 2 mM 2-Mercaptoethanol, stored at 4°C

d) 0.1mg/ml ß-galactosidase standard

0.1mg/ml ß-gal in 0.1 M sodium phosphate (pH 7.5) containing 2 mM 2-Mercaptoethanol, stored at 4°C

e) Stop buffer

1M sodium carbonate in water

f) Substrate solution (per well):

Sodium phosphate buffer	0.1M
ONPG stock	22 µl
100x MgCl solution	1 µl

2.1.8 Antibiotics

100 µg/ml Ampicillin in ddH_2O

10 µg/ml Gentamycin in ddH_2O

30 µg/ml Kanamycin in ddH_2O

2.1.9 Restriction enzymes

BsrGI: obtained from *Bacillus stearothermophilus* GR75 . (New England BioLabs) with the restriction site:

5'……T^GTACA…..3'

3'……ACGTG^T…..5'

2.1.10 Bacterial Strains

E.coli DH10B: DH10B™ (Invitrogen) cells are highly efficient E. coli, perfect for most applications. DH10B™ E. coli is available in both electro-competent and chemically competent formats. The high transformation efficiency makes them ideal for generating cDNA or genomic libraries.

E.coli DB3.1: DB3.1™ (Invitrogen) competent cells are suitable for propagation of plasmids containing the *ccd*B gene. They are designed for use with the Gateway® Vector Conversion System. Used for propagating Gateway® destination, pDONR™, and supercoiled pENTR™ vectors.

2.1.11 Vectors

2.1.11.1 Gateway entry vector

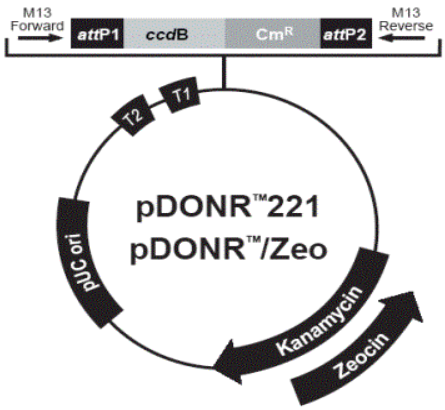


Figure 2.1: pDONR™ 221

This vector was used to generate the entry clones required to shuttle the cDNA into destination vectors.

This is a Gateway™ system compatible donor vector, obtained from Invitrogen. This plasmid was used to generate entry vectors, which were further used to shuttle the cDNA into destination vectors. The ORF is cloned by BP reaction between the two recombination sites *att*P1 and *att*P2. The *ccd*B gene present in the death cassette inhibits the replication of this plasmid in many bacterial strains. The plasmid encodes resistance for kanamycin, which is used for the selection of positive clones, while the chloramphenicol resistance gene is replaced by the ORF during BP reaction.

2.1.11.2 Gateway destination vectors

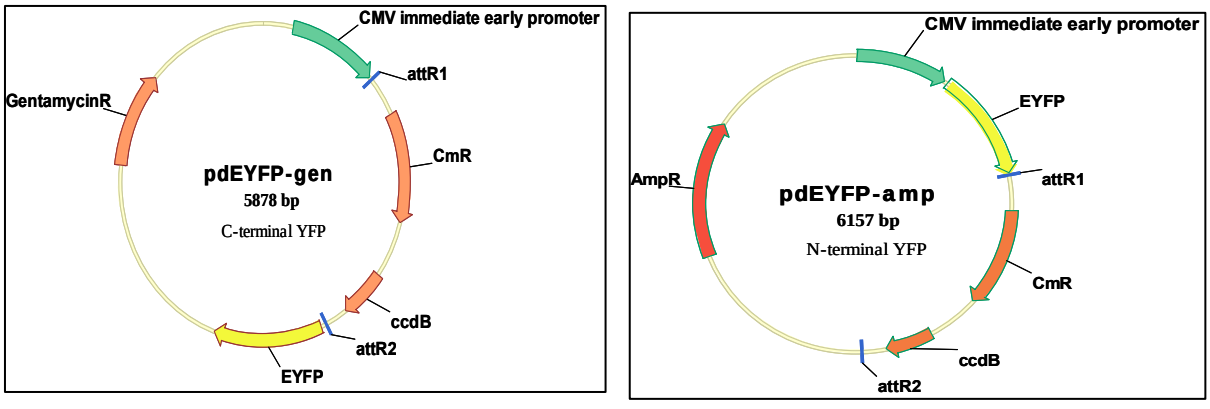


Figure 2.2: pdEYFP-gen and pdEYFP-amp

These destination vectors were used to express YFP tagged proteins in mammalian cells.

Expression vector encoding YFP was obtained from BD Clontech and modified by Wiemann et.al; to generate pdEYFP-gen and pdEYFP-amp. The pdEYFP-gen (encoding gentamycin resistance) generates a fusion protein with YFP at the C-teminal of the protein, while the pdEYFP-amp (encoding ampicillin resistance) generates a N-terminally tagged protein. The ORF is cloned by LR reaction between the two recombination sites *att*R1 and *att*R2. Both vectors contain the death cassette with *ccd*B gene, which facilitates the selection of positive clones. The transcription is driven by CMV promoter.

2.1.12 Cell lines

Cell line	Source	Catalog number	Type	origin	Growth medium
HEK-293	ATCC	CRL-1573	normal epithelial	Human - embryonic kidney	DMEM +10% FBS
NIH3T3	ATCC	CRL-1658	normal fibroblast	mouse	DMEM +10% NCS
BT474	ATCC	HTB-20	Epithelial – ductal carcinoma	Human - breast	DMEM +10% FBS
SKBR3	ATCC	HTB-30	Epithelial – adenocarcinoma, metastatic	Human - breast	McCoy's 5a + 10% FBS
HeLa	DKFZ Tumor bank	810015	Epithelial – carcinoma	Human - cervix	RPMI 1640 + 10% FBS

2.2 Methods

2.2.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a technique for the amplification of minute amounts of DNA, so that enough working material is generated for analysis and further use. PCR has also become a crucial tool in several other molecular biology approaches like site directed mutagenesis, generation and modification of DNA sequences and vectors, and also in forensic and diagnostic applications. Every PCR requires some starting material - the template DNA (10 ng) - from which the gene of interest or the sequence of interest needs to be amplified, specific primers – small oligonucleotide sequences (15-30 bp) that can bind to the template and initiate elongation, dNTPs for new strand synthesis, a DNA polymerase - usually a heat resistant DNA polymerase and finally the right buffer system in which the reaction can take place efficiently.

The first step in the Gateway system is to clone the gene of interest into an entry vector. In our case the cDNA sequences which needed to be modified need to be first amplified from the vector in which it was cloned and then the amplified sequence needed to be modified by the addition of 3´and 5´attachment sites (*att*B) by performing a second PCR.

2.2.1.1 Generation of Entry clones

Generation of entry clones involves a two step PCR followed by BP reaction. The first PCR in this system is done using gene-specific primers. The reaction parameters are listed below.

Template DNA (10-50ng)	1µl
Forward primer (from 10pmol stock)	1µl
Reverse primer (from 10pmol stock)	1µl
dNTPs (10mM)	1µl
10x reaction buffer	5µl
Enzyme (expand high fidelity PCR system	1µl
Water	40µl
Total reaction volume	50µl

The above reaction mixture was placed in a thermal cycler and a PCR was performed according to the following program

95°C	2 min	
95°C	15 sec	13 x
58°C	15 sec	
68°C	1 min	
68°C	10 min	
4°	hold	

If the PCR was not successful, a ramping PCR was performed with the following program

95°C	2 min	
95°C	15 sec	13 x
62°C minus 2°C per cycle	15 sec	
68°C	1 min	
68°C	10 min	
4°	hold	

Gateway PCR2

In this PCR the amplified ORF from the first PCR was used as a template and the primers were designed with *att*B sites so as to form a product that is flanked by attachment sites (*att*B) and is gateway compatible. The PCR was performed with following parameters.

Template DNA (product of PCR1, diluted 1:5 or undiluted)	1µl
Forward primer (st*att*B1-PageR1, 10 pmol)	1µl
Reverse primer (st*att*B2-PageS1, 10 pmol)	1µl
dNTPs (10mM)	1µl
10x reaction buffer	5µl
Enzyme (Expand high fidelity PCR system)	1µl
Water	40µl
Total reaction volume	50µl

This reaction mixture was placed in a thermal cycler and a PCR was performed according to the following program.

95°C	2 min	
95°C	15 sec	13 x
60°C	15 sec	
68°C	1 min	
68°C	10 min	
4°	hold	

The resulting products were analysed on an agarose gel.

2.2.1.1.1 BP reaction

The product of the two step Gateway PCR was then cloned into Entry vector $p^{DON}201$ by performing a BP reaction.

5x BP reaction buffer	2µl
PCR product	6µl
Entry vector (pDON201)	1µl
BP clonase	1µl
Total reaction volume	10µl

This reaction mixture was then incubated overnight at 25°C on a heating block or a thermal cycler. On the following day, the reaction was stopped by adding 1µl of Proteinase-K (2µg/µl) and 20µl H_2O and incubating at 37°C for 10 min.

The DNA was then precipitated by adding

Pellet paint	2µl
3M sodium acetate	3µl
Isopropanol	30µl
Ethanol (100%)	60µl

The mixture was then incubated for 20 min at -20°C followed by centrifugation at 13000 rpm for 15 min. The supernatant was discarded and the pellet was washed once with

70% ethanol. The pellet was dried and then dissolved in 5µl of water. 1µl of this DNA was used for transformation into bacteria (DH10B) by electroporation.

2.2.1.1.2 LR reaction

Once the Entry clone was generated, it could now be used to clone the gene of interest into the destination vector of choice by performing a second site specific recombination reaction – the LR reaction.

5x LR reaction buffer	2µl
Destination vector (~150ng)	1µl
Entry clone (~100ng)	1µl
Water	3.875µl
Topoisomerase	0.125µl
LR clonase	1µl
Total reaction volume	10µl

This reaction mixture was then incubated overnight at 25°C on a heating block or a thermal cycler. On the following day, the reaction was stopped by adding 1µl of Proteinase K (2µg/µl) and 20µl H_2O and incubating at 37°C for 10 min. The DNA was then precipitated by adding

Pellet paint	2µl
3M sodium acetate	3µl
Isopropanol	30µl
Ethanol (100%)	60µl

The mixture was then incubated for 20 min at -20°C followed by centrifugation at 13000 rpm for 15 min. The supernatant was discarded and the pellet was washed once with 70% Ethanol. The pellet was dried and then dissolved in 5µl of water. 1µl of this DNA was used for transformation into bacteria (DH10B) by electroporation.

2.2.2 Preparation of electro competent cells

On the day before the preparation of competent cells 1-2 litres of sterile water and 20 ml of sterile 10% glycerol was prepared and the bacterial strain (DH10B or DB3.1) was inoculated in 50-100 ml of LB medium without antibiotic and cultured overnight at 37°C on a shaker (200-220 rpm) to be used as an inoculum on the following day. From the culture that had grown overnight 5 ml was used to inoculate 500 ml of LB medium without antibiotic and incubated at 37°C in a shaker at 220 rpm. $O.D_{600}$ of the culture was measured every 30 min until it reached 0.5. During this time, the sterile water, 10% glycerol and the centrifuge were all cooled to 4°C. Once the $O.D_{600}$ of the culture reached 0.5, it was decanted into centrifuge tubes and centrifuged at 4000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in 300 ml of ice-cold sterile water. The suspension was again centrifuged at 4000 rpm for 15 min at 4°C and the supernatant was discarded. The pellet was resuspended in 300 ml of ice-cold sterile water and centrifuged again at 4000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended in 10 ml of ice cold 10% glycerol. The suspension was centrifuged a 4000 rpm for 15 min at 4°C and the supernatant was discarded. The pellet was now resuspended in 1-1.5 ml of ice cold 10% glycerol and 25µl aliquots were transferred into 0.5 ml Eppendorf tubes, pre-cooled on dry-ice. The tubes were then quickly transferred to an ethanol-dry-ice bath or liquid nitrogen to quick-freeze. The cells were stored at – 80°C for further use.

2.2.3 Transformation of bacteria by electroporation

Competent cells were thawed on ice. The DNA and electroporation cuvettes were cooled on ice before use. SOC medium was warmed to 37°C. The electroporation voltage was set to 1.7. Once the cells thawed, they were transferred to the chilled cuvette and 1-3µl of plasmid DNA was added to the cells. The cuvette was tapped well to mix the DNA and the cells and care was taken to avoid any bubbles. The cuvette was then placed in the electroporator and pulsed. 200µl of warm SOC medium was quickly added to cells and the suspension was transferred to fresh lableled Eppendorf tubes. The tubes were incubated at 37°C for 30 min on a shaker at 220 rpm. Later, the cell suspension was spread on LB agar plates with appropriate antibiotic and incubated overnight at 37°C in an incubator.

2.2.4 Isolation of plasmid DNA (Mini-prep)

In order to isolate plasmid DNA from bacteria in small amounts for further analysis, the mini-prep protocol was used. Plasmid DNA was isolated using the Qiaprep spin Mini-prep kit, according to the manufacturer's specifications. All centrifugations were performed in an Eppendorf tabletop micro-centrifuge at 13000 rpm at RT unless otherwise indicated.

A single bacterial colony from the LB agar plate with the appropriate antibiotic was inoculated into 3 ml of LB medium with the appropriate antibiotic. The culture was incubated overnight at 37°C with shaking (210–230 rpm). 1.5 ml of the bacterial culture was taken into a microfuge tube and centrifuged at 14000 rpm for 30-60 seconds. After discarding the supernatant the pellet was re-suspended in 250µl of re-suspension buffer (P1). Immediately 250µl of lysis buffer (P2) was added and the tubes were inverted 4-6 times to mix. 350µl of neutralizing buffer (N3) was added and the tubes were inverted 4-6 times. The tubes were centrifuged at 14000 rpm for 10 minutes at RT. The supernatant was transferred on to the column, provided by the manufacturer, and centrifuged at 14000 rpm for 1 min at RT in a tabletop centrifuge. The flow through was discarded and the column was washed with 750µl of buffer PE and centrifuged at full speed (14000 rpm) for 1 min. The flow through was discarded and centrifuged again at full speed for 1 min to remove any residual ethanol. The DNA was eluted from the column by adding 50µl water and centrifuging at 14000 rpm for 1 min at RT. DNA was stored at – 20°C until further use.

2.2.5 Large scale preparation of plasmid DNA (Maxi prep)

To prepare large amounts of plasmid DNA, the maxi prep protocol was used. Plasmid DNA was isolated using the Qiagen Plasmid Maxi Kit according to the manufacturer's instructions. All centrifugations were performed in a fixed rotor centrifuge at RT unless otherwise indicated.

A single bacterial colony from an LB agar plate with the appropriate antibiotic was inoculated into 100 ml of LB medium with the appropriate antibiotic and incubated overnight at 37°C with shaking (210-230 rpm). The culture was centrifuged at 7000 rpm for 5 minutes to pellet the cells in 50 ml falcon tubes. The pellet was re-suspended in 10 ml of re-suspension buffer (P1). To the suspension, 10 ml of lysis buffer (P2) was added and mixed gently by inverting the tubes 4-6 times. The tube was incubated at RT for 5 min and 10 ml of pre-chilled neutralization buffer (P3) was added to the suspension. Immediately, the suspension was mixed gently by inverting the tube 4-6 times and incubated on ice for 10 minutes and then

centrifuged at maximum speed for 20 minutes in a fixed rotor centrifuge. Meanwhile a Qiagen Q100 tip was equilibrated with 10 ml of equilibration buffer (QBT). After the lysate was centrifuged, the supernatant was carefully transferred into the equilibrated Q100 tip. The flow through was discarded and the cartridge was then washed twice with 30 ml wash buffer (QC). The cartridge was then placed into a fresh falcon tube and the DNA was eluted using 15 ml elution buffer (QF). 10.5 ml of Isopropanol was added to the eluted sample and mixed immediately to precipitate the DNA. The DNA was then pelleted by centrifuging for 30 minutes at RT at maximum speed. The pellet was then washed with 70% ethanol and transferred into a 1.5 ml microfuge tube and centrifuged at maximum speed for 15 minutes. After removing the supernatant the pellet was washed again with 70% ethanol to remove any traces of salt. The tubes were centrifuged at 14000 rpm for 15 minutes at RT. The supernatant was discarded and the pellet was dried. The pellet was dissolved in appropriate volume of 10mM Tris HCl pH 7.0 or Millipore H_2O and stored at –20°C until further use.

2.2.6 Measuring the concentration of DNA

The concentration of the DNA/RNA obtained was determined by measuring the absorbance at 260 nm using a Spectrophotometer (Anthelie, SECOMAM). The purity of the Plasmid DNA can be determined by the ratio of Abs_{260} to Abs_{280}. A ratio of 2:1 indicates highest purity. 5 µl of DNA was diluted in 95 µl of TE or water and the absorbance was recorded at 260 nm. The concentration of the DNA was then calculated using the following formula .

$$\textit{Concentration of DNA (µg/ml)} = 50 \times O.D_{260} \times \textit{dilution factor}$$

2.2.7 Restriction digest

Restriction digests were performed in a final volume of 20µl. For analytical digests 150 - 200 ng of the eluted plasmid DNA, 2µl of 10x buffer-2 (NEB), 0.2µl of 100x BSA (NEB) with 1µl of BsrG1 enzyme were mixed in an Eppendorf tube and the volume was made up to 20µl with Millipore H_2O. The mixture was incubated at 37°C for a minimum of 2 hrs to overnight. The digests were then checked on agarose gels.

2.2.8 Agarose gel electrophoresis

For 1% agarose gels, 1g of agarose and the electrophoresis buffer (1x TAE) were combined and heated in a microwave until agarose was dissolved. The solution was poured into the gel cast. Once the gel solidified, it was transferred into the electrophoresis chamber with 1x TAE buffer. Mini gels were run at 80 -100 volts and midi gels at 100 -120 volts for 60-90 minutes. Gels were analysed by Ethidium bromide staining.

2.2.9 Cell culture

2.2.9.1 Sub-culturing and maintenance of mammalian cells

Cells were cultured routinely in T75 flasks. On the day of requirement, cells were washed once with 5 ml of warm PBS and 3 ml of trypsin was added to the cells so as to cover the cells uniformly. Cells were incubated for 2-5 min at 37°C to aid detachment from the surface of the flask. The cells were observed under the microscope to confirm detachment. 7 ml of complete medium (with FBS) was added to the cells to stop trypsinisation. Cells were pipetted up and down several times to ensure a single cell suspension and to avoid clumps. Cells were then counted and transferred into a new flask with 10-12ml of appropriate medium. The flasks were incubated in the incubator (37°C, 5% CO_2).

2.2.1.1 Cell counting using a *Neubauer* chamber

The cells were counted using a *Neubauer* hemocytometer. Equal volumes of cells and 0.4% Trypan blue solution (100µl of cell suspension and 100µl Trypan blue) were mixed together in a microcentrifuge tube and left undisturbed for 3 minutes. Trypan blue is a dye which stains dead cells blue, while the live cells remain unstained. The chamber of the hemocytometer was filled with the stained cell suspension by capillary action. The total number of cells in the four marked squares (Figure 2.6) was counted using a microscope. Each chamber of the hemacytometer is divided into nine 1.0mm squares. A cover glass is supported over these squares so that the total volume over each square is 0.0001ml or 10^{-4} ml (length x width x height; i.e., 0.1cm x 0.1cm x 0.01cm). Since $1cm^3$ is equivalent to 1ml, the cell concentration per ml will be the average count per square x dilution factor x 10^4.

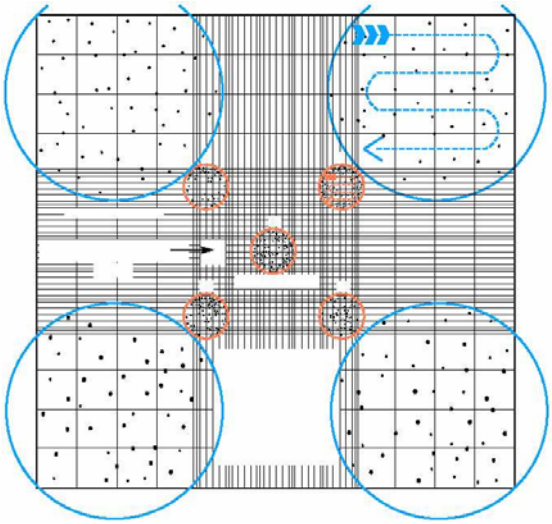


Figure 2.3: Counting cells with *Neubauer* hemocytometer
Cells stained with trypan blue were loaded onto the hemocytometer and the total number of cells in the four big squares was counted. The number of cells/ml of cell suspension is then calculated as described.

2.2.1.2 Transfection of mammalian cells

Liposome mediated transfection

Introduction of DNA into mammalian cells can be efficiently done by using artificial liposomes. In this technique, cationic lipids forming micellar structures called Liposomes are allowed to form complexes with DNA to create lipoplexes which have a negatively charged surface due to the DNA bound on its surface[78]. These structures fuse with the cell membrane after interactions with surface proteoglycans, many of which are positively charged. The complexes are then internalised by endocytosis, resulting in the formation of a double-layer inverted micellar vesicle. During the maturation of the endosome into a lysosome, the endosomal wall might rupture, releasing the contained DNA into the cytoplasm and potentially towards the nucleus. DNA imported into the nucleus might result in gene expression.

Transfection protocol using Effectene for 96 well format

On the day before transfection, cells were trypsinised, counted and seeded at a density so as to obtain a 60% confluence on the following day. Cell numbers were optimised and numbers varies depending on the cell line. On the day of transfection 40-100ng of plasmid DNA was diluted in 30µl of EC buffer and 0.8µl of Enhancer was added and incubated for 5 min at RT. After the incubation, 1µl of Effectene transfection reagent was added to the

EC/Enhancer/DNA mixture and incubate for another 10 min at RT. When many wells needed to be transfected a master mix the appropriate volume of EC/Enhancer and EC/Effectene was prepared and incubated with DNA accordingly. During the incubation, cells were washed once with PBS and 100µl of fresh complete growth medium with serum and antibiotics was added to the cells. After incubation of the EC/Enhancer/DNA/Effectene mixture for 10min, the complexes were transferred to the cells. Cells were incubated for 24-48 hrs at 37°C with 5% CO2 to allow good expression.

2.2.10 ß-galactosidase assay

Cells were seeded in 96 well plates on the day before transfection. On the following day cells were transfected with 100ng of ß-galactosidase reporter plasmid DNA using Effectene transfection reagent according to the protocol. Cells were allowed to express ß-galactosidase for 24-48 hrs. After 48hrs cells were washed once with 1x pre-warmed PBS for 5 min. PBS was aspirated and 10µl of lysis buffer was added to each well. Cells were briefly frozen on dry ice and thawed at RT. The freeze-thaw step was repeated once again to ensure better lysis. After lysing the cells 100µl of substrate solution was added to cells and mixed thoroughly by vortexing. The plate was incubated at 37°C for 30 min and 50µl of stop buffer was added to each well to stop the reaction. Measurement was done at $O.D_{490}$ in a spectrophotometer.

2.2.11 Protein extraction from mammalian cells

Cells were placed on ice and the medium was removed. Cells were washed once with ice-cold PBS. Appropriate volume of mPER lysis buffer was added to the cells and incubated 5 min at RT. Cells were scraped from the plate using a cell scraper and transferred to a fresh eppendorf tube. The sample was vortexed briefly and centrifuged at 13000 rpm for 30 min at 4°C. The lysate was carefully transferred to a fresh tube. A small aliquot of the lysate was used for protein quantification and the rest was stored at –80°C.

2.2.12 Protein quantification

2.2.12.1 Measurement of protein concentration at UV 280

The lysates were diluted 1:20 in PBS or measured directly in a spectrophotometer and the UV absorbance was measured at UV_{280} in a spectrophotometer. The following formula was used to calculate the protein concentration.

$$\textit{Concentration (mg/ml)} = (1.55 \times A_{280}) - 0.76 \times A_{260})$$

2.2.12.2 Estimation of protein concentration using BCA (Bicinchonic acid) method

Protein quantification with BCA was performed using the Micro BCA protein assay kit from Pierce biotechnologies. The BCA assay is based on the Biuret reaction in which Cu^{2+} is converted to Cu^{1+} in alkaline conditions by a protein resulting in a deep purple colour. This reaction is the basis of the BCA assay where the BCA reacts with the Cu^{1+} ions and forms the colour.

Bovine serum albumin (BSA) was used as a standard in this assay. BSA was diluted in PBS at a concentration ranging from 0.5µg/ml to 200µg/ml. PBS used for preparing standards was used as a blank. 150µl of each of the standards and the blank were pipetted into a 96 well plate at least in duplicates. 5µl of the sample was diluted in 150µl of PBS in duplicates in the same 96 well plate. 12ml of Reagent A and 12.5ml of Reagent B and 0.5ml of Reagent C were mixed freshly and 150µl of this mixture was added to each well containing the standards and the samples. The plate was covered and incubated at 37°C for 2 hrs and allowed to cool to room temperature before measuring the Abs_{562} in a spectrophotometer. The amount of protein was quantified using the standard curve.

2.2.13 Poly Acrylamide gel electrophoresis (PAGE) and Western blotting

Polyacrylamide gel electrophoresis (PAGE) is an analytical technique used to separate and characterise proteins. A solution of acrylamide and bisacrylamide is polymerised. Acrylamide alone forms linear polymers. The bisacrylamide introduces cross links between polyacrylamide chains. The 'pore size' is determined by the ratio of Acrylamide to bisacrylamide, and by the concentration of Acrylamide. A high ratio of bisacrylamide to Acrylamide and a high Acrylamide concentration cause low electrophoretic mobility. Polymerization of acrylamide and bisacrylamide monomers is induced by ammonium

persulfate (APS), which spontaneously decomposes to form free radicals. TEMED, a free radical stabilizer, is generally included to promote polymerisation.

2.2.13.1 SDS- Poly acrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) is an amphipathic detergent. It has an anionic head group and a lipophilic tail. It binds non-covalently to proteins, with a stoichiometry of around one SDS molecule per two amino acids. SDS causes proteins to denature and disassociate from each other (excluding covalent cross-linking). It also confers negative charge. In the presence of SDS, the intrinsic charge of a protein is masked. During SDS PAGE, all proteins migrate toward the anode (the positively charged electrode). SDS-treated proteins have very similar charge-to-mass ratios, and similar shapes. During PAGE, the rate of migration of SDS-treated proteins is effectively determined by molecular weight. Proteins were routinely analysed using denaturing (SDS) poly acrylamide gels and followed by Coommasie staining or Western blotting. The BIORAD mini-Protean system was used for all electrophoresis applications. The BIORAD semidry blotting system was used for western blotting.

Preparation of Gels

The gel casting stand was assembled and 12.5% polyacrylamide separating gel was prepared according to the volumes given below (enough for 4 gels)

Separating gel (12.5%) :

Acrylamide/bis-acrylamide (1:37.5)	8.3 ml
4x running buffer	5 ml
10% SDS	0.2 ml
ddH2O	6.4 ml
Ammoniumpersulfate	100 µl
TEMED	6.7 µl

The solution was mixed thoroughly and immediately transferred in between the plates in the gel casting stand. The separating gel was pipetted up to the top of the glass plate leaving 3cm at the top of the plate for the stacking gel. The solution was then covered with 100µl

water saturated butanol to prevent drying. The gel was allowed to polymerise for ~30min. In the meantime the stacking gel was prepared accordingly.

Stacking Gel (4%) :

Acrylamide/bis-acrylamide (1:37.5)	1.33 ml
4X stacking buffer	2.5 ml
10% SDS	0.1 ml
ddH2O	6 ml
Ammoniumpersulfate	100 µl
TEMED	5 µl

Once the separating gel polymerised, the butanol was removed and the stacking gel solution was poured on the separating gel till the chamber was filled. The desired comb was placed in between the plates in the stacking gel solution. The stacking gel was allowed to polymerise for ~ 30 min. Polymerised gels were either immediately used for electrophoresis or stored at 4°C until further use.

Poly Acryl amide Gel Electrophoresis (PAGE)

Cell lysates were boiled at 98°C for 3 min and 30µg-100µg of total protein was loaded on to the gel in most cases into the slots. When cells were lysed directly in Laemmli buffer up to 20µl of the sample was loaded on to the gel along with 5µl of prestained protein marker. The voltage was set to 120v and 500mA and the proteins were allowed to separate for 1hr 30 min.

2.2.13.2 Western Blotting

After electrophoresis the proteins separated on the gel were transferred to a PVDF membrane by semidry blotting. PVDF membranes were first put in methanol and then transferred to anode solution II. Four Whatman filter papers were immersed in the 1x anode solution I and placed on the blotting apparatus followed by 3 filter papers soaked in anode solution II. The membrane was then placed on the filter papers followed by the gel. Six filter papers dipped in cathode solution were then placed on the gel. The blotting apparatus was assembled accordingly and the transfer was performed at 23V for 1 hr.

2.2.13.3 Antibody incubations and detection

After the transfer, the blot was washed once with water and stained in Ponceau solution for 2 min. The blot was destained in water for additional 5 min and directly used for antibody incubation. Blocking was done in blocking buffer for 1hr at RT followed by incubation with primary antibody for 1hr (at RT) – overnight (at 4°C) with gentle shaking. The membrane was washed 3 times with wash buffer, 10 min each. The secondary antibody labelled with HRP was diluted in blocking buffer and the blot was incubated for 1hr at RT with gentle shaking followed by 3 washes for 10 min each with wash buffer. The blot was then incubated with appropriate amount of substrate solution for 2 min. The excess substrate was drained and the membrane was put in a polythene sheet for detection. The signal was detected by exposing the membrane to an X-ray film and the film was developed in an X-ray developing machine.

2.2.14 Co-Immunoprecipitation

Co-Immunoprecipitation was performed using the Profound mammalian Co-Immunoprecipitation kit from Pierce Biotechnology. The protocol is briefly described below. All steps were performed at RT and centrifugation was done at 3500 rpm unless otherwise mentioned.

2.2.14.1 Antibody immobilisation

The antibody coupling gel and other reagents provided by the manufacturer were equilibrated to room temperature. 100µl of antibody coupling gel was transferred into a spin column that was placed in microfuge tube. The gel slurry was then washed 2 times with 0.4ml of coupling buffer (PBS + 0.1%Triton X-100) by inverting and gently shaking the tube 4-5 times and centrifuging the tubes briefly. 200µg of purified antibody against the bait protein diluted in coupling buffer to a final volume of 400µl was added to the coupling gel. The beads were resuspended well by inverting the column and shaking gently. 5µl of 5M sodium cyanoborohydride was added to every 100µl of diluted antibody added to the gel and the tubes were inverted 4-5 times to mix well. The coupling was performed by incubating the columns for 4 hrs at RT or overnight at 4°C on an end to end shaker. The tubes were then centrifuged to remove the antibody solution and washed once by adding 400µl of coupling buffer, inverting the tubes 10 times and centrifuging again. The gel was then quenched by adding 400µl of quenching buffer and inverting the tubes 10 times followed by centrifugation.

400µl of quenching buffer was added to the gel and 4µl of 5M sodium cyanoborohydride was added to the slurry. The slurry was incubated at RT for 30 min on an end to end shaker. The tubes were centrifuged and the flow through was discarded. The gel was then washed 4 times with 400µl of washing buffer and two times with 400µl of coupling buffer. Finally the columns were stored at 4°C with the gel resuspended in 400µl of coupling buffer.

2.2.14.2 Preparation of cell lysates

1 x 10^6 cells were seeded in 10cm dishes and incubate overnight at 37°C. On the following day, cells were transfected and incubated for 48 hrs before lysis to allow good expression of the bait protein. Cells were washed once with ice cold PBS and then 500µl of mPER lysis buffer was added to the cells in the petri plate. Cell were incubated at RT for 5 min in the lysis buffer and scraped with a cell scraper and the lysate was collected in an Eppendorf tube. The tubes were centrifuged at 13000 rpm at 4°C for 10 min to get rid of the debris. The clear lysate was then carefully transferred into fresh tubes. The total protein concentration of the lysate was estimated using the BCA method and at least 500µg of total protein was used for the co-immunoprecipitation.

2.2.14.3 Co-Immunoprecipitation

Five hundred micrograms of total protein was diluted in the coupling buffer to a final volume of 400µl and then added to the column containing the gel with immobilised antibody. The tubes were inverted 4-5 times and incubated for 2hrs at RT on an end to end shaker. The tubes were centrifuged and the flow through was collected for further analysis and stored at –80°C. The columns were washed 3 times with 400µl of coupling buffer by inverting the tubes 10 times followed by centrifugation. The wash fractions were collected and kept aside for further analysis. The protein complexes were eluted from the antibody coupled gel by adding 50-100µl of elution buffer and tapping the tubes gently to allow resuspension of the gel in the elution buffer. The columns were then centrifuged and the elution fractions were collected for further analysis. The flow through, wash and elution fractions were further analysed by Western blotting.

2.2.15 Immunofluorescence

Cells were seeded in the appropriate medium at a density so as to obtain 60% confluence on the following day in 6 well plates with cover slips. These were either coated with poly-L-Lysine or left untreated and incubated at 37°C with 5% CO_2 in an incubator for 16-24 hrs. On the following day cells were washed once with PBS (37°C) and fixed with 4% PFA for 15 min at RT. After fixation cell were washed 2 times with PBS for 5 min each. Cells were then permeabilised with 0.2% TritonX-100/PBS for 10 min at RT followed by washing with PBS. Cells were incubated in a blocking solution (3%BSA in PBS or Fx signal enhancer, Invitrogen) for 30 min. Cover slips were then incubated with primary antibody diluted appropriately in 3% BSA/PBS for 30 min at RT to overnight at 4°C depending on the antibody. Cells were then washed 3 times with PBS and then incubated for 30 min at RT in the dark with the fluorescent labelled secondary antibody diluted appropriately in 3%BSA/PBS. Cells were washed 3 times with PBS and then incubated with DAPI diluted in PBS for 5 minutes. Cells were washed 2 times with PBS and rinsed once in Millipore water. The cover slips were then mounted on the glass slides with a drop of mounting medium (Prolong gold anti-fade) and allowed to dry overnight at RT in the dark before making images.

2.2.16 Flow cytometry

Cells were trypsinised and transferred to polystyrene FACS tubes and centrifuged at 1000 rpm for 5 min at RT. The medium and trypsin were then decanted and cells were washed once with PBS. PBS was removed from cells after centrifugation for 5 min at 1000 rpm at RT. Cells were then fixed with 1-2% PFA for 10 min at 37°C and then centrifuged at 1000 rpm for 5 min at RT. The PFA was decanted and cells were washed once with PBS as described above. Permeabilisation was done with ice cold 90% Methanol and cells were incubated on ice for 30 min or at –20°C overnight. Methanol was removed by centrifugation and the cells were washed once with PBS. Cells were then washed once with wash buffer (0.5% BSA in PBS). The primary antibody was diluted appropriately in wash buffer and cells were incubated with 100µl of antibody dilution at RT for 30 min. The cells were washed once with 1ml wash buffer and incubated for 30 min at RT in the dark with the appropriate dilution of the secondary antibody diluted in wash buffer. Cells were washed once with 1ml wash buffer and then resuspended in wash buffer and measured with a flow cytometer.

2.2.16.1 Protocol for automated ERK1/2 activation assay on FACS

Cells were trypsinised counted and seeded in 24 well plates in 500µl of appropriate culture medium with 10% FBS and other supplements. at a density so as obtain 50-60% confluence (32000 cells/well for 293 cells and 12000 cells/well for NIH3T3 cells) on the following day. Care was taken that the cells were evenly distributed in the well and no clusters or clumps were formed. After incubation at 37°C for 24 hrs cells were transfected with 40-200ng of plasmid DNA per well with Effectene according to the protocol already described on a Packard liquid handler. Cells were then incubated for 24 hrs at 37°C to allow expression. On the following day, the culture medium was removed and cells were washed once with PBS.

Fresh culture medium with 1% FBS was added to the cells and then incubated for another 24 hrs at 37°C, in order to synchronize the cells and also reduce the basal phosphorylation of ERK1/2. On the following day cells were washed once with PBS and trypsinised in 50µl trypsin for 2 min at 37°C. Trypsinisation was stopped by adding 50µl of culture medium containing 20% FBS and incubated at 37°C for 5 min at 37°C. Exactly after 5 min, 100µl of 2% PFA was added to fix the cells. Cells were incubated in PFA for 10 min at 37°C and then transferred to a U-bottomed, polypropylene, 96 well plate. Immunostaining was performed on the Packard liquid handler. The 96 well plate with cells was then centrifuged at 1350 rpm for 5 min to pellet the cells. The PFA was aspirated and cells were washed once in 150µl wash buffer (0.5% BSA in PBS) by centrifugation as described above. Permeabilisation was done by incubating cells with ice cold 90% methanol for 30 min on ice followed by one wash with wash buffer. The cells were then resuspended in 50µl of primary antibody dilution (anti-phospho ERK1/2, diluted 1:100 in wash buffer) and incubated at RT for 30 min. Cells were washed once by adding 150µl of wash buffer and the centrifuged. The wash buffer was aspirated and the cells were resuspended in 50µl of secondary antibody dilution (APC labelled, goat anti-rabbit IgG diluted 1:250 in wash buffer). Cells were then incubated at RT for 30 min in the dark. Cells were washed once by adding 100µl of wash buffer and the centrifuged as described. The wash buffer was aspirated and the cells were resuspended in 50µl of wash buffer and measured on FACS Calibur equipped with a plate reader.

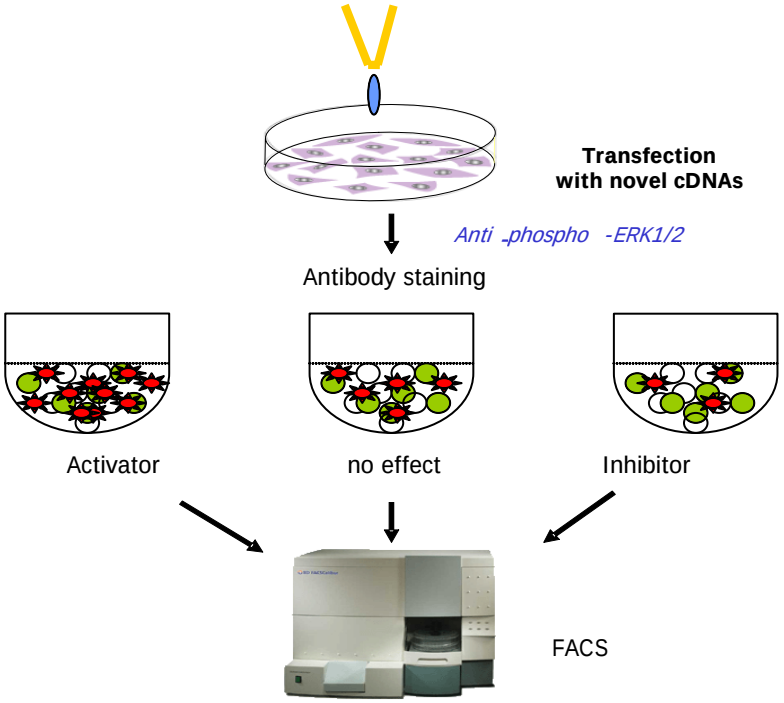


Fig 2.5: Schematic representation of the principle of the MAPK assay. Cells transfected in 24-well plates with YFP tagged cDNAs were allowed to express the protein for 24 hrs and then treated according to the assay protocol. Cells are then stained with phospho-ERK1/2 antibody. The level of phospho-ERK1/2 in the transfected cells is then measured in a FACS

2.2.16.2 Analysis of FACS data

Data generated by the FACS from each experiment was analysed statistically to infer the significance of the results obtained. In brief, a robust smoothed local regression analysis was performed on the data that was generated by the FACS. The raw data was normalize to remove spurious correlations between cell size and the fluorescent channels used to detect transfection and ERK1/2 phosphorylation and to correct for instrument-specific shifts in the measurement intensities. The level of ERK1/2 phosphorylation in transfected cells was compared to that in the non-transfected cells in each well. Continuous shifts of fluorescence intensities of APC (ERK1/2 phosphorylation) in non-transfected cells to higher or lower values in transfected cells were considered an effect (Equation 1). As a measure of effect size, a *z-score* was calculated from the slope Δ of the smoothed local regression function m for mildly perturbed cells (Equation 2). Under the assumption of normality a *p-value* was obtained from the fitted model to indicate the significance of the observed effect. The z-score for an item indicates how far and in what direction that item deviates from its population mean, expressed in units of its population's standard deviation. Since the slope of the regression function is symmetric around zero, here the z-score could be directly obtained

though division by an assay-wide scaling parameter δ_0 which is an estimated of the population's variance (Equation 3).

$$y = y_0 + m(x - x_0) + \varepsilon \quad (1)$$

$$\Delta = \hat{m}'(x_t) \quad (2)$$

$$z = \frac{\Delta}{\delta_0} \quad (3)$$

The p-value is the probability, with a value ranging from zero to one, that the observed values can be drawn from a random sample distribution. A p-value close to zero indicates that the observed values are unlikely to be obtained by chance and hence the location of two distributions most likely differs. Large p-values closer to 1 imply that there is no detectable difference in the two distributions. A p-value of 0.05 is a typical threshold used. The results obtained after the analysis were represented as dot plots, histograms or other user friendly graphical output that could be easily used and interpreted.

Cells transfected with YFP were fixed and stained with phospho-ERK1/2 antibody according to the assay protocol. The transfection (YFP intensity) and the ERK1/2 activation (APC intensity) were measured in a flow cytometer. The data was used to generate a dot plot showing transfection efficiency (x-axis) and ERK1/2 phosphorylation (y-axis) (Fig: 2.6). In the dot plot, the transfected and the non-transfected cells are distinguished by setting an arbitrary demarcation at YFP intensity value of ~200 (--- start of transfection, Fig: 2.3). This demarcation is defined based on the auto fluorescence of non transfected cells that lies within this value (not shown). Proteins that do not effect the ERK1/2 phosphorylation (for eg; YFP) are assumed to show a z-score of zero and positive or negative values indicate activation or inhibition. The z-score for YFP in this experiment was 0.005, suggesting that it does not affect the ERK1/2 activation. The data from all the replicate measurements of YFP were analysed in a generalized local regression function introducing an additional plate-specific factor and p-values were obtained. All the data obtained from FACS based detection of the ERK1/2 activation assay was analysed similarly.

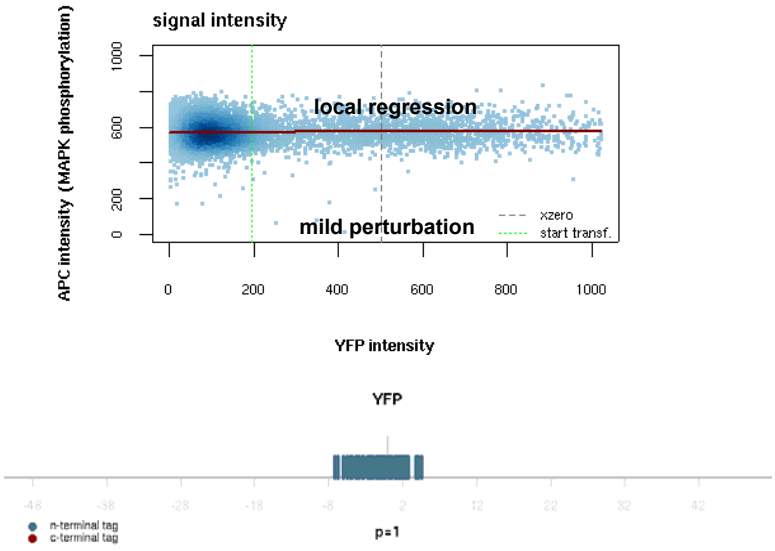


Figure 2.6: Analysis of FACS data. Data generated from one well in a 96 well plate is represented as a dot plot. The dot plot shows the start of transfection, level of expression and also the level of ERK1/2 activation. A line representing the linear regression is also displayed, indicating the effect of the clone on ERK1/2 activation. A graph showing all the replicates and their effect with relevance to the p-value.

2.2.17 Treatment of cells with cell cycle blocking reagents

HEK-293T cells were grown in DMEM with 10% FBS for 16-24 hrs. Cells were washed once with 1x PBS and then fresh medium with cell cycle blocking reagents was added to cells and incubate for16-24 hrs before analysing the cells. Cells were blocked in G0/G1 phase by treating with Differentiation inducing factor-3 (DIF-3, 30µM) and in the G2/M phase with vincristine sulphate (10µM).

2.2.18 Cell cycle analysis by BrdU incorporation

The BrdU incorporation experiments were performed using the BrdU flow kit from BD biosciences. All buffers used were supplied by the supplier unless otherwise mentioned in the protocol. HEK-293T cells were grown in DMEM with 10% FBS for 16-24hrs and treated accordingly with cell cycle blocking reagents or left untreated for further 24 hrs. On the day of staining, cells were pulsed with 10µM BrdU diluted in culture medium for 1 hr. Then the cells were trypsinised and washed once with PBS and fixed in 100µl cytofix/cytoperm buffer for 15 min at RT. After the incubation, cells were washed once with 1ml of perm/wash buffer and then resuspended in 100µl of cytoperm plus buffer for 10 min on ice and washed once with 1ml perm/wash buffer. Cells were then refixed in 100µl of cytofix/cytoperm for 5 min at

RT and washed once in 1ml of perm/wash buffer. The cells were resuspended in 100µl DNAse solution (300µg/ml, diluted in PBS) for 1 hr at 37°C followed by washing once with 1ml of perm/wash buffer. Staining with the anti-BrdU antibody was performed by resuspending cells in perm/wash buffer containing the anti-BrdU antibody (diluted 1:50) and incubating at RT for 20 min. The cells were then washed once with 1ml of perm/wash and resuspended in 1ml of staining buffer. Cells were either analysed immediately by FACS or stored at 4°C.

2.2.19 Antibody generation and Characterisation

2.2.19.1 Peptide selection

Antibodies against the protein of interest were raised by immunizing rabbits with selected peptides. Peptides were selected using a software available at the Invitrogen web site (www.invitrogen.com). Care was taken to choose a unique sequences which did not overlap with sequences from other proteins by performing a protein blast for every selected peptide. The peptide was also chosen from hydrophilic, accessible and highly antigenic regions of the protein. At least four peptides were selected for each protein and given for immunisation. The company synthesised the peptide and used it for immunisation and provided us with appropriate amount of peptide that would be needed for purification by affinity chromatography.

2.2.19.2 Selection of Rabbits

The pre-immune sera of the chosen rabbits were delivered in order to check for any non-specific antibody binding. Cell lysates from 293 cells transfected with the YFP-tagged fusion proteins were used to check non-specific binding. The membranes with both transfected as well as non-transfected cell lysates were incubated the pre-immune sera. Only those rabbits which did not show any non-specific binding were chosen for immunization.

2.2.19.3 Affinity chromatography

Affinity chromatography was the method of choice for the purification of antibodies from the sera. Affinity chromatography (AC) is a technique enabling purification of a biomolecule with respect to biological function or individual chemical structure. The

substance to be purified is specifically and reversibly adsorbed to a ligand (binding substance), immobilized by a covalent bond to a chromatographic bed material (matrix). Samples are applied under favourable conditions for their specific binding to the ligand. Substances of interest are consequently bound to the ligand while unbound substances are washed away. Recovery of molecules of interest can be achieved by changing experimental conditions to favour desorption. AC media are commonly used for applications such as purification of fusion proteins, mono- and polyclonal antibodies, and glycoproteins.

2.2.19.4 Peptide coupling

We used ECH-activated Sepharose as a matrix to couple the peptide. The ECH-activated Sepharose contains free carboxyl groups at the end of long flexible spacer, to which the free amino groups of the peptides can couple forming a carbodiimide bond. The Sepharose is supplied in 20% ethanol. The Sepharose was washed several times in distilled water (pH 4.5) and 0.5M NaCl. The peptides (~10mg) were either dissolved in a 20% dioxane and then made up to 1ml with water or directly dissolved in water and the pH was adjusted to 4.5. The peptide solution was mixed with the Sepharose slurry and mixed well by inverting the column several times. The ratio between the peptide solution and the matrix was kept at 1:0.5. In order to facilitate the formation of a carbodiimide bond a carbodiimide (EDC, 100mg) dissolved in water with a pH 4.5 was added drop wise to the matrix with peptide and mixed by inverting. The mixture was incubated overnight at 4°C on a rotator with gentle end to end rotation. The pH of the mixture was monitored during the first hour of incubation and adjusted to 4.5 if necessary with 0.1M NaOH. On the following day, the slurry was washed 3 times with solutions having alternating pH of low (0.1M Acetate buffer, pH 4.0 in 0.5M NaCl) and high (0.1M Tris/HCl, pH 8.0 in 0.5M NaCl). The slurry was then washed with distilled water. The column was then stored in PBS with sodium azide (0.2%) at 4°C until further use.

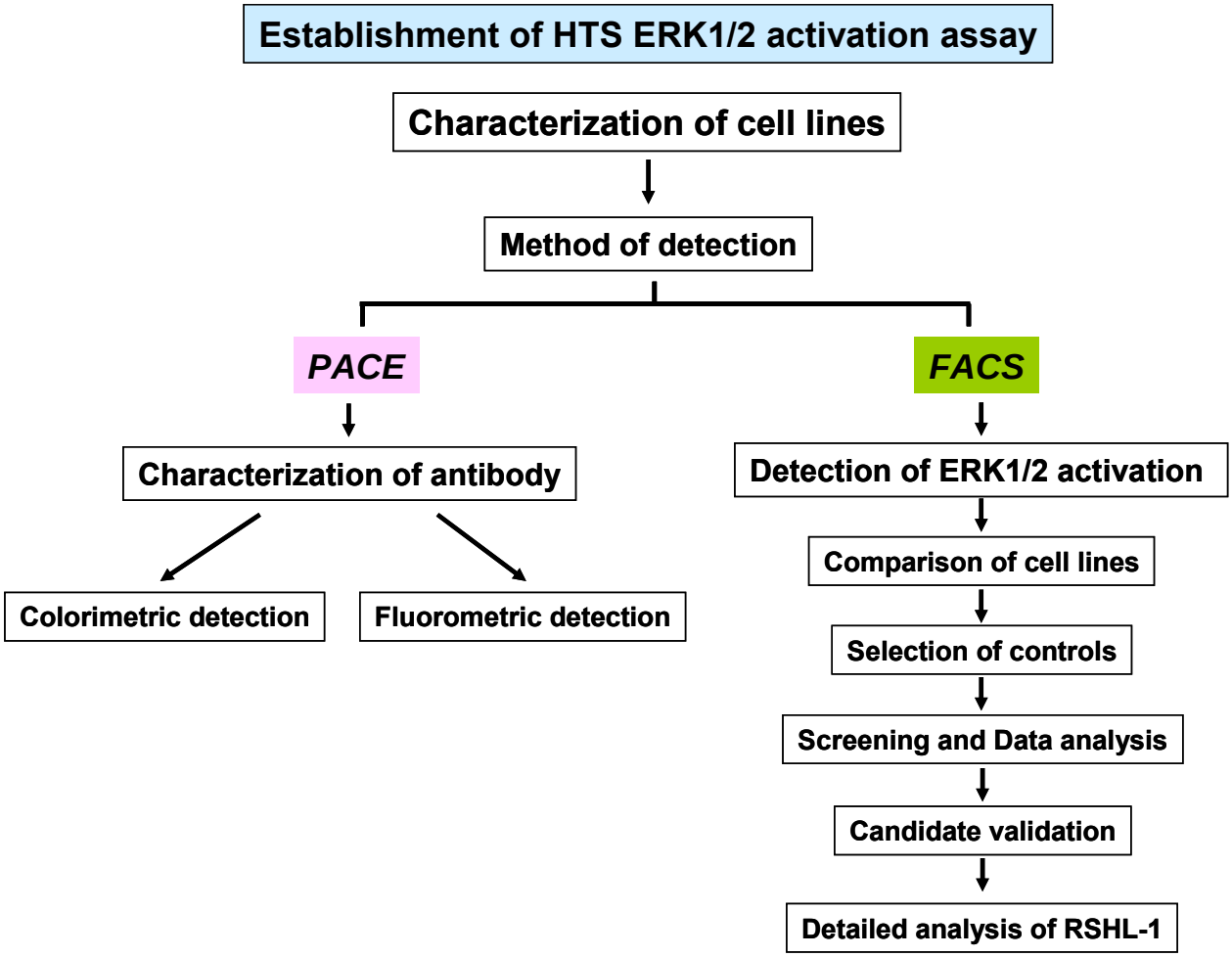
2.2.19.5 Antibody purification

On the day of purification, the sera were thawed and the column was equilibrated to room temperature. The beads were washed with PBS and approximately 50ml of the serum was added to the beads coupled with the peptide. The mixture was incubated overnight at 4°C on a rotator with gentle end to end mixing. The mixture was then centrifuged and the supernatant was separated from the beads. The beads were then washed with PBS and antibody was eluted

with 20 mM Tris pH 7.5 , 150 mM NaCl. Fractions (1ml) were collected and their protein concentration was measured using a spectrophotometer with UV Abs_{280}. The fractions with absorbance 280 between 0.5 and above were used to check the presence of the desired specific antibody. Cell lysates from 293 cells transfected with the YFP-tagged protein were separated by PAGE and then transferred on to PVDF membranes by western blotting. The selected fractions were used on these membranes and the presence of the antibody was confirmed by detection of a signal corresponding to the YFP-tagged protein on the X-ray film. These fractions were then collected and stored with 50% glycerol at -80°C for further use.

3 Results

Schematic representation of assay establishment and screening



The establishment of the ERK1/2 activation assay involves the selection of appropriate cell lines and antibodies to be used in the assay. Once the cell lines and the antibodies had been characterised, the method of detection was optimised for the assay conditions and HTS requirements. After optimisation of the method of detection, the screening was performed. The candidates resulting from the screen were validated to confirm the effects. Detailed functional analysis was performed with one of the candidates from the screen.

3.1 Characterisation of ERK1/2 activation in different cell lines

The cell line that can be used to monitor the ERK1/2 activation or perturbations in the pathway needs to be well characterised for the activation state and regulation of ERK1/2 activation by known stimuli (e.g. FBS, EGF) and inhibitors (for example: U0126). The main characteristic of an ideal cell line for the assay would be the ability to modulate ERK1/2 pathway in such a way that when the cells are serum starved, the level of phospho-ERK1/2 should decrease to a basal level and then increase significantly upon stimulation with specific growth factors or serum. Ensuring that the ERK1/2 activation state can be modulated and monitored using well known stimuli and inhibitorsit should be feasible to define a normal response to a particular stimulus or inhibitor in the cell line and thus be able to detect perturbations in this response as an “effect” on the ERK1/2 activation.

Two human breast cancer cell lines (BT474 and SKBR3), a human cervical carcinoma cell line (HeLa), a human embryonic kidney cell line (HEK-293T), and one murine fibroblast cell line (NIH3T3) were selected for initial characterisation studies. All the cell lines were deprived of serum or growth factors in the medium for at least 24 hrs prior to addition of EGF or treatment with the inhibitor, respectively. It was observed that the BT474 cells had reduced level of phosphorylated ERK1/2 prior to stimulation with EGF (Fig: 3.1 a) which increased significantly upon stimulation. Cells treated with the specific MEK1/2 inhibitor, U0126, showed reduced ERK1/2 phosphorylation after stimulation with EGF. Unlike BT474 cells, the SKBR3 cells showed ERK1/2 activation even without stimulation with EGF (Fig: 3.1 b), and the level of activation did not increase with EGF stimulation. The MEK1/2 inhibitor, U0126, did not induce any reduction in the ERK1/2 activation in these cells. HeLa cells showed a high basal level of activated ERK1/2 without stimulation with EGF (Fig: 3.1 c). The level of activation increased only slightly after stimulation, and the activation of ERK1/2 was reduced when cells were treated with the U0126. The HEK-293T cells showed a low level of ERK1/2 activation prior to stimulation with EGF. Stimulation with EGF induced an increase in the activation of ERK1/2. However, cells treated with U0126, did not show an increase in ERK1/2 activation even after stimulation with EGF (Fig: 3.1 d). The NIH3T3 cells showed signs of cytotoxicity when cells had been incubated in serum free medium for 24 hrs. Hence, these cells were incubated in medium with 1% FBS or serum free medium for 24 hrs prior to stimulation. NIH3T3 cells also showed decreased level of ERK1/2 activation upon serum deprivation (Fig: 3.1 e). EGF stimulation resulted in significant increase in ERK1/2 activation and treatment with U0126 resulted in inhibition of ERK1/2 activation after EGF stimulation.

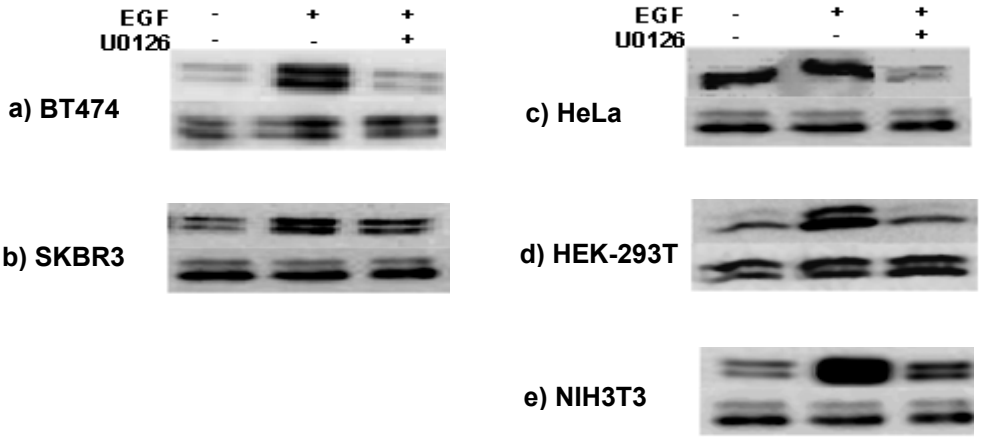


Figure 3.1: Characterisation of different cell lines for ERK1/2 activity. Cells were seeded in 6 well plates and incubated for 24hrs in complete growth medium (DMEM) with 10% FBS at 37°C. On the following day, the medium was removed and cells were washed once with PBS and incubated in serum free medium or medium with 1% FBS (NIH3T3) for 24 hrs and treated with U0126 for 30 min or left untreated before stimulating with EGF (25ng/ml) for 10 minutes. Cells were then lysed and 30µg of total protein was loaded per lane. Western blotting was performed with phospho-ERK1/2(phospho-p44/42) antibody. **Lane 1:** serum starved. **Lane 2:** Stimulated with EGF. **Lane 3:** Treated with U0126 and then stimulated with EGF

Out of the five cell lines selected for characterisation, BT474 cells, HEK-293T cells and NIH3T3 cells were found to be suitable for the assay. In these cell lines the ERK1/2 activation could be regulated by conditions like serum deprivation, stimulation, and treatment with an inhibitor. They showed a reproducible stable pattern of ERK1/2 activation in which the level of activated ERK1/2 was reduced upon serum deprivation and increased significantly upon stimulation with EGF. Treatment with U0126 prior to stimulation with EGF leaded to inhibition of ERK1/2 phosphorylation by MEK1/2. The SKBR3 and HeLa cells had abnormal level of ERK1/2 activation which could not be regulated by external stimuli or conditions like serum deprivation and hence were considered unsuitable for the assay.

3.2 Comparison of transfection efficiency

Detection of ERK1/2 activation in fixed cells after over-expression of an uncharacterised protein should be sensitive and specific. The change in the activation state of ERK1/2 that is measured was always in comparison to the non-transfected cells. Therefore, it was essential to estimate the transfection efficiency of a particular cell line that was used in the assay.

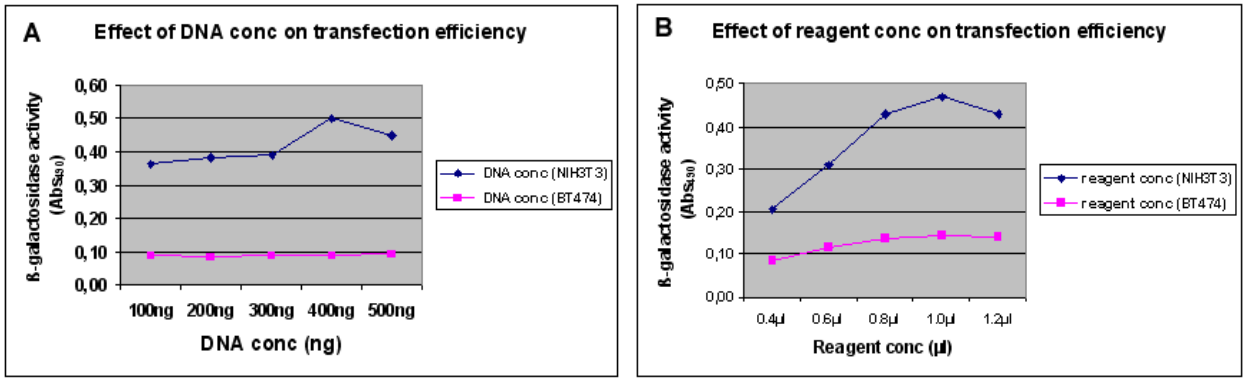


Figure 3.2: Comparison of transfection efficiency. Cells were transfected with varying amounts of plasmid DNA ranging from 100-500ng (A) and Lipofectamine 2000 reagent ranging from 0.4µl – 1.2µl (B). One day after the transfection, cells were lysed and the ß-Galactosidase activity was measured by the addition of ONPG as a substrate. The Abs_{490} was measured in a plate reader in a 96 well plate format. NIH3T3 cells showed higher transfection efficiency than BT474 cells. A DNA concentration of 400ng/well and a reagent concentration of 1µl/well resulted in highest transfection efficiency for NIH3T3 cells.

The BT474 cells, NIH3T3 cells, and the HEK-293 cells were selected for further characterisation. HEK-293T cells were not used for the establishment of the ELISA based assay because of their weak adherent property. Therefore, initially the transfection efficiency of NIH3T3 cells and BT474 cells was compared. Both cell lines were seeded at equal density. The reagent concentration (Lipofectamine2000) and the DNA concentration (ß-galactosidase reporter plasmid) were varied from 100 to 500ng / well and 0.4µl to 1.2µl / well respectively (Fig: 3.2, A and B). A reagent volume of 1µl/well and 400ng of DNA resulted in the highest transfection efficiency for NIH3T3 cells under these experimental conditions (Fig: 3.2, A). The value corresponding to the ß-galactosidase activity (ß-gal activity, Abs_{490}), which is proportional to the number of transfected cells was found to be between 2.0 and 5.0 for NIH3T3 cells and that of the BT474 cells ranged between 2.5 and 3.5 (Fig: 3.2, B). Under the given experimental conditions NIH3T3 cells showed better transfection efficiency and hence were used in assay development.

3.3 PACE

3.3.1 Testing of HTS criteria with PACE

Activation of ERK1/2 occurs via phosphorylation of Thr183/Tyr185 and Thr202/Tyr204 on ERK1/2 respectively by MEK1/2. Detection of activated ERK1/2 can be done by using

commercially available phospho-specific antibodies that specifically recognise the phosphorylated form of ERK1/2 and do not cross react with non-phosphorylated ERK1/2. Their specificity and sensitivity in detecting the phosphorylated ERK1/2 has been well characterised for Western blot applications [79, 80]. The same antibodies were used to detect differences in levels of phosphorylated ERK1/2 for the characterisation of cell lines using Western blotting (Fig: 3.1). The compatibility of the antibodies for detection of phosphorylated ERK1/2 in fixed cells has already been checked and reported in different cell lines [81]. However, the specificity and sensitivity of the antibody differs between cell types and conditions used. Hence, the specificity, sensitivity and the appropriate dilution of the phosphorylated ERK1/2 antibody using NIH3T3 cells for the ERK1/2 activation assay conditions were identified first.

3.3.2 Determination of optimal dilution of phospho-ERK1/2 antibody for PACE

NIH3T3 cells were seeded in 96 well plates and treated according to the PACE protocol. In order to determine the optimal dilution of phospho-ERK1/2 antibody, a rabbit polyclonal anti-phospho ERK1/2 antibody from Cell signalling technology, was diluted in a range from 1:100 to 1:1000 (Fig 3.3). Cells were stained with primary and secondary antibody (Ab +1 +2), just the primary antibody (Ab +1 -2), only secondary antibody (Ab -1+2) or were left unstained (Ab -1 -2).

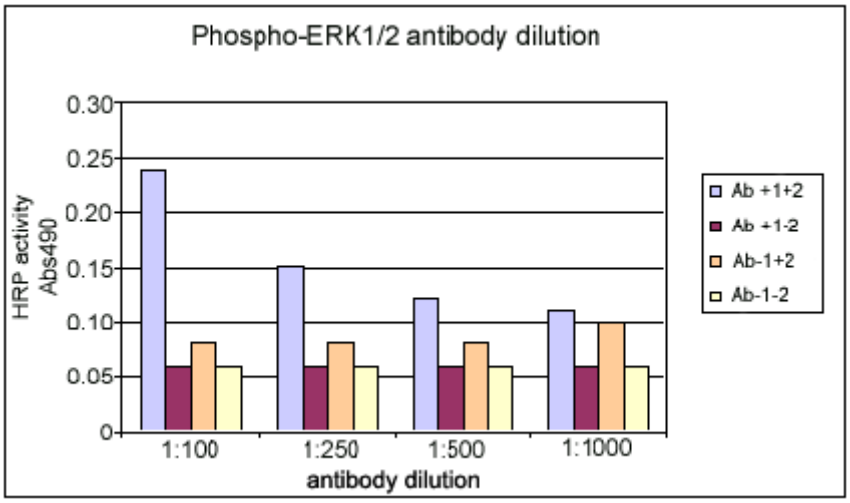


Figure 3.3: Determination of optimal antibody dilution for PACE. NIH3T3 cells were seeded in 96 well plates, incubated overnight at 37°C and then treated according to the PACE protocol. Cells were then stained with different dilutions of rabbit phospho-ERK1/2 antibody. The dilution of 1:100 was found to be optimal for PACE.

Wells without cells showed no immuno-reactivity and the phospho-ERK1/2 level (Abs_{490}) of these wells was between 0.05 and 0.1 for all the dilutions of primary antibody. The wells incubated with secondary antibody alone showed a signal between 0.07 and 0.09. This was only a little higher than the values obtained from wells which had not been stained with antibodies. A signal with the phospho-ERK1/2 level of ~ 0.24 (Abs_{490}) was observed at a primary antibody dilution 1:100 (Fig 3.3). However the signal decreased when the phospho-ERK1/2 antibody was further diluted.

3.3.3 Specificity of phospho-ERK1/2 antibody

It is pivotal that the phospho-specific antibody reacts only with phospho-ERK1/2 and does not cross-react with the non-phosphorylated form. To validate that the selected antibody met this criterion, cells were serum starved and then either stimulated with EGF or left unstimulated and stained with ERK1/2 and phospho-ERK1/2 antibodies, respectively. Level of ERK1/2 did not differ between stimulated and non-stimulated cells (Fig 3.4). Phospho-ERK1/2 levels increased in cells that were stimulated in comparison to the non-stimulated cells showing that the phospho-ERK1/2 antibody was specifically recognising the phosphorylated form of ERK1/2.

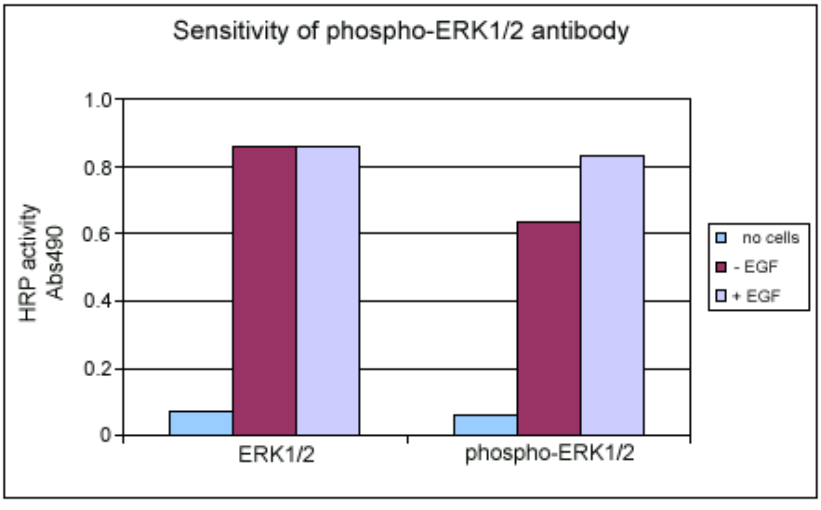


Figure 3.4: Determination of specificity of phospho-ERK1/2 antibody. NIH3T3 cells were serum starved for 24 hrs and were either stimulated with EGF (25ng/ml) for 10 min or left untreated. Cells were stained according to the PACE protocol with ERK1/2 and phospho-ERK1/2 antibodies to detect any cross reactivity and non-specific binding of the antibody. Level of ERK1/2 remained unchanged after stimulation and the level of phospho-ERK1/2 increased slightly after stimulation.

3.3.4 Determination of sensitivity of PACE

The sensitivity of the Phospho-specific Antibody Cell based Elisa (PACE) can be defined as the ability to detect subtle differences in levels of ERK1/2 phosphorylation. When the cell number is kept constant in all wells, the level of ERK1/2 should also remain constant. However, during the staining procedure of the PACE protocol, loss of cells can occur due to several washing steps involved. The loss of cells from wells does not occur uniformly from all the wells and hence can result in varying cell numbers and thus impart variations in the level of ERK1/2 in different wells of the same experiment. These variations were normalised by a crystal violet staining assay which gives a value at Abs_{595}, for the total number of cells present in a given well at the time of measurement of the ERK1/2 activation. A ratio (Abs_{490}/Abs_{595}) was calculated for the crystal violet binding (Abs_{595}) value and the phospho-ERK1/2 value (Abs_{490}) to normalize these differences in ERK1/2 levels that were actually imparted by differences in cell numbers. Once this variation was normalised, it could be confirmed that the differences observed in ERK1/2 phosphorylation levels did indeed reflect the actual levels of the activated protein in the cells. The sensitivity of PACE was determined by staining cells, under different conditions, according to the PACE protocol.

3.3.4.1 Detection of ERK1/2 activation by PACE using HRP labelled secondary antibody

In order to determine the sensitivity of PACE under the given assay conditions, phosphorylation of ERK1/2 was detected by staining fixed cells with anti-phospho-ERK1/2 antibody followed by a secondary antibody labelled with HRP. Confluence level of the cells was found to affect the activation of ERK1/2. When cells were > 60% confluent at the time of the assay, the activation of ERK1/2 after serum stimulation could not be observed clearly. The cell number of 7,000 NIH3T3 cells that were seeded per well in a 96 well plate was found to be optimal for the assay conditions. Cells were treated according to the PACE protocol and stained with ERK1/2 and phospho-ERK1/2 antibodies, and a HRP labelled secondary antibody was used for detection. The signal was measured at Abs_{490} in a plate reader. The level of ERK1/2 appeared to vary slightly under the different conditions of the experiment before normalisation for cell number (Fig: 3.5 a). This variation, however, was minimised after normalisation (Fig: 3.5 b). The levels of phospho-ERK1/2 did not differ significantly between the normalised and the un-normalised values. Cells stimulated with EGF showed an increase in phospho-ERK1/2 level (Abs_{490}/Abs_{595} = 6.2) when compared to the serum deprived (untreated) cells (Abs_{490}/Abs_{595} = 4.8). The difference in phospho-ERK1/2 level between the untreated and stimulated cells was

comparable to the Western blot results obtained from the same cell line (Fig: 3.1 e). However, the signal intensity in Western blotting experiments was much higher than the signal intensities observed in PACE for the corresponding samples.

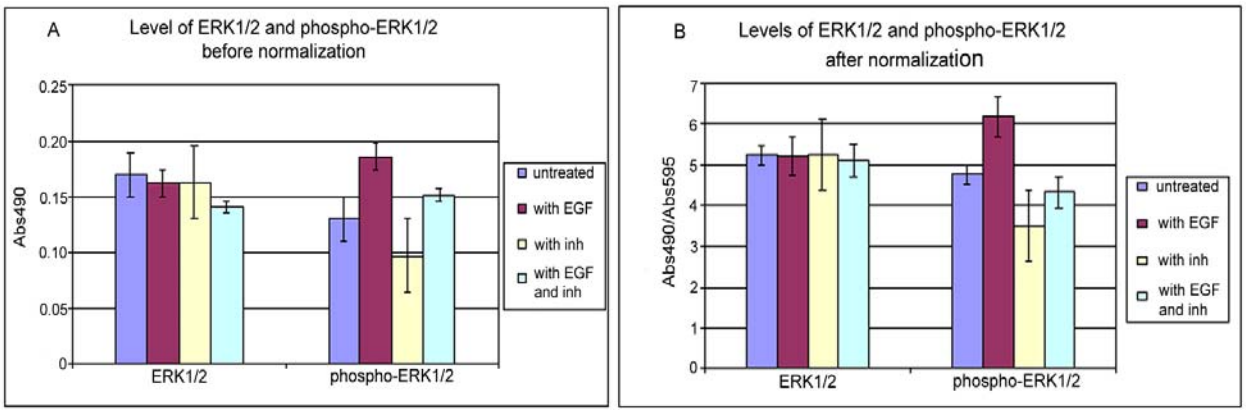


Figure 3.5: Detection of ERK1/2 and p-ERK1/2 in NIH3T3 cells using HRP labeled secondary antibody. NIH3T3 cells were seeded in 96 well plates and incubated overnight at 37°C and serum starved for 24 hrs prior to treatment with U0126 for 30 min or left untreated before stimulation with EGF (25ng/ml) for 10 min. Cells were stained according to the PACE protocol with ERK1/2 antibody and the phospho-ERK1/2 antibodies. A HRP labeled secondary antibody was used. Differences in cell numbers between wells were corrected using crystal violet staining. (a) After correction for cell number, the variation in ERK1/2 levels was normalized and slight differences in the level of phospho-ERK1/2 could be seen (b).

The low sensitivity of the HRP based detection could be attributed to the kinetics of the enzymatic reaction. A HRP molecule that is linked to the secondary antibody reacts with the substrate resulting in the formation of a coloured product. This reaction is linear in which the conversion is fast and reaches saturation quickly. This may be a drawback of the HRP based colorimetric detection which fails to present subtle differences generated from such an approach. Hence, I next tried to detect these differences using a fluorescently labelled secondary antibody instead of HRP.

3.3.4.2 Fluorometric detection of phospho-ERK1/2 using $Alexa_{568}$ labelled secondary antibody

Cells were stained with ERK1/2 and phospho-ERK1/2 antibodies followed by a anti-rabbit $Alexa_{568}$ labelled secondary antibody, according to the PACE protocol. The levels of ERK1/2 and phospho-ERK1/2 were estimated by measuring the relative fluorescence units (RFU). Variations in levels of ERK1/2 between different conditions were observed which, however, were not significant. As with the HRP labelled antibody the signal for level of

phospho-ERK1/2 increased after stimulation and the signal for cells that had been treated with inhibitor did not show an increase in the level after stimulation with EGF (Fig: 3.6).

The signal intensities for the level of phospho-ERK1/2 before and after stimulation were similar to those with HRP, and did not differ significantly. The results did not correlate with the data obtained by Western blotting (Fig: 3.1), where differences in the level of phospho-ERK1/2 between starved and stimulated cells could be clearly seen.

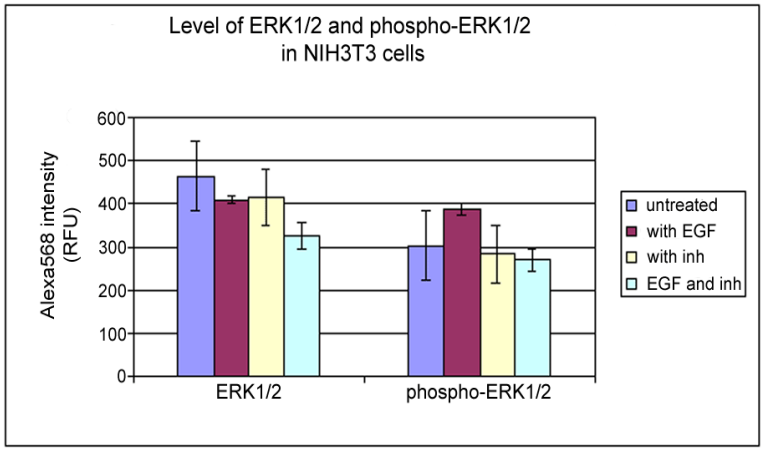


Figure 3.6: Detection of ERK1/2 and p-ERK1/2 in NIH3T3 cells using $Alexa_{568}$ labeled secondary antibody. Cells were stained according to the PACE protocol with ERK1/2 antibody and phospho-ERK1/2 antibody. Differences in phospho-ERK1/2 levels before and after stimulation and due to the treatment of cells with U0126 were not significantly different. The differences in phospho-ERK1/2 levels were not in correlation with the Western blot results.

3.3.5 In – cell detection of YFP with PACE

In the case of PACE, the effect of over-expression of YFP/CFP tagged proteins on the ERK1/2 phosphorylation in a defined cell system should be monitored. The level of phospho-ERK1/2 and the expression of the YFP/CFP tagged protein of interest need to be simultaneously measured in every well with fixed cells. Hence, the detection of transfected cells was checked by transfecting NIH3T3 cells with YFP or CFP expression constructs and fixed 24 hrs later in 96 well plates as recommended for the measurement of fluorescence in the plate reader. Expression of YFP/CFP was measured in a series of wells. Relative fluorescence values obtained from six wells, each transfected with YFP/CFP are shown here (Fig: 3.7). Wells without cells and wells with untransfected cells were also measured as controls. The RFU obtained from wells without cells, untransfected cells and cells transfected with YFP/CFP were similar. The YFP/CFP expressed in cells could thus not be detected by the Fluorometer.

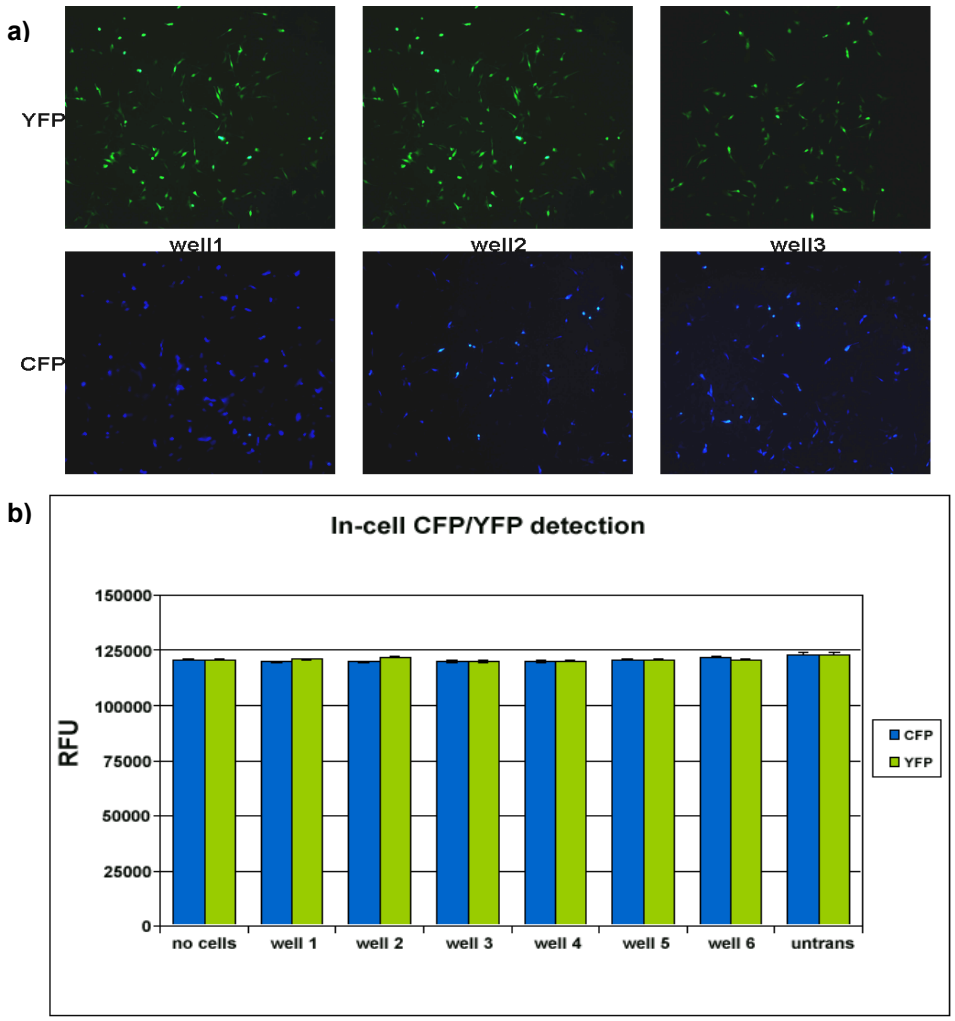


Figure 3.7: Fluorometric detection of YFP/CFP in NIH3T3 cells. a) Images showing transfected cells in wells that were used for detection of YFP and CFP in a fluorometer. Wells 1, 2 and 3 represented in the graph are shown in the figure. b) NIH3T3 cells were transfected with YFP or left untransfected. After 24hrs, the plate was measured using a fluorescent plate reader to measure the transfection (YFP/CFP level). Empty wells also showed high fluorescence. Wells without any cells or those that were untransfected showed equally high fluorescence values as the wells with YFP/CFP transfected cells.

PACE was finally regarded not to be suitable for the establishment of an ERK1/2 activation assay. Initially, the signals obtained from the HRP based detection were low and the differences in the phospho-ERK1/2 level between serum starved and stimulated cells were not significant. A fluorescently labelled secondary antibody was used instead of the HRP labelled antibody in order to improve the sensitivity. However, the difference in the phospho-ERK1/2 level between serum starved and stimulated cells did not improve and moreover the expression of YFP/CFP was not detectable.

3.4 Fluorescence Activated Cell Sorter (FACS)

3.4.1 FACS based detection of ERK1/2 phosphorylation

Due to the limitations of plate reader based measurement, alternative methods for the detection of ERK1/2 activation had to be considered. One promising method was Fluorescence Activated Cell Sorter (FACS). FACS based detection offers several advantages over the detection using a plate reader. A plate reader measures the total fluorescence from each well and thus gives an averaged value for both transfected and non-transfected cells and the auto-fluorescence background from the well surface. The information regarding the activation state of ERK1/2 in transfected and non-transfected cells could not be differentiated thus leading to the loss of sensitivity. In contrast, FACS based detection provides a single cell resolution and allows monitoring the level of expression and other parameters in each cell. Differences in ERK1/2 phosphorylation and the level of expression of the protein of interest can thus both be monitored in every cell individually. Additional information regarding cell morphology, transfection efficiency, level of expression and ERK1/2 activation can all be acquired by FACS based detection. Data generated by FACS, however needed to be analysed carefully by applying statistical tools which impart significance to the data and filter out artefacts, thereby enhancing the quality of the results obtained. Measurement of ERK1/2 activation by FACS required several modifications to the assay protocol. Cells needed to be trypsinised and then stimulated in suspension followed by fixation and staining. The assay protocol was modified and adapted accordingly for FACS based detection. The stimulation was done by FBS and not by EGF as FBS induced ERK1/2 activation through different growth factors and receptors compared to EGF, which acts primarily through the EGFR and its downstream pathway.

3.4.2 Detection of ERK1/2 activity in NIH3T3 and HEK-293T cells

The detection of ERK1/2 activation in NIH3T3 and HEK-293T cells was compared. HEK-293T cells had not been tested in PACE because of their weak adherent property that would have led to loss of cells during the procedure of serum starvation and staining. However, in FACS based detection, cells were trypsinised, stimulated and stained in suspension and hence their weak adherence was not a problem. Non-transfected cells treated and stained according to the assay protocol were measured in a FACS Calibur and dot plots were generated with YFP intensity on the x-axis and ERK1/2 phosphorylation on the y-axis. Each plot was divided into

two regions R1 and R2 by a horizontal line, based on expected ERK1/2 phosphorylation levels (APC intensity) (R2 = 0 – 600 and R1 = 600 – 1000) in cells that were serum starved and stimulated with FBS, respectively (Fig: 3.8).

Serum starved NIH3T3 cells showed APC intensities (ERK1/2 phosphorylation) from 300 – 800 units. From a total of 2,557 cells measured, 2,196 cells were in the region R2 corresponding to the low levels of ERK1/2 phosphorylation and 361 cells were present in the region R1 (Fig: 3.8, 1 a). When stimulated with 10% FBS for 5 min, the phosphorylation of ERK1/2 increased and out of the 2,860 cells measured, 2,766 cells were found in the region R2 and 94 cells in the region R1 (Fig: 3.8, 1 b). In case of the HEK-293T cells, out of the 7,404 cells measured in the serum starved sample, 6,971 cells were found in the region R2 and 433 cells were in region R1 (Fig: 3.8, 2 a). In the sample with serum stimulated cells a total of 6,081 cells were acquired out of which 3,957 cells lay in the region R2 and 2,124 cells were in the region R1 (Fig: 3.8, 2 b). These results show that phosphorylation of ERK1/2, after stimulation with FBS, is more efficient in NIH3T3 cells than the HEK-293T cells. Nevertheless, the activation of ERK1/2 upon stimulation was also detectable in HEK-293T cells.

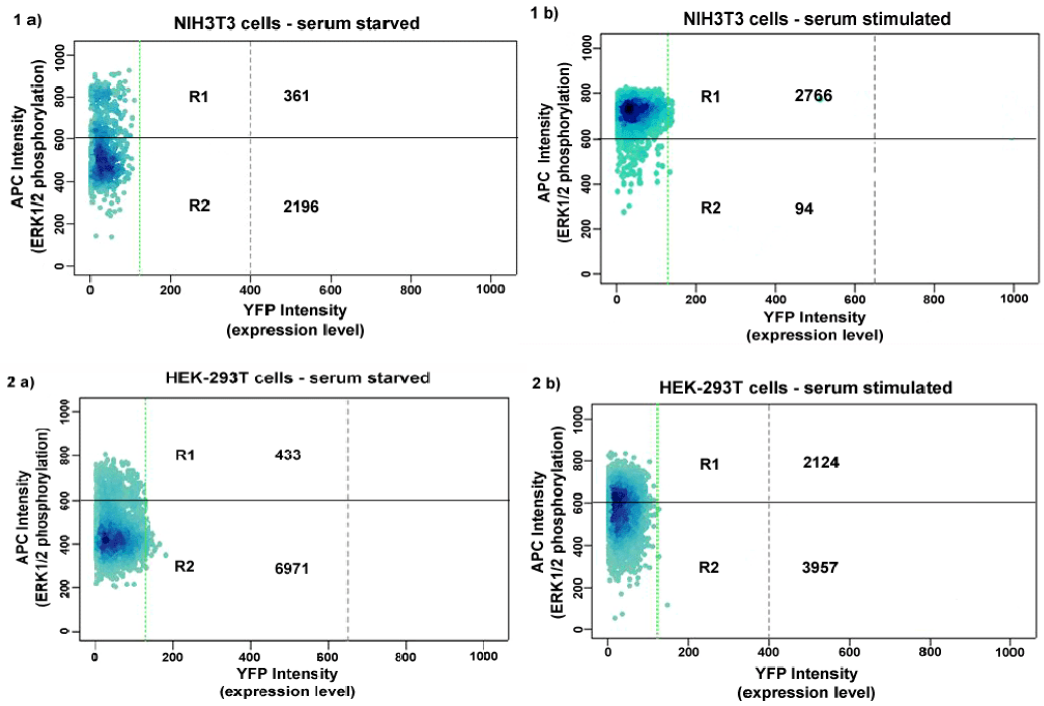


Figure 3.8: Detection of ERK1/2 phosphorylation in NIH3T3 and HEK-293T cells. ERK1/2 phosphorylation was measured in a FACS. The results are represented in a dot plot that is divided into two regions R1 and R2 by a horizontal line at 600 units. Region R1 corresponds to the phospho-ERK1/2 level of serum starved cells and region R2 corresponds to the range of fluorescence intensities (ERK1/2 phosphorylation) exhibited by cells stimulated with 10% FBS. Serum starved NIH3T3 cells mostly lay in the region R2 (2,196) (1a) and after stimulation most of the cells lay in the region R1 (2,766) (1b). Most of the serum starved HEK-293T cells also lay on the region R1 (6,971) (2a) and after stimulation the main population shifted upwards with 2,124 cells in the region R1. Activation of ERK1/2 was more efficient in NIH3T3 cells and the activation of ERK1/2 was clearly detectable in HEK-293T cells.

3.4.3 Comparison of cell number and transfection efficiency of NIH3T3 and HEK-293T cells

In addition to the biological effect, other parameters like the cell number and transfection efficiency are essential for a HTS assay. The transfection efficiency of mini-prep DNA is relatively lower than maxi-prep DNA. However, in the systematic screen, mini-prep DNA of the expression plasmids, prepared on at automated liquid handler, was used to transfect cells as manual maxi-preparations would not be feasible in high numbers. For NIH3T3 cells 12,500 cells/well, and for HEK-293T cells 33,000 cells/well were plated in a 24 well plate. The cells were transfected with 135 ng of mini-prep DNA/well of a set of randomly selected clones in order to compare the cell numbers acquired and the transfection efficiency in both cell lines. Cells were serum starved for 24 hrs and then stimulated and processed according to the automated assay protocol (see Materials and Methods). The final acquired cell number after staining ranged from 60 to 5,000 cells (Fig: 3.9, 1a) with a majority of wells having cell numbers between 1,000 and 4,000 for NIH3T3 cells. The transfection efficiency differed greatly between wells, with most of the wells having a transfection efficiency of less than 10 % (Fig: 3.9, 1b).

For HEK-293T cells, number of cells per well ranged from 2,000 to 13,000 with majority of well having cell numbers between 7,000 and 12,000 (Fig: 3.9, 2a) and the transfection efficiency ranged from 30 to 100% with the average between 50% and 80% (Fig: 3.9, 2b). As a consequence, NIH3T3 cells were not used for the screen due to the low transfection efficiency and low cell numbers acquired after staining. An effort to increase the transfection efficiency of NIH3T3 cells failed and resulted in high cytotoxicity. In addition, HEK-293T cells are of human origin, which would be advantageous when screening human proteins, and in view of a possible later extension of the assay to RNAi.

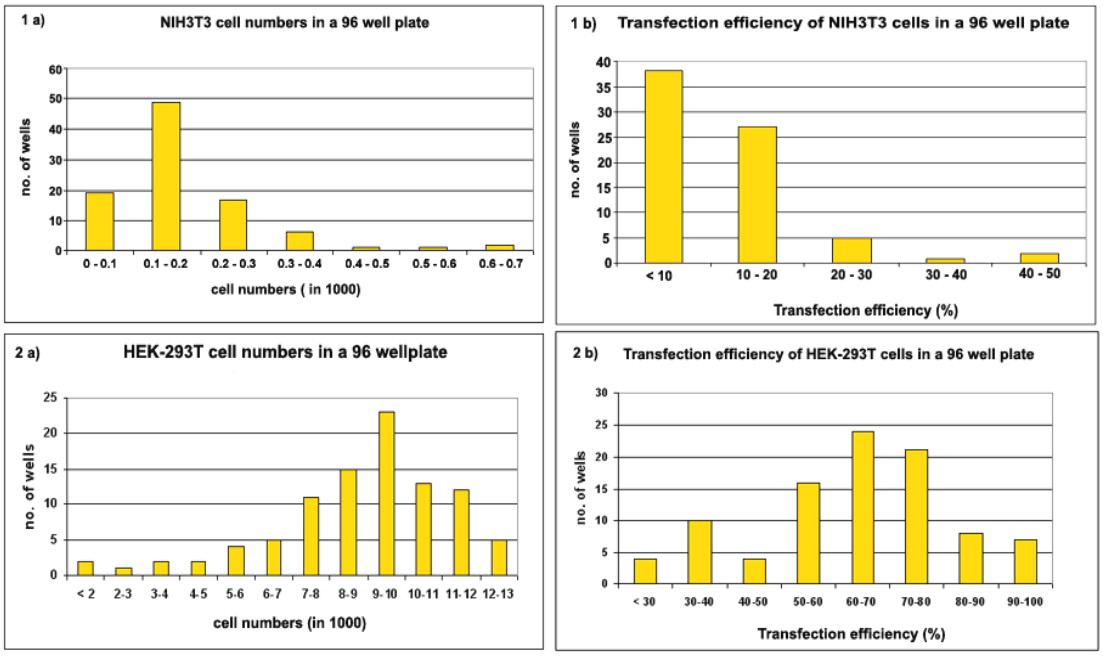


Figure 3.9: Comparison of acquired cell numbers and transfection efficiency of NIH3T3 and HEK-293T cells. NIH3T3 and HEK-293T cells were seeded in 24 well plates (12,500 cells/well and 33,000 cells/well respectively), transfected with 135 ng of mini-prep DNA/well and then processed according to the automated assay protocol. After staining, cells were measured in a plate reader in FACS Calibur. NIH3T3 cells had low acquired cell numbers (100 – 400/well (1a) and transfection efficiency (10-20%), (1b) when compared to HEK-293T cells (7,000-12,000 cells/well), (2a) with 50-80% transfection efficiency (2b).

3.5 Effect of control proteins on ERK1/2 phosphorylation

The screening was to be performed with 200 novel cDNAs (see Supplements) which had been cloned into expression vectors containing a YFP tag at the N or C terminal ends of the protein, respectively. Hence, it was first necessary to prove that the tag, YFP, alone did not have any effect on ERK1/2 activation when over-expressed. It was also essential to select proteins that are known to activate or inhibit ERK1/2 phosphorylation as control proteins that could be used in the screen with every experiment.

Table 1: List of characterised proteins used as controls in the assay

Name	Accession No.	Function
YFP		No function in mammalian cells (not expressed in mammalian cells)
Phospholipase C delta 4 (PLCD4)	BC006355	Activation of several signalling pathways, overexpression induces ERK1/2 activation
MAP Kinase Kinase2 (MEK2)	BC018645	Dual specificity kinase, activates ERK1
Dual specificity protein phosphatase 10 (DUSP10)	BC063826	Dual specificity protein phosphatase, inactivates MAPKs
Annexin A1 (ANXA1)	BC001275	Anti-proliferative, sustained ERK activation
Cystathionine gamma lyase (CGL)	BC015807	Over-expression induces sustained ERK activation

Well characterised proteins were selected and the respective coding regions cloned into expression vectors with N-and C- terminal YFP tag. The tagged proteins were then transfected into HEK-293T cells and their effect on ERK1/2 phosphorylation was monitored by performing the FACS based ERK1/2 activation assay. The assay was performed under both serum starved and stimulated conditions. Over-expression of a protein that activated ERK1/2 should lead to increased levels of phospho-ERK1/2 in serum starved conditions, where the transfected cells should show higher phospho-ERK1/2 levels than the non-transfected cells. In contrast, proteins that inhibit ERK1/2 activation upon over-expression, should only show the effect when serum starved cells were stimulated. The transfected cells, in this case, should show lower level of phospho-ERK1/2 than non-transfected cells. It was essential to observe the behaviour of control proteins under both these conditions in order to decide the conditions under which the assay was stable. The effect of the proteins on ERK1/2 phosphorylation was determined by calculating a z-score and the significance of this effect was given by a p-value (see Materials and Methods).

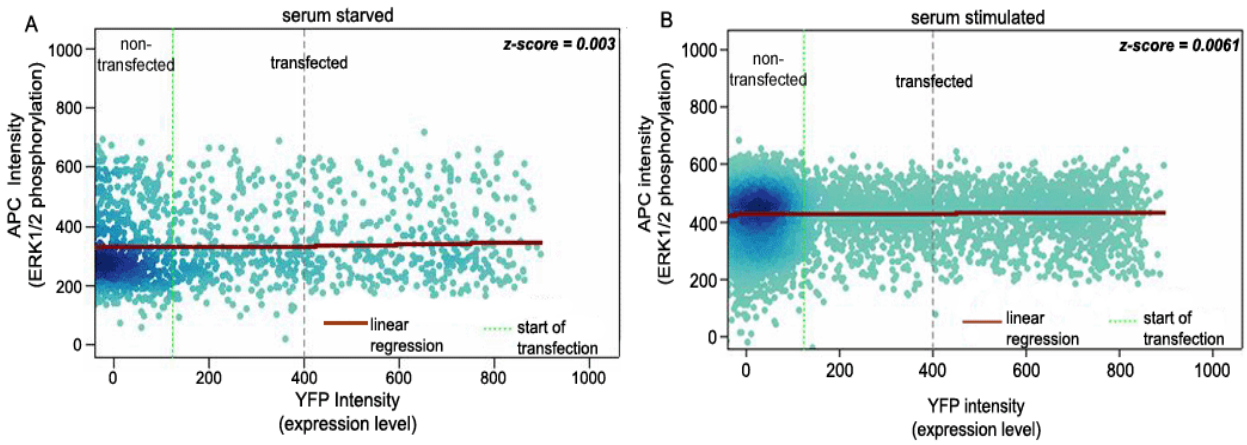


Figure 3.10: Effect of YFP on ERK1/2 phosphorylation. NIH3T3 and HEK-293T cells were transfected with YFP, allowed to express the protein for 24hrs, and then serum starved for 24 hrs. Finally, cells were either stimulated with 10% FBS for 10 min or left untreated. Level of ERK1/2 phosphorylation was detected using phospho-ERK1/2 antibody followed by measurement in FACS. YFP did not have any effect on ERK1/2 phosphorylation in both serum starved and stimulated conditions. Cells transfected with YFP and non-transfected cells showed the same level of ERK1/2 phosphorylation.

The effect of YFP on ERK1/2 phosphorylation was determined by comparing the level of phospho-ERK1/2 in non-transfected cells to that in transfected cells. YFP alone did not show any effect on ERK1/2 phosphorylation (Fig: 3.10). Untransfected cells and YFP transfected cells both showed a similar level of ERK1/2 activation. After stimulation with 10% FBS, the level of phospho-ERK1/2 increased and the shift in the APC intensity was similar for both transfected and non-transfected. Over-expression of YFP tagged MEK2, the upstream activator of ERK1/2,

did not show any effect on the activation of ERK1/2 in the assay conditions (Fig: 3.11). The YFP tagged dual specificity protein phosphatase, DUSP10, was used as a control for proteins that inhibit ERK1/2 phosphorylation. The N-terminal YFP fusion protein showed a significant decrease in ERK1/2 phosphorylation upon over-expression in both serum starved and stimulated conditions (Fig: 3.11, 2a, b), while the corresponding C-terminal YFP fusion protein did not show an inhibiting effect on ERK1/2 phosphorylation (Fig: 3.11, 2c, d). YFP-tagged Annexin A1, ANXA1, was chosen as a control for activation of ERK1/2. Over-expression of ANXA1 has been reported to induce sustained activation of ERK1/2 [82]. However, ANXA1 could not induce activation of ERK1/2 under the assay conditions (Fig: 3.11, 3).

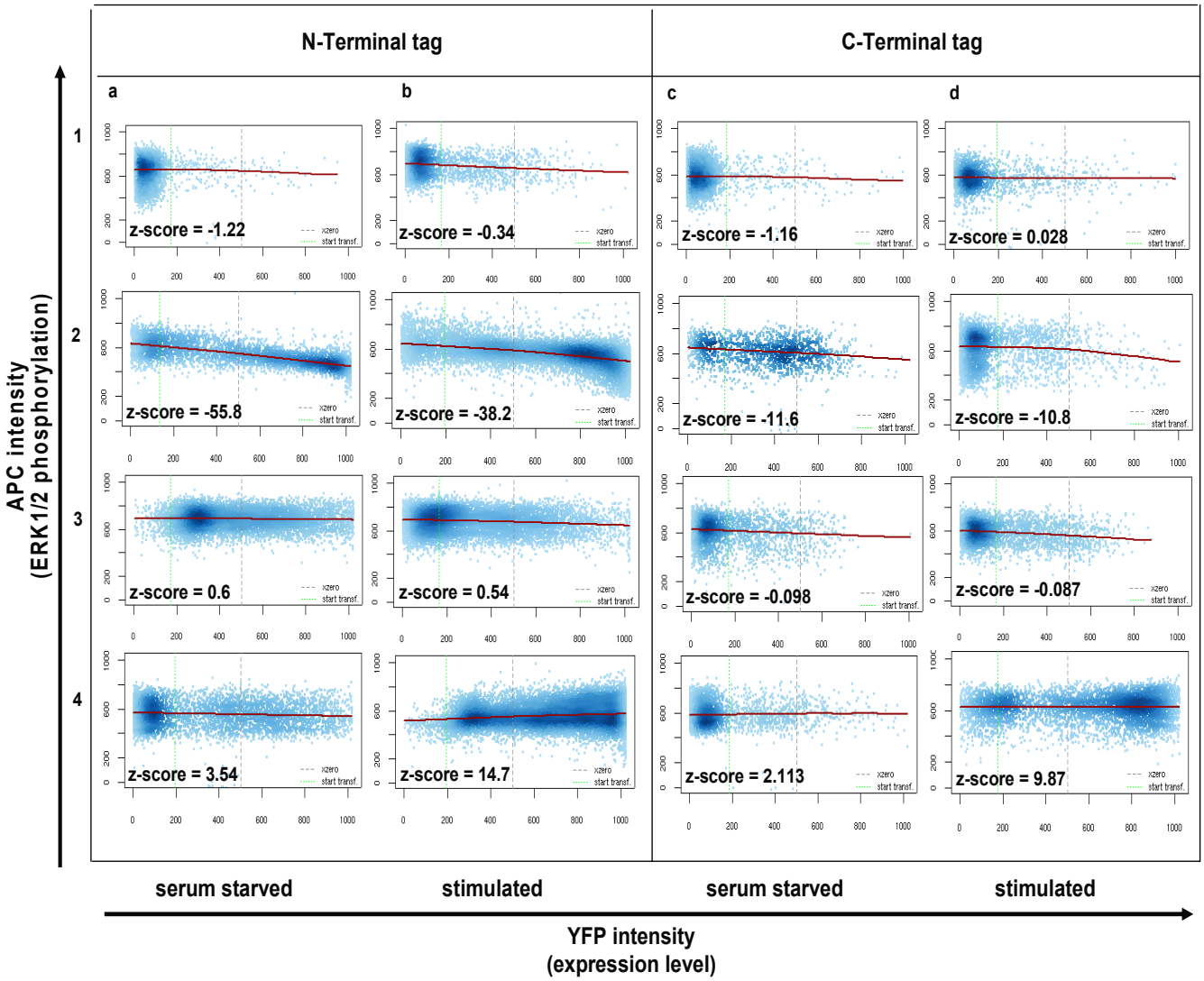


Figure 3.11: Effect of N/C-terminal YFP tagged control proteins on ERK1/2 phosphorylation 1) Over-expression of YFP tagged MEK1, an upstream kinase of ERK1/2, did not have any effect on ERK1/2 phosphorylation. 2) Over-expression of N-terminal YFP tagged DUSP10 effectively decreased ERK1/2 phosphorylation in transfected cells. 3) YFP tagged *Annexin A1* over-expression does not show any effect on ERK1/2 activation. 4) *Phospholipase – C delta 4* tagged with YFP shows slight activation of ERK1/2 when over-expressed.

Over-expression of Phospholipase c delta 4 (PLCD4) was shown to upregulate members of ERK1/2 pathway and in turn induce ERK1/2 activation [83]. The YFP tagged PLCD4, when over-expressed, induced an increase in ERK1/2 phosphorylation as expected. This increase was evident in serum stimulated cells with both N- and C-terminal YFP tagged PLCD4 (Fig: 3.11, 4a, c). In serum starved cells, YFP tagged PLCD4 did not induce an increase in ERK1/2 phosphorylation.

In summary, YFP was chosen as a neutral control protein that does not affect ERK1/2 activation and also proves that over-expression itself does not affect the cell system used in the assay. We observed that orientation of the YFP tag relative to the ORF of interest affects the functionality of a protein. For example; the N-terminal YFP tagged DUSP10 showed the inhibitory effect on ERK1/2 activation, whereas the C-terminal YFP tagged DUSP10 did not. In consequence, the N-terminal YFP tagged DUSP10 was chosen as a positive control for inhibition. PLCD4 showed a slight activating effect on ERK1/2 phosphorylation and was used as an activator control for the assay. The effects brought about by the control proteins were similar in both serum starved and stimulated cells. Cells that had been stimulated with 10% FBS were intact, and a higher number of cells were recovered after the staining procedure as compared to cells that had been serum starved. Many of these died during starvation and, as a consequence, the number of cells recovered was rather low. Both, activators and inhibitors could be clearly identified in cells stimulated with FBS and hence the assay was performed with cells stimulated with 10% FBS.

3.6 Screening and candidate selection

The mini-prep plasmid DNA for controls and the novel cDNAs was distributed in a 96 well plate in a particular order (DNA plate, Fig: 3.12 a) and then used for transfection. The DNA was mixed with transfection reagents in a mixing plate from which the complexes were used for transfecting cells seeded in 4 x 24 well plates (assay plate). Four 24 well plates were used in order to achieve high cell numbers for each experiment as more cells (33,000 cells/well) could be seeded per well in a 24 well plate as compared to the 96 well plate where only 10,000 cells could be used. After transfection the cells from 4 x 24 well plates were trypsinised, fixed and transferred to one 96 well plate for staining and measurement (acquisition plate). The transfection and staining procedures were automated. After staining, each plate was measured in a FACS Calibur with a HTS loader for 96 well format.

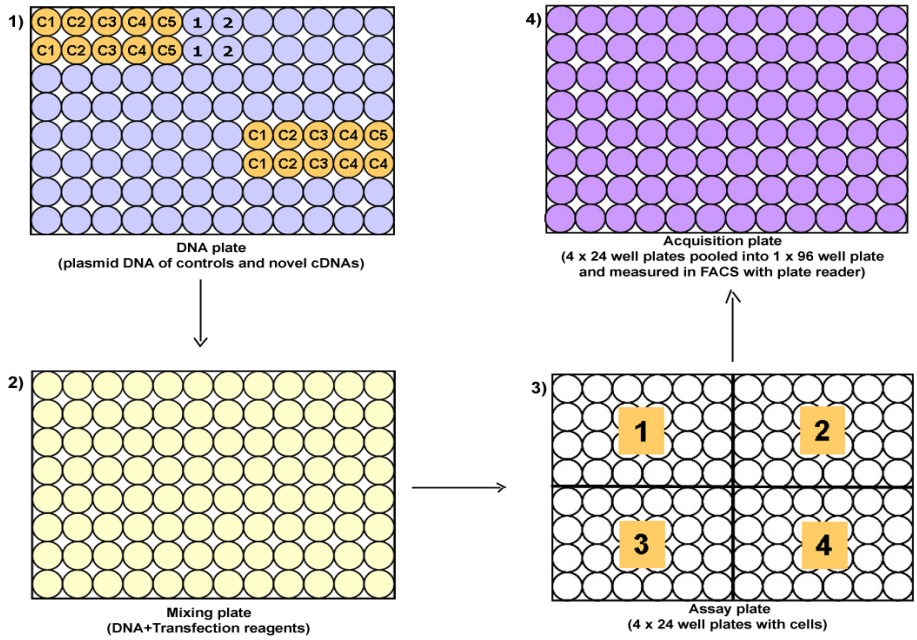


Figure 3.12 a: Schematic representation of the assay pipeline 1) Mini-prep DNA for the controls and the Gateway expression clones of the novel cDNAs was normalised and distributed systematically in a 96 well plate. 2) The DNA from DNA plate was mixed with the transfection reagents in a separate plate in the same order 3) The DNA-transfection reagent complexes were transfected into cells seeded in 4 x 24 well plates (representing one 96 well plate) in the same order of distribution as in the DNA plate. 4) Cells were treated according to the assay protocol and transferred to one 96 well plate for staining and measurement.

The data obtained was analysed (see Materials and Methods), their z-scores and p-values were calculated and then represented in a user-interface (Fig: 3.12 b). Data from one screening plate (one 96 well plate) was presented as a colour coded graphical format mimicking a 96 well plate (Fig: 3.12 b, A). Wells in blue indicated that the protein in that well had an inhibitory effect on ERK1/2 activation while those with red indicated an activating effect. Wells with proteins that had no effect on ERK1/2 activation were represented in white. In addition, each screening plate was represented as a box plot showing the effect of the ORF in each well (Fig: 3.12 b, B). The z-score of the neutral control protein, YFP, was considered to be zero and the deviation from this z-score towards an increase or decrease was regarded as an effect. The box plot provided a quick overview of the results from one 96 well plate that showed well numbers on x-axis and the z-score on the y-axis (Fig: 3.12 b, B). Information regarding the number of replicate wells for each ORF, and the effect of both the N- and C-terminal YFP tagged ORF was represented as a bar plot (Fig: 3.12 b, C). Further, the results obtained for each ORF were represented as dot plots with information regarding the expression level of the YFP tagged ORF (YFP intensity) and the level of phospho-ERK1/2 in the transfected as well as the non-transfected cells (Fig: 3.12 b, D).

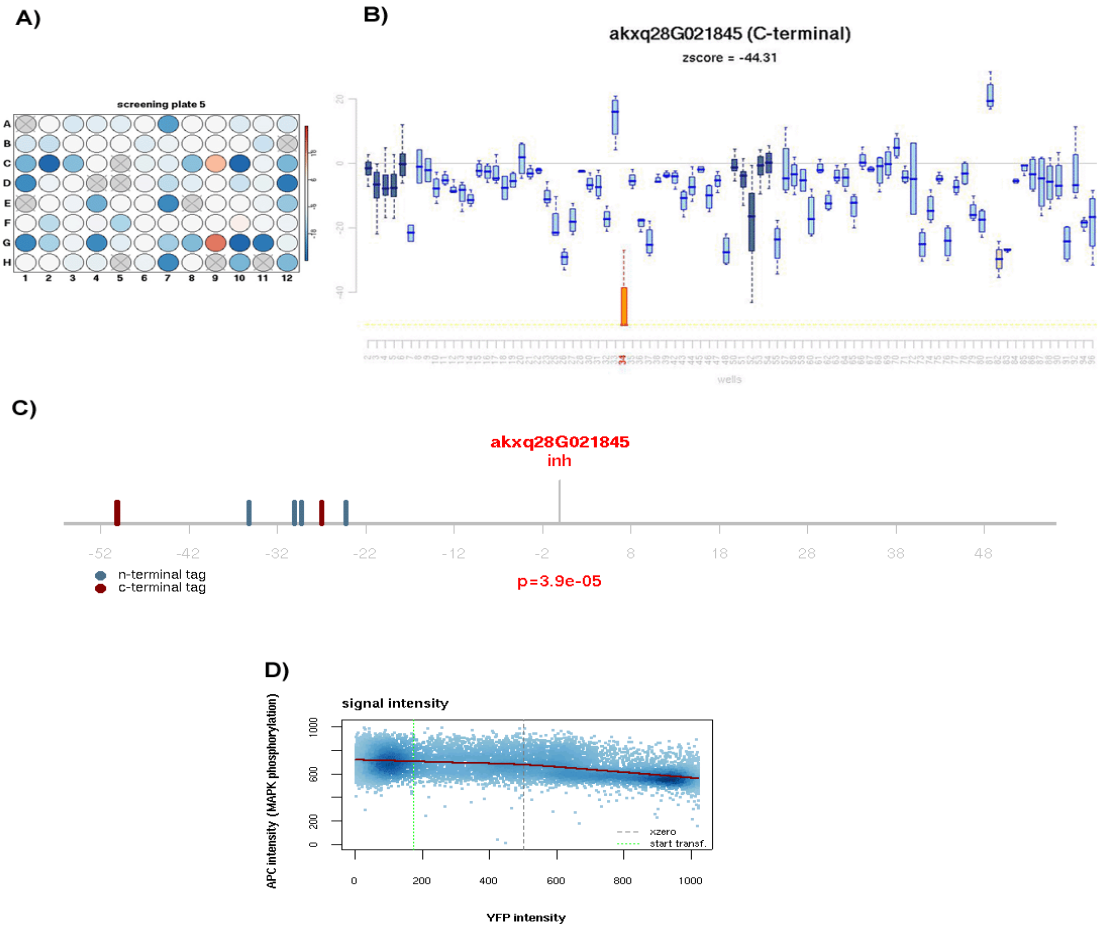


Figure 3.12 b: Data analysis, graphical representation and candidate selection. A) Each screening plate that has been analysed is represented as one 96 well plate with wells that are colour coded from dark blue (inhibitor) to red (activator) with white being the colour code for no effect. B) Each screening plate is further represented as a box plot showing the effects of the individual clones (single wells) on ERK1/2 activation. The z-score is plotted on the y-axis and the well number on the x-axis. This representation of the results gives a quick overview of the experiment. C) Bar plots showing the p-value and the effect of one ORF on ERK1/2 activation with details about the number of replicates with each of the C- or N-terminal YFP tagged ORFs and their effects correspondingly. D) Representation of the effect of one ORF in the form of a dot plot with the expression level (YFP intensity) on x-axis and the ERK1/2 phosphorylation level (APC intensity) on the y-axis.

In order to select effectors of ERK1/2 activation from the screen, the colour coded plate (Fig: 3.12 b, A) was used to initially identify potential candidates. The strength of the effect of the protein on ERK1/2 activation was attributed to the z-score or fold change and the significance of the effect was given by the p-value. Proteins that showed a correlation between the dot plot and the z-score/fold change (effect) were first separated. The significance of the effect was then calculated using the p-value. Only proteins with p-values <= 0.005 and a fold change >= 8 were selected as potential candidates. Localisation of the proteins was checked and those which were mis-localised or differed in localisation depending on C- or N-terminal tags were excluded. Further the proteins that showed the effect only with one of the orientation of the tag (N-or C- terminal tag) were also excluded.

Out of the 200 proteins screened in the assay, 14 candidates were selected according to the above described methodology. These candidates were divided into activators and strong, medium, and weak inhibitors (Fig: 3.13). The dot plots corresponding to the activators (*akxq28i0610741* and *IMAGp998D0110102)* showed a clear increase in ERK1/2 activation in transfected cells and the effect was proportional to the expression level of the protein. The fold change exhibited by the proteins was 15 and 17.1 respectively (Fig: 3.13, A). Twelve out of the 14 potential candidates were classified as inhibitors. Five proteins induced a fold change in ERK1/2 activation in the range from 18 to 24 and were categorized as strong inhibitors (Fig: 3.13, B). The remaining seven candidates were categorized as moderate and weak inhibitors and induced a fold change in the range from 8 to 17 (Fig: 3.13, C, D) respectively.

The effect of the 14 potential candidates on the activation of ERK1/2 was to be confirmed by an independent method for validation purposes. Candidates were therefore subjected to validation by performing Western blotting and detecting the level of phospho-ERK1/2.

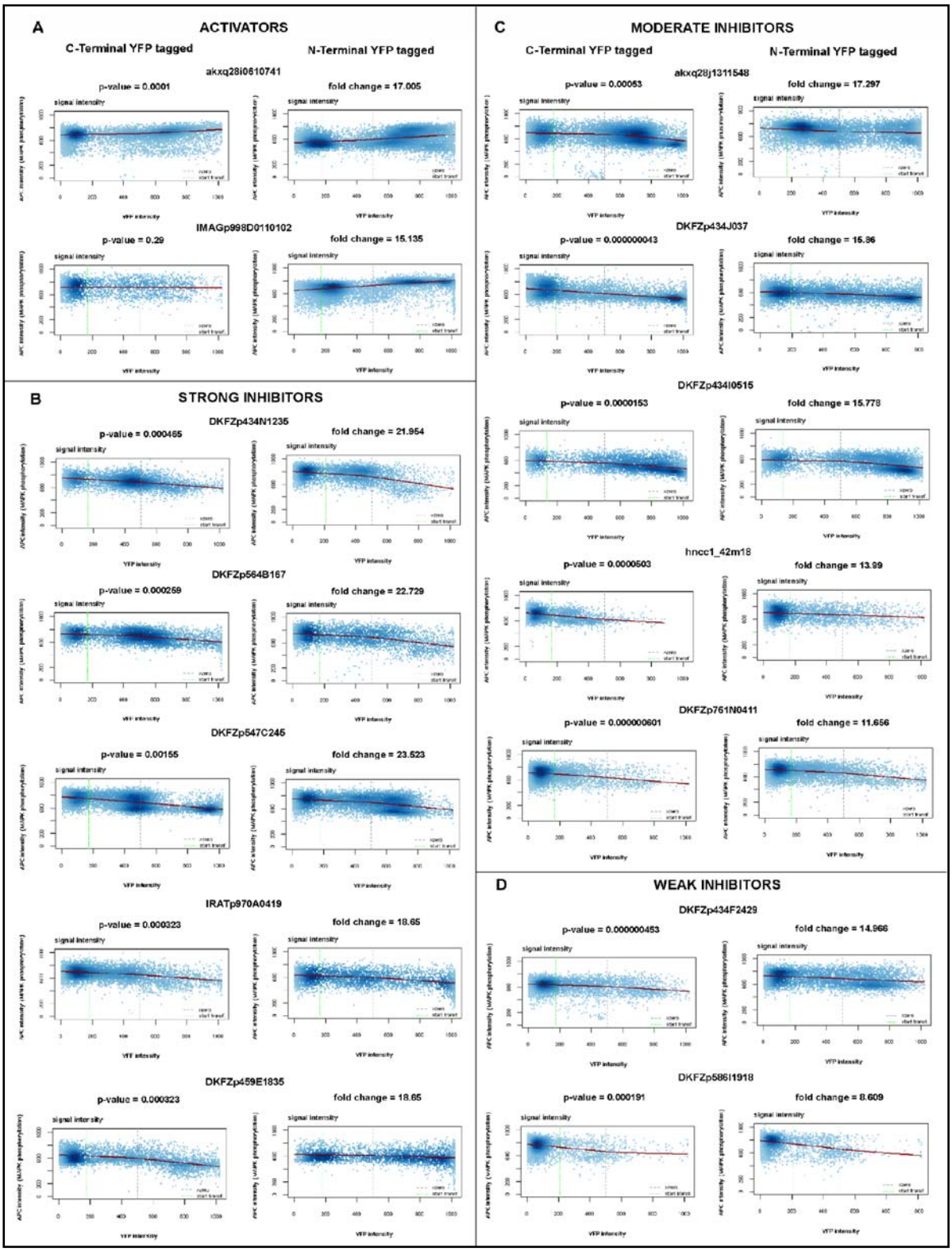


Figure 3.13: Dot plots of 14 preliminary candidates showing their effect of ERK1/2 phosphorylation. From 200 proteins that were screened, 14 potential effectors of ERK1/2 activation were chosen for validation. The dot plots give information regarding their effect on ERK1/2 phosphorylation using FACS based detection. The candidates could be classified into activators and strong, moderate and weak inhibitors. Validation of these candidates was done by western blotting.

3.7 Candidate validation

The YFP tagged candidate proteins were over-expressed in HEK-293T cells and then subjected to the assay conditions. The cells were lysed and the total protein was extracted and quantified. Equal amounts of protein were loaded onto polyacrylamide gels and Western blotting was performed. The membranes were incubated with anti-ERK1/2 and anti-phospho-ERK1/2 antibodies. Mock transfected cells (M) and cells transfected with control proteins (YFP, Phospholipase c Delta-PLCD4, dual specificity protein phosphatase 10 –DSPP) were used as neutral, activating and inhibiting controls respectively. The level of phosphorylated ERK1/2 in the cells transfected with the candidates was compared to the level in cells transfected with the control proteins (Fig: 3.14).

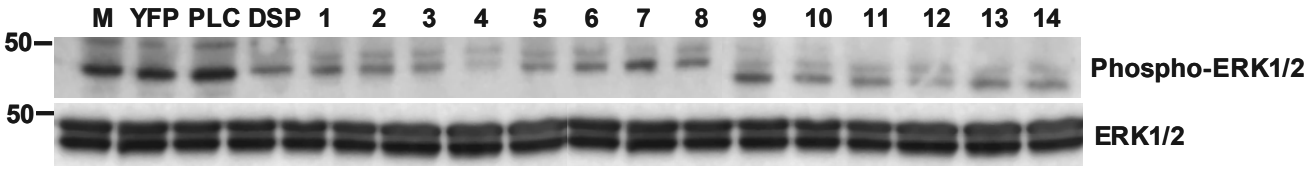


Figure 3.14: Candidate validation by Western blotting. Candidate validation was done by detecting the level of phospho-ERK1/2 in cells transfected with the candidates by Western blotting. Cells were allowed to express the respective proteins for 24 hrs and then treated according to the assay protocol. Western blotting was performed and the membranes were incubated with ERK1/2 and phospho-ERK1/2 antibodies. Mock transfected cells (M) and cells transfected with control proteins (YFP, Phospholipase c Delta-PLC, dual specificity protein phosphatase 10 –DSP) were used as neutral, activating and inhibiting controls. Level of phospho-ERK1/2 was compared between the controls and the candidates.

The level of phospho-ERK1/2 in mock as well as in YFP transfected cells was similar. PLCD4 transfected cells showed higher level of phospho-ERK1/2 in comparison to mock and YFP (Fig: 3.14). As expected, cells transfected with DUSP10 showed very low level of phospho-ERK1/2. The intensity of the phospho-ERK1/2 bands of the control proteins was used to compare the level of phospho-ERK1/2 in the cells transfected with the candidate proteins. Depending on the level of phospho-ERK1/2, the candidates were graded (+ to ++++) from weak to strong effectors. The correlation between the Western blot results, z-score and the effect seen in the FACS dot plots was taken into consideration for the validation of the candidates.

A list of the 14 potential candidates and their effect on ERK1/2 activation in the Western blotting experiment are listed below (Table: 3). Candidate proteins that were graded with a (+) were excluded (Table 3, marked in red) and the rest of the candidates were confirmed as modulators of ERK1/2 activation and were regarded suitable for further detailed functional analysis.

Table 3: List of candidate proteins selected for validation and their effect on ERK1/2 phosphorylation. (see validation table)

	DKFZ ID	Gene name	Effect in ERK1/2 assay	Effect in Validation
1	DKFZp459E1835	Fun domain containing 2, FUNDC2	inhibitor	++
2	DKFZp564B167	Brain protein 44	inhibitor	++
3	IRATp970A0419	Interferon alpha inducible protein 27, IAP27	inhibitor	+++
4	DKFZp434N1235	Solute carrier family 25	inhibitor	++++
5	hncc1_42m18	Melanoma inhibitory protein 2, MIA2	inhibitor	++
6	IMAGp998D0110102	Cell division cycle 23, CDC23	activator	+
7	akxq28i0610741	PAS domain containing 1, PASD1	activator	++
8	DKFZp547C245	Reticulon 4, RTN4	inhibitor	+
9	DKFZp761N0411	Structural maintenance of chromosome 6 like 1, SMC6	inhibitor	+
10	DKFZp434J037	SNF-1 like kinase 2	inhibitor	++
11	DKFZp434F2429	Rho guanine nucleotide exchange factor 3	inhibitor	++
12	DKFZp586I1918	LIM domain and actin binding 1	inhibitor	+++
13	akxq28j1311548	Translocon-associated protein delta	inhibitor	++
14	DKFZp434I0515	Radial spoke head like-1	inhibitor	++

Table 4: Validation table

Clone ID	Gene name	Accession No	localisation N-terminal	localisation C-terminal	Effect on ERK1/2 activation	p-value	Annotation/Interpro
DKFZp459E1835	FUN14 domain containing 2, FUNDC2	BC108657	Mitochondria	Mitochondria	Inhibitor	0.000323	IPR007014 Fun14 domain containing 2
DKFZp586I1918	LIM domain and actin binding 1	AL136911	Cytoplasm and nucleus	unknown	inhibitor	0.000191	No specific hits
DKFZp564B167	Brain protein 44	AL110297	mitochondria	mitochondria	inhibitor	0.000259	No specific hits
IRATp970A0419	Interferon -alpha inducible protein 27	BC015492	Endosomes, Lysosomes	unknown	inhibitor	0.00492	IPR002091 Aromatic amino acid permease
DKFZp434N1235	Solute carrier family 25	AL136857	cytoplasm	mitochondria	inhibitor	0.000465	IPR002113 Adenine nucleotide translocator 1
hncc1_42m18	MIA2 melanoma inhibitory protein	AF390175	cytoplasm	Cytoplasm and nucleus	inhibitor	5.03E-05	IPR000532 Glucagon/GIP/secretin/VIP
akxq28i0610741	PAS Domain containing 1, PASD1	BC040301	cytoplasm and nucleus	Nucleus	activator	1.00E-04	IPR000700 PAS-associated domain
DKFZp434J037	SNF1-like kinase 2	AL136891	Nucleus - speckles	Nucleus - speckles	inhibitor	4.30E-08	No specific hits
DKFZp434F2429	Rho guanine nucleotide exchange factor (GEF) 3	AL136832	Cytoplasm and nucleus	Cytoplasm and nucleus	inhibitor	4.53E-07	IPR001331 Guanine-nucleotide dissociation stimulator
akxq28j1311548	Translocon-associated protein delta	BC003371	Endoplasmic reticulum	Endoplasmic reticulum	inhibitor	0.00053	IPR009779 Translocon-associated, gamma subunit
DKFZp434I0515	Radial spokehead-like 1, Rshl1	AL136761	Cytoplasm and nucleus	Cytoplasm and nucleus	inhibitor	1.53E-05	IPR001156 Peptidase S60, IPR006802 Radial spokehead-like protein

3.8 Detailed functional analysis of Radial spoke head like 1 (Rshl1)

Out of the validated candidates that were obtained from the ERK1/2 activation, the Radial spoke head like 1 (*Rshl1*) protein was identified as an inhibitor of ERK1/2 activation. In addition, the transcript had been found downregulated in kidney cancer patients by tumour gene expression profiling [40]. In a proliferation screen done in the department, Rshl1 was identified as an inhibitor of proliferation[84]. These findings from previous studies suggested the involvement of Rshl1 in cell cycle regulating processes and therefore instigated me to further investigate the function of this protein.

3.8.1 Localisation of YFP-tagged Rshl1

To start with the functional analysis of Rshl1, the localisation of the YFP-tagged Rshl1 was determined in HEK-293T cells. The localisation had previously been determined only in the monkey cell line Vero (http://www.lifedb.de), and it was necessary to confirm the results also in the cell line that had been utilised in the MAPK assay. C- and N-terminal YFP tagged Rshl1 was expressed in HEK-293T cells for localisation experiments. Rshl1 localised to the cytoplasm and nucleus of HEK-293T cells (Fig: 3.15), verifying the results obtained in the Vero cell line. The orientation of the tag did not affect the localisation of Rshl1.

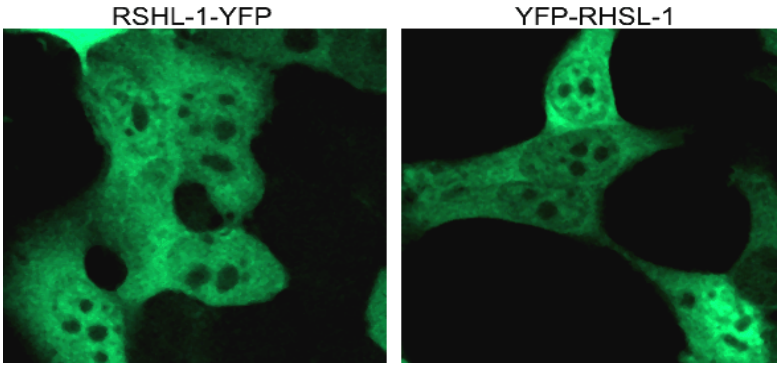


Figure 3.15: Localisation of YFP tagged Rshl1. HEK-293T cells were transfected with YFP tagged Rshl1 on cover slips in 6 well plates. After 24 hrs, cells were fixed with 4% PFA and images were taken with a confocal laser scanning microscope at 63x magnification. YFP tagged Rshl1 localises to the cytoplasm and nucleus of HEK-293T cells. The orientation of the tag did not affect the localisation.

3.8.2 Analysis of ERK1/2 activation in HEK-293T cells by immunofluorescence

To confirm the inhibition of ERK1/2 by Rshl1, HEK-293T cells transfected with the YFP-Rshl1 and YFP alone were treated according to the assay protocol and stained with the phospho-ERK1/2 antibody. Cells transfected with YFP-Rshl1 (Fig: 3.16, 1a) showed low level

of phospho-ERK1/2 as compared to the untransfected (Fig: 3.16, 1b) as well as the YFP transfected cells (Fig: 3.16, 2).

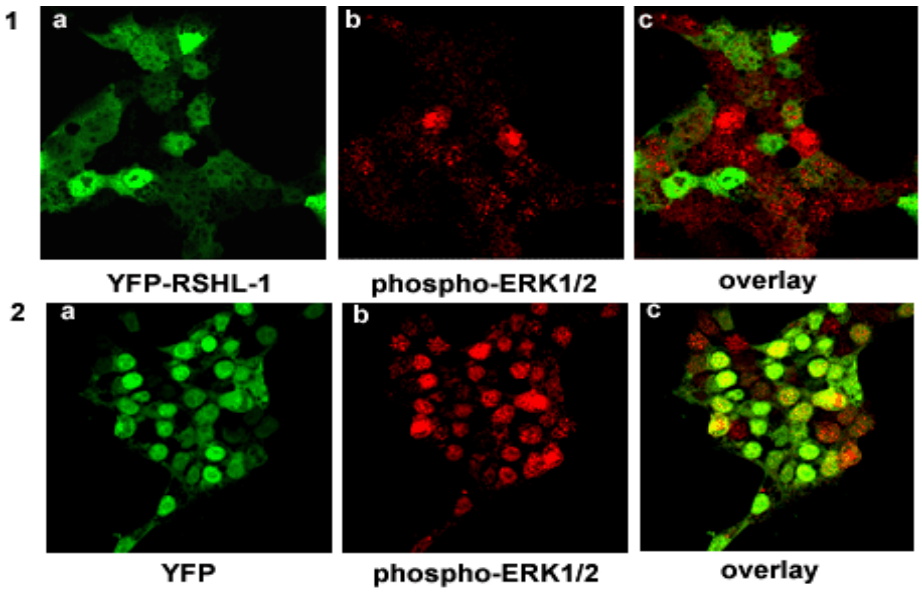


Figure 3.16: Cells transfected with YFP-Rshl1 show low levels of phospho-ERK1/2. HEK-293T cells were grown on cover slips and then transfected with YFP-tagged Rshl1. After 24 hours, the cells were serum starved for one day and then stimulated with 10% FBS for 5 min. Cells were fixed and stained with phospho-ERK1/2 antibody. Cells transfected with YFP-tagged Rshl1 showed lower levels of phospho-ERK1/2 compared to cells transfected with YFP.

3.8.3 Effect of YFP tagged Rshl1 over-expression on cell cycle

Previous results [84] indicate that over-expression of Rshl1 leads to inhibition of proliferation. However, details on the mechanism leading to this observation had not been unravelled. In order to probe into these details a cell cycle analysis of HEK-293T cells over-expressing Rshl1 was performed.

3.8.3.1 Cell cycle analysis of cells over-expressing Rshl1

In order to analyse whether over-expression of Rshl1 would affect the cell cycle progression in HEK-293T cells, G0/G1 synchronized cells were checked for their capacity of BrdU incorporation. Cells were first synchronized by DIF-3 treatment and then release from growth arrest by adding fresh medium without DIF-3 and then pulsed with BrdU for 4 hours. Samples were taken every hour and stained with anti-BrdU antibody. The measurement was done using a FACS Calibur. Acquired cells were separated into transfected and untransfected cells by setting appropriate gates (Fig: 3.17 a) and each population was then analysed for the

incorporation of BrdU. The ratio between the number of cells in S-phase (BrdU positive, UR and UL in Fig: 3.17a) and those not in the S-phase (LL and LR in Fig: 3.17a) was plotted for both transfected and non-transfected cells from each sample. The ratio between BrdU positive cells and BrdU negative cells in mock transfected cells (UL/LL, Fig: 3.17a) was 0.7 after one hour of FCS stimulation. The ratio increased to 1.64 after 4 hrs of stimulation, indicating the increased entry of G0/G1 arrested cells into S-phase upon serum stimulation. However, in cells transfected with YFP-Rshl1 the ratio between BrdU positive and negative cells (UR/LR, Fig: 3.17b) was 1.1 after 1 hour FCS stimulation and 1.3 after 4 hrs of FCS stimulation. At the same time, untransfected cells in the same sample (UL/LL, Fig: 3.17b) showed ratios of 1.66 and 2.5 for 1hr and 4hrs after stimulation, respectively. These results indicate that the untransfected cells in the YFP-Rshl1 transfected sample moved steadily into S-phase after FCS stimulation whereas the transfected cells were blocked in the G0/G1 phase even after 4 hrs, or delayed in their cell cycle progression. The graph (Fig: 3.17 b) shows that untransfected, mock and YFP transfected cells showed a steady increase in the number of cells entering the S-phase after releasing the cells from G0/G1 phase.

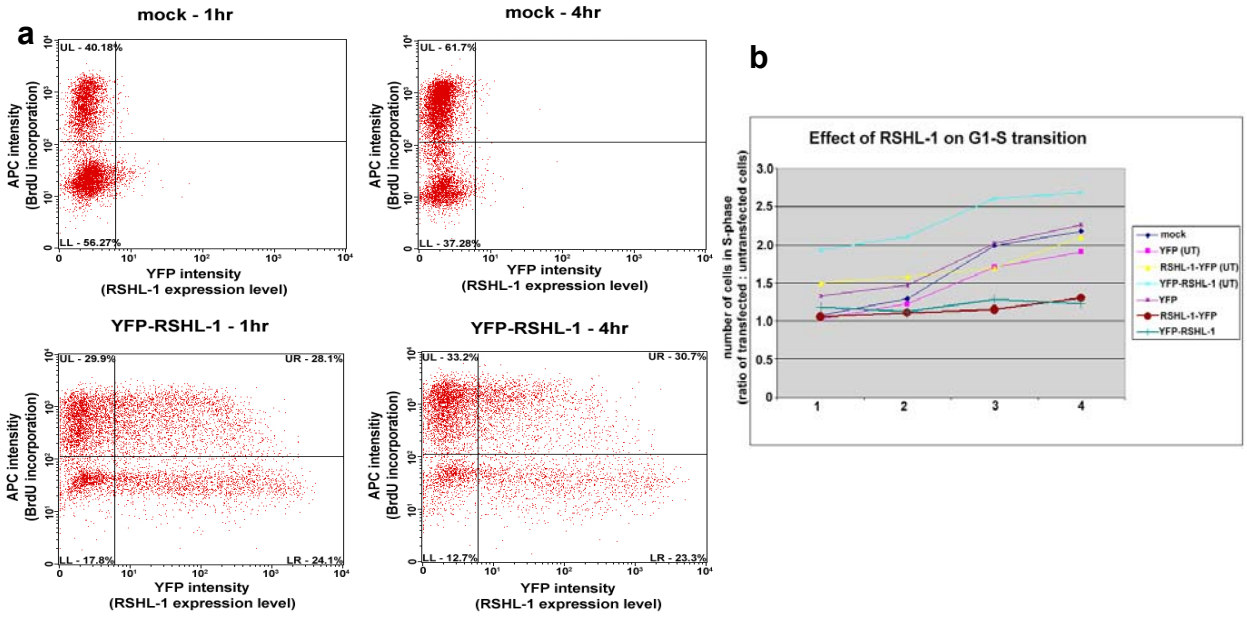


Figure 3.17: Effect of Rshl1 on G1-S transition. HEK-293T cells were grown for 1 day in DMEM and then transfected with C-terminal and N-terminal YFP tagged Rshl1, YFP or with empty vector (mock). One day later cells were treated with DIF-3 for 16-24 hrs to induce G0/G1 arrest. On the next day, arrested cells were released by induction with medium without DIF-3 and with 10µm BrdU for 4 hrs. Samples were taken every hour and stained with anti-BrdU antibody and the number of cells in S-phase was measured in FACS. Cells transfected with YFP-tagged Rshl1 do not enter the S-phase or show a delay in entry into the S-phase when compared to the mock and YFP transfected or untransfected cells (UT) (b).

3.8.3.2 Effect of YFP tagged Rshl1 over-expression on cell cycle regulating proteins

Cell cycle progression is regulated by cyclins and cyclin dependant kinases (CDKs). Proteins that inhibit these kinases, CDK inhibitors (CKI), inhibit cyclin dependent kinases and delay/arrest cell cycle progression. In order to check which proteins are involved in Rshl1 mediated cell cycle arrest, the expression levels of these proteins in cells arrested in G0/G1 phase and the pattern of expression after releasing the cells from the arrest by serum stimulation for 4 hours was monitored. The level of cyclin D1 was found to be unaffected by Rshl1 in arrested as well as cells released from the arrest (Fig: 3.18).

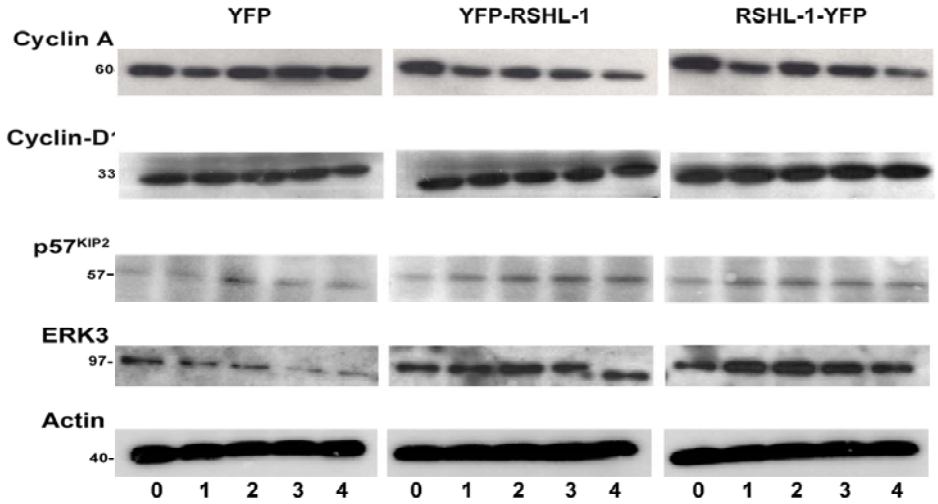


Figure 3.18: Effect of Rshl1 on cell cycle regulating proteins. HEK-293T cells were grown for 1 day in DMEM and then transfected with C-terminal and N-terminal YFP tagged Rshl1, YFP. One day later cells were treated with DIF-3 for 16-24 hrs to induce G0/G1 arrest. On the next day, arrested cells were released by induction with medium without DIF-3 and with 10µm BrdU for 4 hrs. The level of cyclins D remained same during this period in YFP and YFP tagged Rshl1 cells. The level of ERK3 and $p57^{KIP2}$ was higher in cells transfected with YFP tagged Rshl1 when compared to the mock and YFP transfected cells and the level of cyclin A was also decreased.

However, the level of cyclin A was found to decrease slightly after release from arrest in YFP-Rshl1 transfected cells. The level of $p57^{KIP2}$ was found to be higher in cells transfected with Rshl1 in comparison to the YFP transfected cells and the level of $p57^{KIP2}$ increased over time. The level of one of the ERK3 isoforms (~60kDa) was also found to be higher in cells transfected with Rshl1(Fig: 3.18).

3.8.4 Identification of proteins interacting with Rshl1

Identification of interacting partners of proteins is essential for the elucidation of their respective biological function. This would give information regarding the role of any protein in

its natural biological context and help to define the specific pathway or cellular processes in which the protein is involved. Identification of the interacting partners of Rshl1 was, therefore, the next step in its functional characterisation.

3.8.4.1 Detection of interacting proteins with help of an antibody array

Interacting partners of RHSL-1 were discovered using the antibody microarray technology (in a collaboration with Dr. Birgit Guilleaume). Antibodies against 67 different proteins linked to the MAPK pathway, cell cycle regulation, and Apoptosis (i.e. the MAPK pathway, Apoptosis, cell cycle antibody sampler kits from BD biosciences, see materials and Methods) were spotted on a Nexterion slide H surface to generate an antibody microarray. HEK-293T cells were transfected with YFP-Rshl1. After 2 days cells were lysed and the lysate was incubated on the antibody microarray. Interactions were then detected using a biotinylated anti-GFP antibody followed by $Alexa_{562}$ labeled Streptavidin. Spots corresponding to CDK2, pan-ERK, MEK1, p70, Caspase-7 and Histone-H3 antibodies gave positive signals (Fig: 3.17) indicating that these proteins interact with Rshl1.

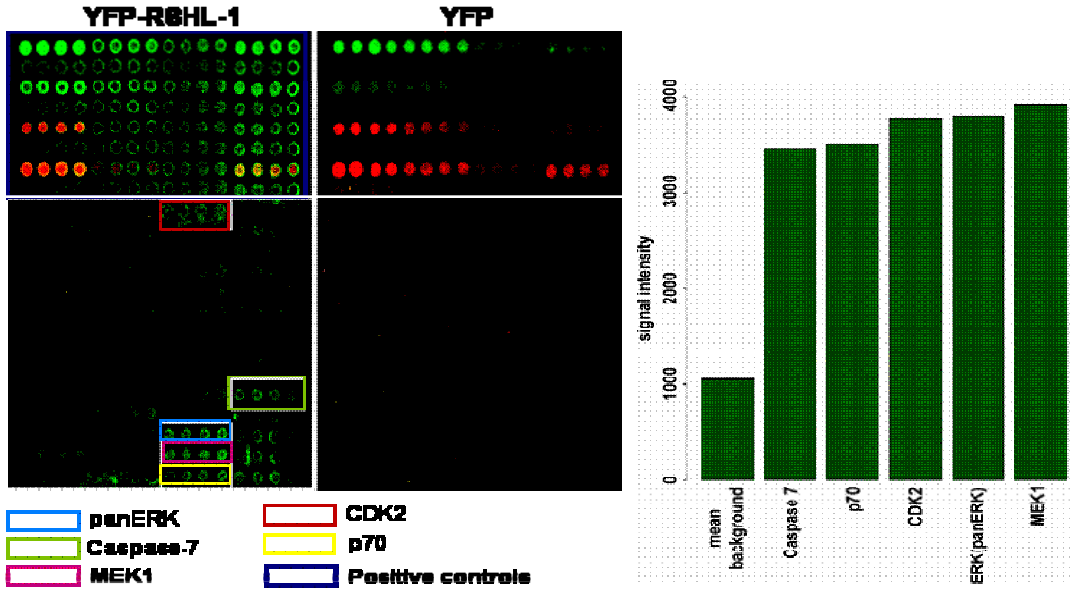


Figure 3.19: Detection of protein interactions of Rshl1. Antibodies directed against 67 different proteins and positive controls (Streptavidin, goat anti-mouse IgG, each conjugated with fluorescent dye) were spotted in quadruplicate on Nexterion slide H micro arrays. The horizontal white line separates positive control spots (above) and antibody samples. Cells over expressing the Rshl1-YFP fusion protein were lysed and incubated with the array. The interaction was detected by incubation with biotinylated YFP/CFP-antibody and $Alexa_{532}$ conjugated Streptavidin (left). A duplicate micro array was incubated with lysate of cells over expressing the N-YFP tag alone (centre) and no interaction was detected by incubation with biotinylated YFP/CFP-antibody and $Alexa_{532}$ conjugated Streptavidin. Signals were read with a fluorescent scanner (ScanArray ExpressHT, Perkin Elmer). Signal intensities (green channel) for interacting proteins, which are framed (left), with signals three times greater than the mean background are plotted (right). The mean background is the local mean background of all spots.

3.8.4.2 Confirmation of interaction partners by co-immunoprecipitation

In order to confirm the interactions that had been identified on the antibody array, YFP tagged and overexpressed Rshl1 was immuno-precipitated with a GFP specific antibody, following the protocol described in Materials and Methods (section 2.2.14). The precipitated proteins were eluted and separated in a polyacrylamide gel and analysed by Western blot for the presence of interacting partners that had been identified in the antibody array experiment.

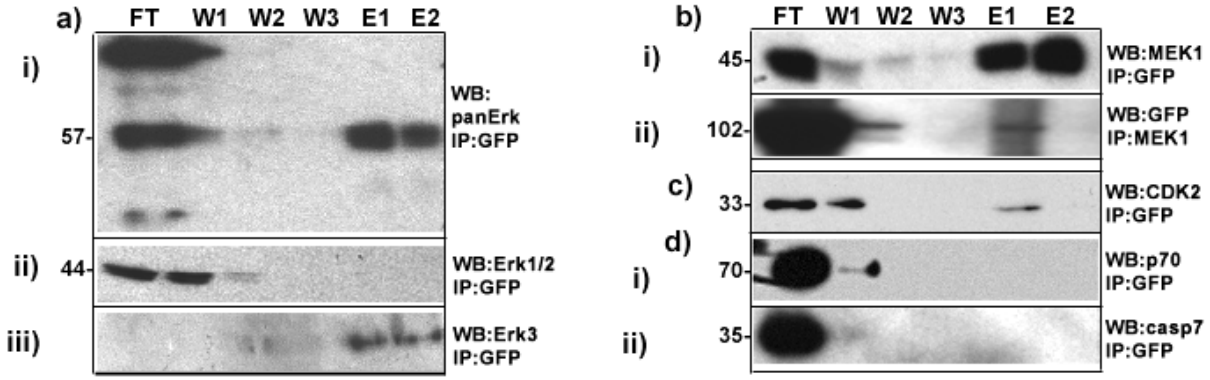


Figure 3.20: Confirmation of interacting partners of Rshl1 by co-immunoprecipitation. The results from antibody chip regarding the interacting partners of Rshl1 were confirmed by performing a co-immunoprecipitation. HEK-293 cells were transfected with YFP-RHSL-1 and incubated for 48 hrs at 37°C to allow expression. Cells were then lysed accordingly, and 500µg -1mg of total protein was loaded on to a mini-column with sepharose beads coupled with anti-GFP antibody. The co-immunoprecipitation was performed according to the protocol. The precipitated protein was eluted and loaded on to a poly acrylamide gel. Western blotting was done with the respective antibodies. Mek1, ERK3 and CDK2 co-precipitated with YFP-Rshl1 and could be detected on a Western blot. YFP-Rshl1 also co-precipitated with Mek1 when Mek1 was immuno-precipitated.

An antibody against panERK (which recognizes several isoforms of ERK) had been utilized on the antibody array and gave a signal indicating that some isoforms of ERK were interacting with Rshl1. When the same antibody against panERK was used for detection of interacting partners of Rshl1 in Western blotting, a strong band was observed at a molecular weight greater than 50kDa. In an attempt to identify the isoform or isoforms of ERK that interact with Rshl1, antibodies specifically targeting ERK1/2 or ERK3 were used for detection. While the ERK1/2 antibody did not react with protein in the Western blot, a faint band at a molecular weight greater than 50kDa was identified when the membrane was incubated with ERK3 antibody. This molecular weight corresponds to that of an ERK3 isoform (57kDa) (Fig: 3.20 a). When the membrane was incubated with MEK1 antibody, a band was observed at 45kDa corresponding to the molecular weight of MEK1 (Fig 3.20 b) in both elution 1 and elution 2. To confirm the interaction of MEK1 with Rshl1, MEK-1 was precipitated from lysate of cells over-expressing YFP tagged Rshl1 and then Rshl1 was detected with anti-GFP antibody. A band at

102kDa which corresponds to the molecular weight of the YFP tagged Rshl1 (Fig: 3.20 b) was detected. An antibody against CDK2 recognised a protein at 33kDa, which corresponds to the apparent molecular weight of CDK2 (Fig: 3.20 c). Two other proteins, caspase-7 and p70 that had been identified in the antibody array as interacting partners of Rshl1 were not identified by specific antibodies in the co-immunoprecipitation experiments (Fig: 3.20 d).

In summary, the interactions of Rshl1with MEK1, ERK3 and CDK2 were confirmed. However, interactions with caspase-7 and p70 could not be confirmed through co-immunoprecipitation.

3.8.5 Co-localisation studies of YFP-tagged Rshl1

To check if the interaction between Rshl1 and its interacting partners occurs in a specific cellular compartment, co-localisation studies with the YFP tagged Rshl1 and its interacting partners by double immunofluorescence was performed. Further, it was necessary to investigate whether it is possible to elucidate Rshl1 mediated inhibition of ERK1/2 activation via immunofluorescence. HEK-293T cells expressing YFP tagged Rshl1 were stained with respective antibodies for the proteins that had been confirmed as interacting partners. YFP tagged Rshl1(green) localised to the nucleus and the cytoplasm of HEK-293T cells (Fig: 3.21, 1a). ERK1/2 (red) was found to be distributed in the cytoplasm (Fig: 3.21, 1b). When both Rshl1 and ERK1/2 images were overlaid, co-localisation (yellow) was found in the cytoplasm of the transfected cells (Fig: 3.21, 1c). ERK3 was found to be present in structure that suggested cytoskeletal structures (Fig: 3.21, 2b). Thus, YFP-Rshl1 and ERK3 did not show co-localisation (Fig: 3.21, 2c). Cells over-expressing YFP-Rshl1 were also stained with a MEK1 antibody to check for co localisation (Fig: 3.21, 3). Cytoplasmic co-localisation of MEK-1 with YFP-Rshl1 was seen in transfected cells (Fig: 3.21, 3b,c). Finally, co-localisation with CDK2 with YFP-Rshl1 was investigated. CDK2 localised primarily to the cytoplasm in and some cells were identified with CDK2 localising in the nucleus (Fig: 3.21, 4b). Cells expressing YFP-Rshl1 showed co-localisation pattern with CDK2 (Fig: 3.21, 4c). Apart from ERK3, all other interacting partners as well as ERK1/2 were thus shown to also co-localize with YFP-Rshl1 in HEK-293T cells.

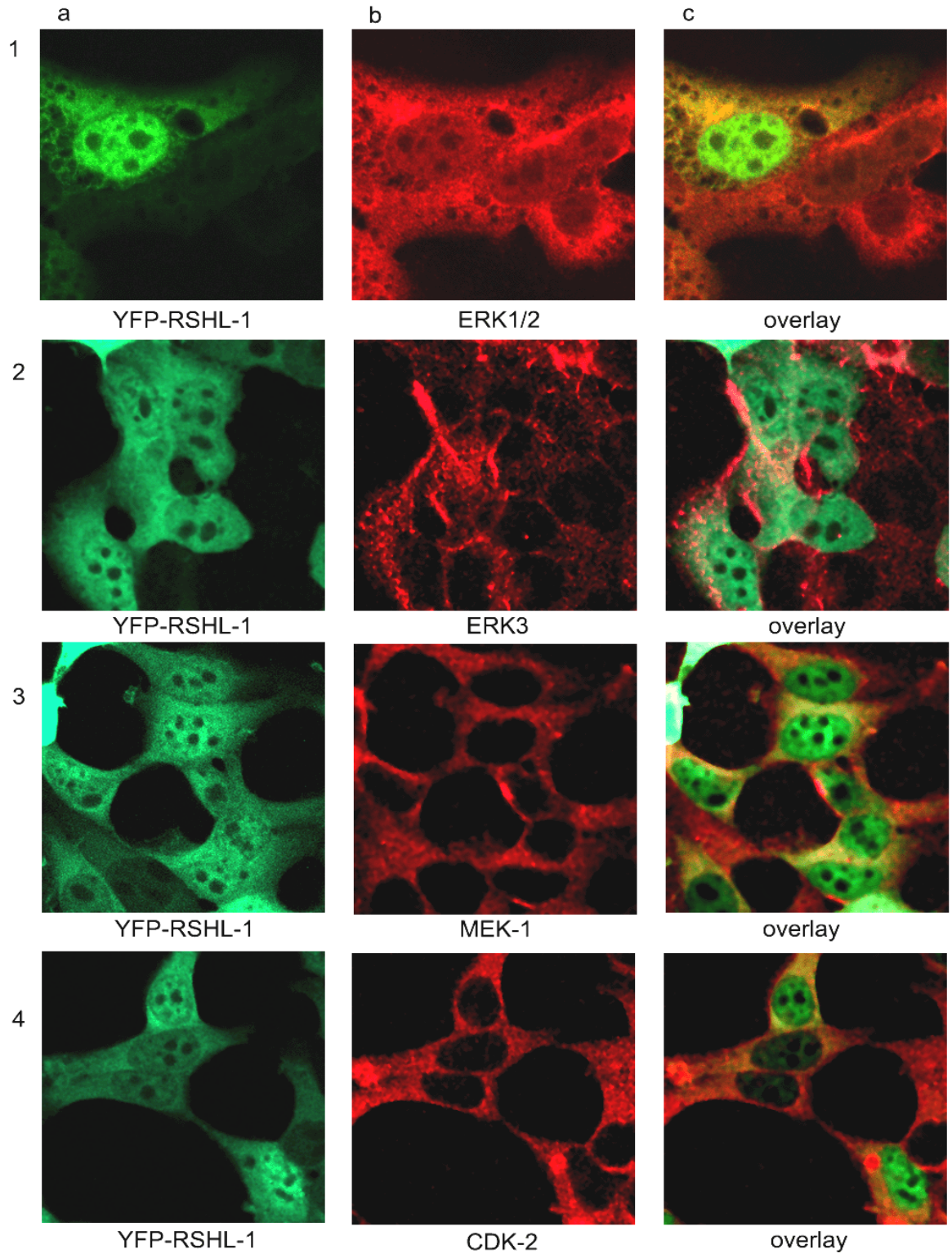


Figure 3.21: Co-localisation of YFP tagged Rshl1. HEK-293T cells were transfected with YFP tagged Rshl1 on cover slips in 6 well plates. After 24 hrs, cells were fixed with 4% PFA and stained with the respective antibodies. Images were taken on a Zeiss confocal laser scanning microscope at 63x magnification. Transfected cells show co-localisation of Rshl1 with ERK1/2 in the cytoplasm (1c). Mek-1 also co-localized with YFP-Rshl1 in the cytoplasm (3c) and CDK2 co-localized with YFP-Rshl1 (4c). However, ERK3 did not show any co-localisation with YFP-Rshl1 under these conditions.

3.8.5.1 Effects of YFP-Rshl1 over-expression

In summary, over-expression of YFP-Rshl1 in HEK-293T cells resulted in the reduction of ERK1/2 phosphorylation (Fig. 3.16). YFP-Rshl1 arrested or delayed the passage of cells from G0/G1 to S-phase (Fig: 3.17) and affected the expression of proteins involved in the regulation of cell cycle. The CDK inhibitor protein $p57^{KIP2}$, and ERK3 were up regulated and cyclin a level was reduced in cells transfected with YFP-Rshl1 (Fig: 3.18). Furthermore, YFP-Rshl1 was found to interact with MEK1, ERK3 and CDK2 using the antibody array technology as well as by co-immunoprecipitation (Figs: 3.19, 3.20). Co-localisation studies indicated that though YFP-Rshl1 does not interact with ERK1/2, it co-localized with ERK1/2 in the cytoplasm. YFP-Rshl1 also co-localized with MEK1 in the cytoplasm. CDK2 co-localized with YFP-Rshl1 primarily in the cytoplasm but was also found in some cells in the nucleus (Fig: 3.20). The results obtained from over-expression studies prompted a further analysis of the localisation, expression pattern and co-localisation of endogenous Rshl1 in HEK-293T cells.

3.8.6 Endogenous Rshl1 localizes to primary cilia, cytoplasm and nucleus

To study the localisation pattern of endogenous Rshl1, a peptide antibody that specifically recognizes Rshl1 was generated, and used to identify the localisation of endogenous Rshl1. Rshl1 contains a predicted radial spoke domain, which is present in proteins that are an integral part of cilia. They form the radial spoke of cilia, which is essential for the movement and intraflagellar transport [85]. Most mammalian cells form cilia in G0-G1/S phase [86]. The primary cilium is assembled in up to 95% of cells in fibroblasts in culture and up to 30% in epithelial cells. Cilia are constituted of a variety of proteins including acetylated tubulin which forms the main part of the shaft of the cilium and also of other cilia related structures like microtubule organizing centre and the centrioles.

In order to check if Rshl1 also localizes to the cilia, HEK-293T cells were grown on cover slips for 2 days until they reached confluence so as to induce the cells to enter G0 (G1) phase. Cells were then stained with anti-Rshl1 and anti-acetylated tubulin antibodies. Immunofluorescent detection of Rshl1 in HEK-293T cells revealed that endogenous Rshl1 indeed co-localizes with acetylated tubulin. Primary cilia were clearly visible after two days in many cells and Rshl1 co-localized with acetylated tubulin at the cilium, but was also present in the cytoplasm and in the nucleus (Fig: 3.22 a).

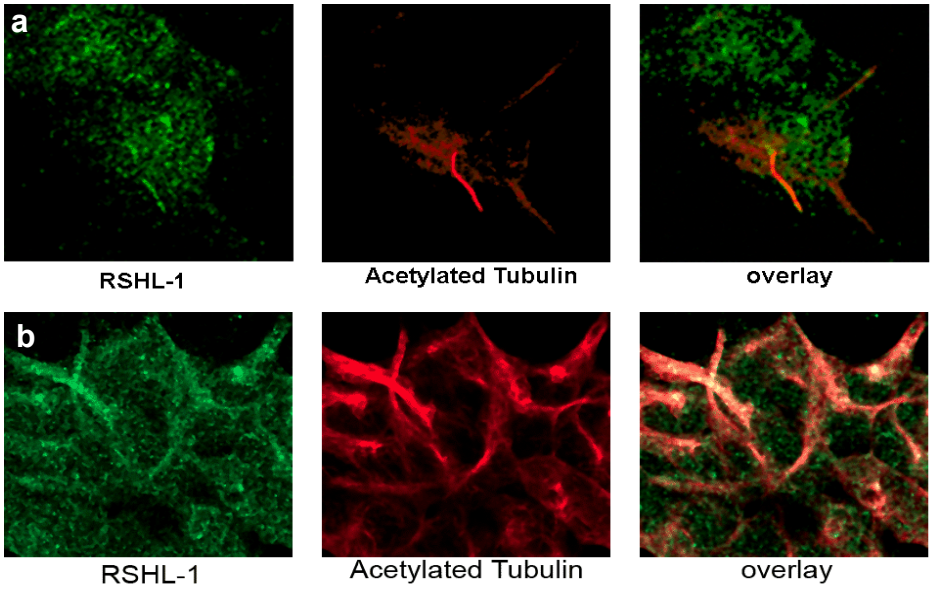


Figure 3.22: Endogenous Rshl1 co-localises with Acetylated tubulin. HEK-293T cells were grown on cover slips for 2 days, and then fixed and stained with a specific antibody against Rshl1 and Acetylated tubulin. Rshl1 co-localised with acetylated tubulin (a marker of primary cilia). It was observed in the primary cilia and also in the nucleus and the cytoplasm in may cells (a). It also clearly co-localised with acetylated tubulin in areas other than the primary cilia (b).

3.8.7 Co-localisation of endogenous Rshl1 with its interacting partners

Results from the antibody array and co-immunoprecipitation indicated that Rshl1 interacts with MEK1, CDK2 and ERK3. The data from the co-localisation studies with YFP tagged Rshl1 show that Rshl1 also co-localizes with ERK1/2, MEK1 and CDK2 when over-expressed. To confirm these results the co-localisation of endogenous Rshl1 with these proteins in HEK-293T cells was analysed using specific antibodies against these proteins. Endogenous Rshl1 and ERK1/2 both localized to the cytoplasm and the nucleus of the cells (Fig: 3.23, 1 c and b). In some cells the distribution of Rshl1 was more in the nucleus. Co-localisation with ERK1/2 was seen primarily in the cytoplasm, and in the nucleus also in a few cells (Fig: 3.23, 1d). ERK3 primarily localized to the cytoplasm (Fig: 3.23, 2b). Co-localisation of ERK3 with Rshl1 could not be observed under these conditions (Fig: 3.23, 2d). MEK1 was located primarily in the cytoplasm of most of the cells (Fig: 3.23, 3b). MEK1 and Rshl1 did not show co-localisation (Fig: 3.23, 3d). CDK2 was found to be present in the nucleus and to a lesser extent in the cytoplasm of HEK-293T cells (Fig: 3.23, 4b). Rshl1 and CDK2 showed co-localisation in the nucleus and the cytoplasm of most cells (Fig: 3.23, 4d).

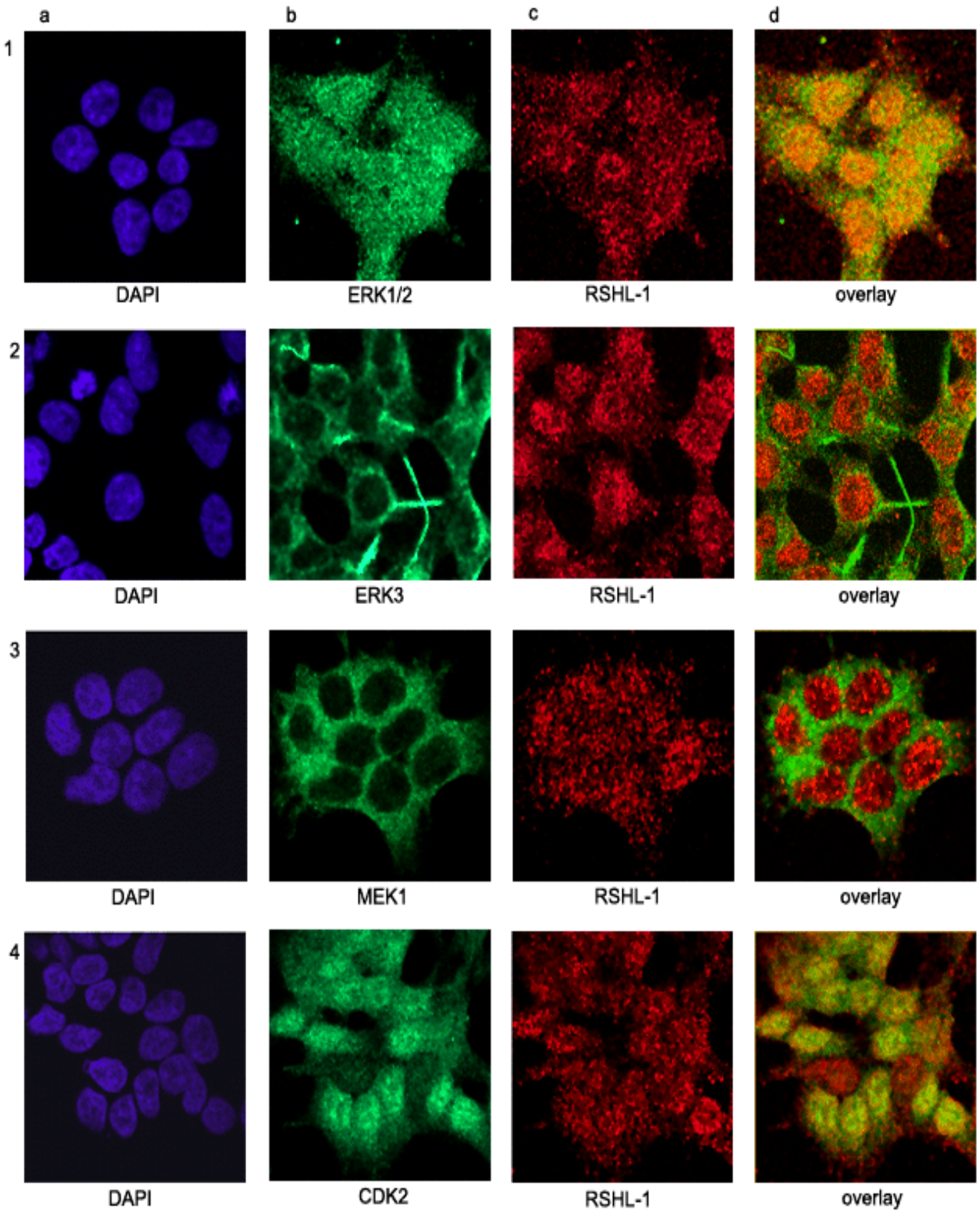


Figure 3.23: Endogenous Rshl1 co-localizes with ERK1/2, Mek1 and CDK2. HEK-293T cells were grown on cover slips in 6 well plates for one day and stained with specific antibodies against Rshl1 and the interacting partners (ERK1/2, ERK3, MEK1 and CDK2). 1) Rshl1 and ERK1/2 both localized to the cytoplasm and nucleus of HEK-293T cells. Co-localisation was observed in the cytoplasm. 2) Cells stained with Rshl1 and ERK3 antibodies showed cytoskeletal staining for ERK3 and cytoplasmic/nuclear staining for Rshl1. Co-localisation was not observed for ERK3 and Rshl1. 3) Mek1 was primarily located in the cytoplasm and Rshl1 did not co-localize with Mek1. 4) Rshl1 and CDK2 showed clear co-localisation in the nucleus of many cells and in the cytoplasm of a few cells.

In conclusion, co-localisation of endogenous Rshl1 was detected with CDK2 and ERK1/2. The pattern of distribution of Rshl1, however, varied between cells in the population. In some cells more nuclear localisation was observed and in others it was uniformly distributed through the cell. This difference in the pattern of distribution led me to investigate if the localisation and in turn the co-localisations of Rshl1 and its interacting partners might change depending on the different phases of the cell cycle.

3.8.8 Co-localisation studies of Rshl1 in G0/G1 arrested HEK-293T cells

HEK-293T cells were treated with Differentiation inducing factor 3 (DIF3), a potent inhibitor of cyclin D expression, which blocks cells in G0/G1 phase of the cell cycle. Upon treatment with DIF-3, cells showed a change in morphology with decreased cytoplasm and partial rounding of cells. Immunofluorescence analysis using the Rshl1 antibody and the antibodies for ERK1/2, ERK3, MEK1 and CDK2 was next performed to check the co-localisation. In addition, cells were stained with DAPI for nuclear staining.

In most of the G0/G1 arrested cells, Rshl1 localized to the nucleus and cytoplasm (Fig: 3.24, 1a). ERK1/2 was located in the cytoplasm as well as in the nucleus (Fig: 3.24, 1b). Co-localisation of Rshl1 and ERK1/2 was observed in the nuclear region (Fig: 3.24, 1d). ERK3 localized only to the cytoplasm (Fig: 3.24, 2b) and did not show any co-localisation with Rshl1 in cells blocked in G0/G1 phase. MEK1 localisation was also limited to the cytoplasm (Fig: 3.24, 3b). MEK-1 and Rshl1 did not show co-localisation (Fig: 3.24, 3d). CDK2 and Rshl1 showed co-localisation primarily in the nucleus though CDK2 was distributed in both the nucleus and the cytoplasm (Fig: 3.24, 4d,b). These results indicate that Rshl1 localises in the cytoplasm and nucleus of G0/G1 arrested cells and co-localises with ERK1/2 , CDK2 in the G0/G1 phase of the cell cycle.

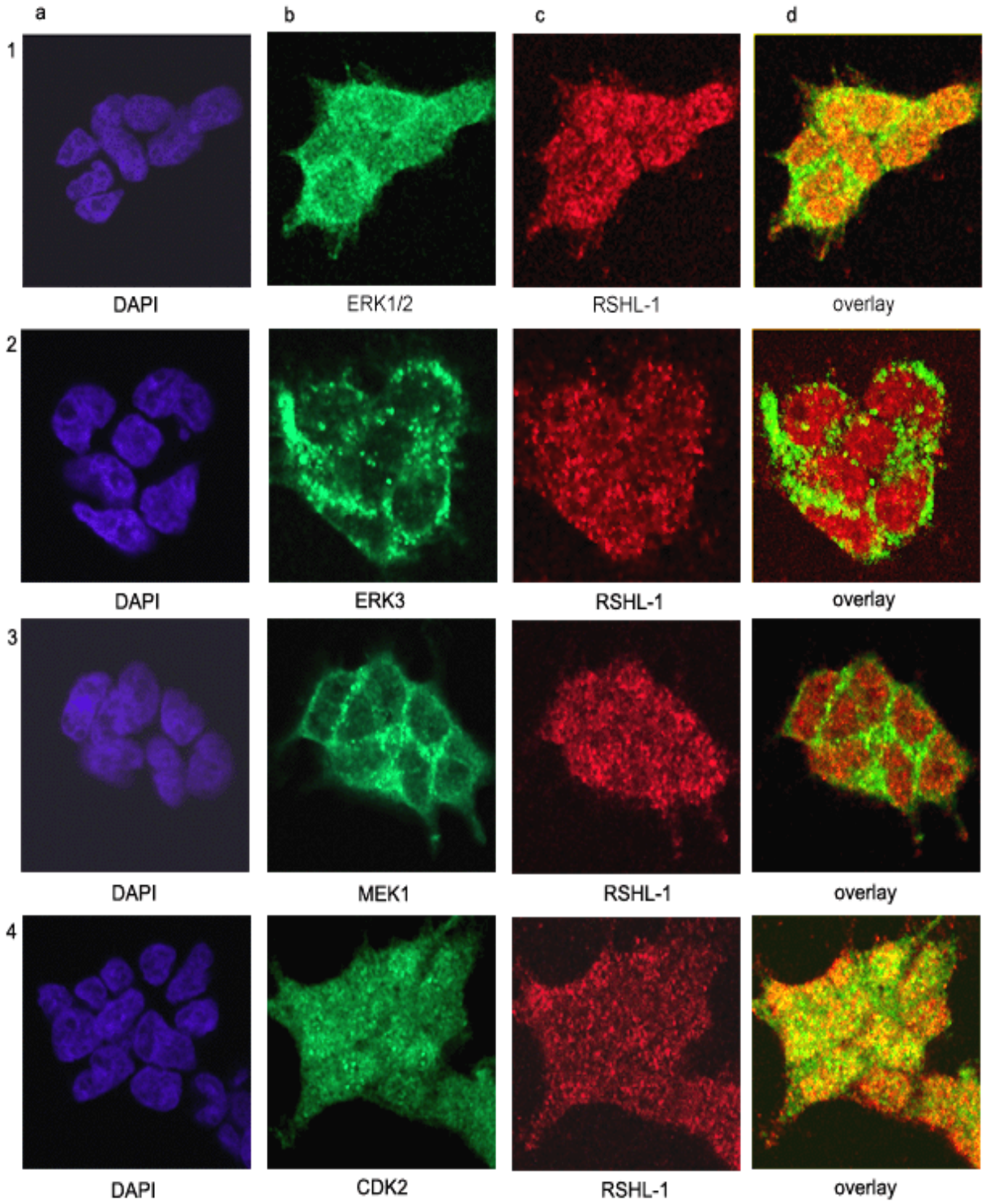


Figure 3.24: Co-localisation of Rshl1 in G0/G1 arrested cells. HEK-293T cells were grown on cover slips for 1 day and then treated with DIF3 for 24hrs in order to block them in G0/G1 phase. Cells were fixed and stained with Rshl1 and antibodies against ERK1/2, ERK3, Mek1 and CDK2. Rshl1 localized to the cytoplasm and the nucleus (1a). Rshl1 showed co-localisation with ERK1/2 in the nucleus but no in the cytoplasm (1d). When stained with both ERK3 and Rshl1 antibodies, I could not observe any co-localisation (2d). Co-localisation with Mek1 was also not observed in G0/G1 arrested cells. However, Rshl1 showed co-localisation with CDK2 in the nucleus and the cytoplasm.

3.8.9 Co-localisation studies of Rshl1 in HEK-293T cells arrested in G2 phase

To block HEK-293T cells in G2 phase of the cell cycle, cells were treated with vincristine sulphate which is a microtubule de-polymerizing agent. As expected, Vincristine treatment induced disruption of the cytoskeleton and cells rounded up. Immunofluorescence analysis using a specific Rshl1 antibody and the antibodies for ERK1/2, ERK3, MEK1 and CDK2 was performed to check the co-localisation. Cells were also stained with DAPI for nuclear staining.

In most of G2 arrested cells, Rshl1 localized to the cytoplasm (Fig: 3.25, 1a). ERK1/2 was located in the cytoplasm (Fig: 3.25, 1b). Rshl1 and ERK1/2 co-localized in the cytoplasm (Fig: 3.25, 1d). ERK3 localized only to the cytoplasm (Fig: 3.24, 2b) and did not show co-localisation with Rshl1 in cells blocked in G2 phase. MEK1 localisation was limited to the cytoplasm with minimal distribution in the nucleus (Fig: 3.25, 3b). However, MEK-1 and Rshl1 showed no co-localisation (Fig: 3.25, 3d). CDK2 and Rshl1 showed co-localisation only in the nucleus though CDK2 was distributed in both the nucleus and the cytoplasm (Fig: 3.25, 4d,b). These results indicate that Rshl1 localizes in the nucleus and cytoplasm of G2 arrested cells and co-localizes with ERK1/2, CDK2 in the G2 phase of the cell cycle in HEK-293T cells.

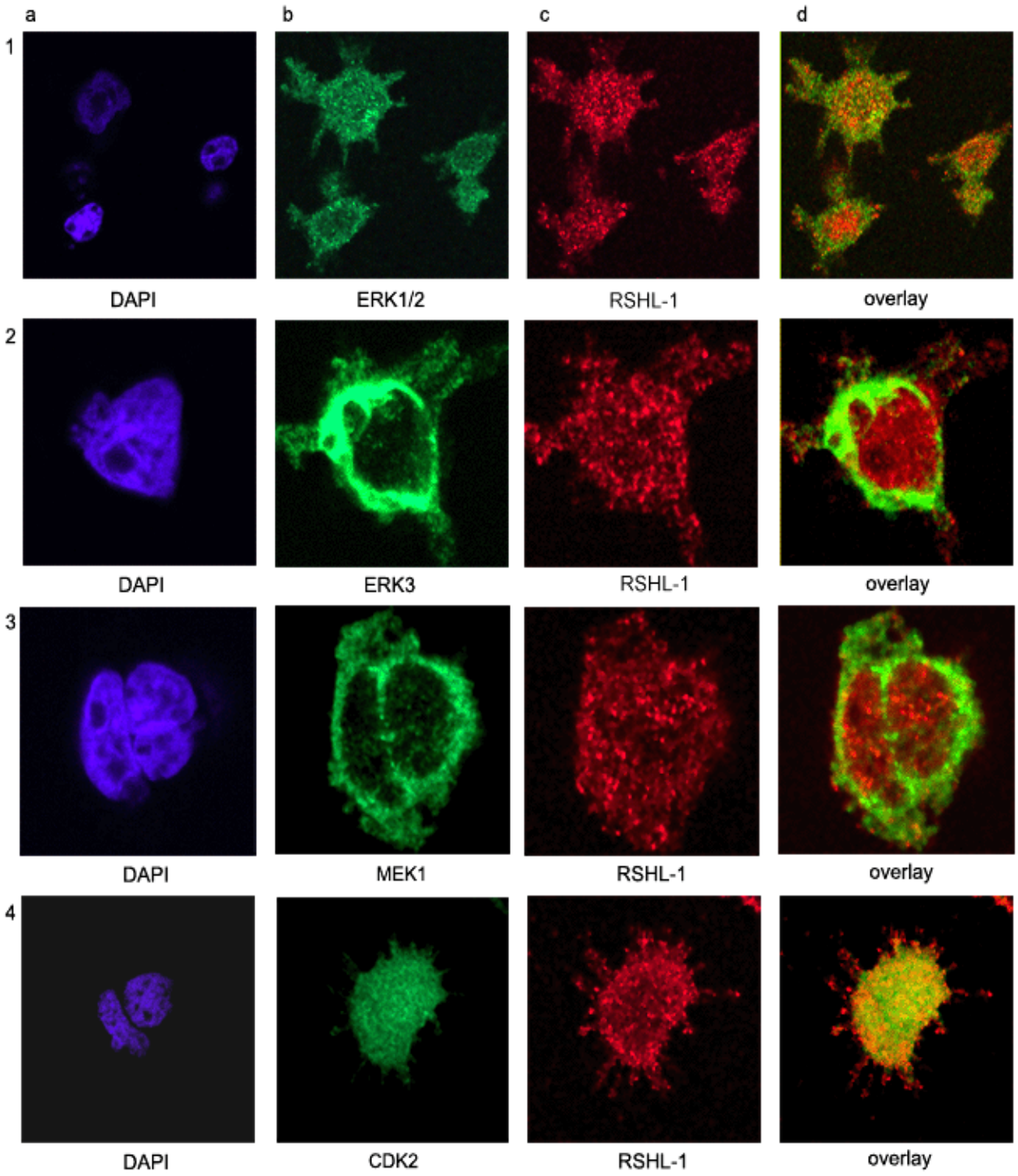


Figure 3.25: Co-localisation of Rshl1 in G2 arrested cells. HEK-293T cells were grown on cover slips for 1 day and then treated with vincristine for 24hrs in order to block them in G2 phase. Cells were fixed and stained with Rshl1 and antibodies against ERK1/2, ERK3, Mek1 and CDK2. Rshl1 primarily localized to the cytoplasm (1a). Rshl1 showed partial co-localisation with ERK1/2 in the cytoplasm (1d). When stained with both ERK3 and Rshl1 antibodies, we could not observe any co-localisation (2d). Co-localisation woth Mek1 was also not observed in G2 arrested cells (3d). However, Rshl1 showed co-localisation with CDK2 in the cytoplasm (4d).

In summary, endogenous Rshl1 localizes to the primary cilia, cytoplasm and nucleus of HEK-293T cells in a cell cycle phase dependent manner. Localisation to the primary cilia and cytoplasm is confined to the G0/G1 phase. The distribution was found to change to the cytoplasm and nucleus in G1/S, and to the cytoplasm in the G2/M phases. Rshl1 co-localized with acetylated tubulin also in structures other than primary cilia that resembled cytoskeletal structures. It co-localized with ERK1/2 in the cytoplasm and with CDK2 in the nucleus as well as in the cytoplasm of unsynchronized cells. Further analysis showed that Rshl1 co-localized with ERK1/2 primarily in the G0/G1 phase mostly in the nucleus, and with CDK2 in the nucleus and cytoplasm in G0/G1 phase and in the nucleus and cytoplasm in the G2/M phase.

Table 4. Interaction and co-localisation studies of Rshl1

Protein	**Interaction Antibody array**	**Interaction Co-ip**	**Co-localisation Studies**	**Cell compartment**
CDK2	+	+	+	Nucleus and cytoplasm
MEK1	+	+	+	Cytoplasm
ERK1/2	+*	-#	+	Cytoplasm
ERK3	+*	+#	-	NA
Caspase-7	+	-	-	NA
p70	+	-	-	NA

* pan ERK antibody is specific for all isoforms of ERK

Western blot with antibodies specific for different ERKs was performed and ERK3 was identified to be pulled down and the interaction was confirmed

Table 5. Cell cycle phase dependent localisation of Rshl1

Cell cycle phase	**Localisation**
G0	Primary cilia and cytoplasm
G1/S	cytoplasm and nucleus
G2/M	cytoplasm

4 Discussion

The identification of genes and proteins that are causally involved in cellular processes leading to disease is one of the remaining challenges after completion of the sequencing of the human genome. With a gene catalogue at hand, tools are being developed to unravel the biological activities of the encoded proteins at a large scale, and high-throughput cellular assays are one potent means in this direction. The focus of this work was to develop and systematically apply a new assay that should identify proteins modulating the ERK1/2 signalling pathway. This assay complements a number of other assays, namely a DNA replication assay (cell proliferation) and an apoptosis assay, that are developed in the department.

Most genes that have a role in disease related cell signalling should induce phenotypic effects when perturbed, and these can be measured in cell based assays. Thus, my challenge had been to develop a strategy that would avoid the considerable time and resources required to first identify the function of the individual proteins, but rather screen previously uncharacterised proteins in a simple phenotypic assay that would allow for the discrimination between proteins that are either involved or not involved in the ERK1/2 signalling pathway.

Automated high throughput assays need to reduce biological activity to simple, easily quantifiable readouts. However, cell signalling in living cells functions within complex networks. As our knowledge of signal transduction expands, it becomes increasingly clear that determining the precise function of any protein is an extremely complex task that requires consideration of many factors. From the standpoint of assay design, it is apparent that the techniques used to assess the impact of a modifier of signal transduction need to take this underlying biological complexity into consideration as much as possible. The ERK1/2 pathway is one such signalling pathway that operates at various levels in different modules in a cell system and may vary from cell system to cell system. Activation of the ERK1/2 pathway mostly has a differentiative/proliferative effect whereas the inhibition is often associated with cell death or apoptosis. Taking the approach of protein over-expression into consideration, the final read out, i.e. the activation or inhibition of ERK1/2, is of significant value and provides information regarding the putative role of the proteins in cellular processes.

High throughput screening for proteins involved in signalling mechanisms can be done by using several techniques like high content screening microscopy, plate reader based methods, flow cytometry etc. Antibody based assays are very useful in this approach and have already been successfully used for such purposes. Development of antibodies specific for activated forms

of several proteins and also of the ERK1/2 proteins has made it possible to detect changes in the activation state in cells e.g. after the over-expression of unknown proteins. Two major advantages of antibody based detection are the specificity and sensitivity of the method. Plate reader based detection methods enable quick measurement of the effect and are rather simple to perform. However, the final readout is the average value from a single well. Subtle differences between individual cells and also patterns of activation or inhibition within a cell population are not detectable. The transfection efficiency of cells is also a major factor influencing the read-out, as low efficiencies induce changes in only a small number of cells. These induced effects could then easily be lost in the noise of the other non-transfected cells. After an initial testing of the plate reader based detection for the MAPK assay, it could be concluded that with additional parameters like transfection and the level of expression of a protein to be measured, apart from the effect itself, the plate reader based detection was unsuitable for this assay. In contrast, FACS based detection offers clear advantages in these respects. The major advantage in this approach is the single cell resolution that is achieved. The level of ERK1/2 activation in each cell can be monitored and any changes in the activation pattern can be put in relation to the expression level of the particular protein that is to be analysed. FACS based detection provides also spatial information on the effect of the protein of interest on the ERK1/ 2 pathway in each individual cell.

4.1 Characterisation of cell lines

In order to establish the ERK1/2 assay, first the ability to modulate the ERK1/2 pathway had to be tested in a number of cell lines, in order to identify at least one that was suitable. I selected five mammalian cell lines in total for this analysis. The cell lines included breast cancer cell lines BT474 and SKBR3, both of which are well characterised for the activation and regulation of the ERK1/2 pathway through EGFR [58]. BT474 and SKBR3 cells were selected as both express high levels of c-erbB2 [87, 88], which is one upstream receptor tyrosine kinase in the ERK1/2 pathway mediated by EGF [89]. Though both cell lines showed a high expression of c-erbB2, they differed in the activation pattern of ERK1/2 pathway. The SKBR3 cells express EGFR at a higher level than the BT474 cells [58] leading to the constitutive activation of the ERK1/2 pathway even without the ligands. The BT474 cells, in contrast, had minimal activation of ERK1/2. I next wanted to exploit this difference in the activation pattern of the ERK1/2 pathway in these cell lines by using the constitutively active SKBR3 cells to screen for inhibitors and the BT474 cells for activators. However, I observed that the SKBR3 cells did show constitutive

activation in serum starved cells (Fig: 3.1b), but did not show a marked increase upon EGF stimulation. Further, treatment with the specific MEK1/2 inhibitor, U0126, did not reduce the phospho-ERK1/2 levels significantly. Thus ERK1/2 activation could not be modulated in this cell line.

The human cervical carcinoma cell line HeLa was then characterised for ERK1/2 activation (Fig: 3.1c). HeLa cells also showed high ERK1/2 phosphorylation even in serum starved conditions which did not further increase with the addition of EGF. This could be explained by the fact that the HeLa cells exhibit auto-activation mechanisms of the ERK1/2 pathway keeping this pathway active even in the absence of specific ligand. In contrast, the mouse fibroblast NIH3T3 cells and the human kidney HEK-293T cells exhibited the expected ERK1/2 activation pattern (Fig: 3.1 d, e). There was minimal ERK1/2 activation in serum starved cells which increased significantly upon EGF treatment. The activation of ERK1/2 could also be inhibited by treatment with U1026 prior to EGF stimulation. The ERK1/2 activation in these cell lines could be modulated by activators or inhibitors effectively and so the BT474, NIH3T3, and the HEK-293T cells were chosen for the further characterisation.

4.2 Method of detection

Levels of phospho-ERK1/2 and transfection (YFP tagged protein expression) are the two parameters that were to be monitored in the assay. Several methods have been described for the detection of ERK1/2 activation. Western blotting is routinely used for analysis. For high throughput screening, fluorescence microscopy, plate reader based methods [81] and flow cytometry [90] have already been described. Detection of ERK1/2 phosphorylation can be done by using specific antibodies against the phosphorylated form of ERK1/2 that are commercially available. The antibody used in the establishment of the assay had already been characterised for its specificity to detect phospho-ERK1/2 in a different experimental setup [90]. Hence, I first tested this antibody for its performance under the assay conditions with the chosen cell lines.

Plate reader based methods are mostly used for high throughput assays. They facilitate fast and simple detection of ERK1/2 activation in multi-well plate formats. I adapted a phospho-specific cell based ELISA (PACE) [81] for the ERK1/2 activation assay. PACE is based in the detection of phospho-ERK1/2 in fixed cells in 96 well plate format. The signal output in this method depends on the number of cells and the amount of antigen present in the cells in each well. It involves a chromogenic reaction with a HRP labelled secondary antibody and OPD as a

substrate. Detection of differences between levels of phospho-ERK1/2 in the samples was, however, not optimal with this method (Fig: 3.5).This could be because the reaction between the HRP and the substrate is not linear and results in accumulation of the signal with time, leading to the loss of information from the subtle differences in ERK1/2 phosphorylation. Thus a fluorescently labelled secondary antibody was used instead, so as to increase the sensitivity of the detection and to obtain an absolute signal for the amount of phospho-ERK1/2 that was present in the cell. However, the differences between the serum starved and the serum-stimulated samples was not detected optimally. Each well from a 96 well plate contained both transfected and non-transfected cells, as the transfection efficiency greatly varied between the different cell lines and was never 100%. In consequence, the signal that was detected from one well was a combined signal from both transfected and non-transfected cells. When the transfection efficiency of a particular plasmid had been low, the signal from the transfected cells was masked by the signal (noise) measured from the non transfected cells, resulting in false negative data. Therefore, information about the efficiency of transfection is important to avoid such false results. However, the detection of transfection (YFP/CFP) in fixed cells using a fluorometer was not achieved in these assay conditions. And even if this had been possible, the over-expression of any protein varies to a great extent between the transfected cells [84], and the average measurement of the protein content thus does not provide information on the specific effects that could well be induced by varying amounts of the protein under investigation.

FACS based detection of phopho-ERK1/2 in fixed cells gives the advantage of a single cell resolution. This method of detection thus provides information about the transfection efficiency, the level of protein over-expression and the level of phospho-ERK1/2 in each cell individually. It consequently allows for a correlation between the level of expression of a particular protein and its effect on ERK1/2 activation. Using this method, the levels of phospho-ERK1/2 could be discriminated between starved and stimulated cells, and also between transfected and non-transfected cells. The information that comes out of such a multi-parameter detection method is critical for the interpretation of results and also to avoid false positive and negative results. In the set-up established during this work, the final detection of signals was done in a 96 well plate format which was convenient for the throughput that is to be expected from a high throughput screen. Having chosen the method of detection the assay protocol was adapted and optimised accordingly and finally a FACS based, automated, high throughput assay was developed to screen for novel proteins involved in ERK1/2 activation.

4.3 Controls in the ERK1/2 activation assay

Over-expression of any protein may or may not affect activation of ERK1/2 under the assay conditions. Every cell has a basal level of ERK1/2 phosphorylation which is detected in the assay, even in the unstimulated cells. Once stimulated, the phosphorylation usually occurs rapidly thus inducing is a shift in the phospho-ERK1/2 level. Differences in this shift that was due to the over-expression of a particular protein was to be detected and determined as an effect. This can be done by comparing the pattern of activation between untransfected and transfected cells. Since not all cells are transfected, also untransfected cells are present in each well and serve as an internal control. This avoided false interpretation of effects that could have been caused not by the over-expressed proteins but rather be due to systematic errors (errors in seeding, confluence, cell clumps, plate effects, etc.).

As all the proteins chosen for the screen were YFP-tagged, an influence of the tag alone on ERK1/2 activation had to be excluded. YFP as such has no function in mammalian cells and was not expected to have any effect on ERK1/2 activation. Indeed YFP was found to not affect the activation pattern of ERK1/2 both in serum starved as well as stimulated conditions (Fig: 3.10). Then, control proteins were selected with know activating or inhibiting effects on ERK1/2 activation (Table: 1). MEK2, which specifically phosphorylates ERK2, did not show the expected increase in the activation of ERK1/2 in the assay conditions. This could be due to the presence of the relatively large tag (YFP, 27kD) at the C/N terminus, which could lead to the masking of binding sites and rendering the fusion proteins inactive. Alternatively, this could be a consequence of a different biological context, i.e. the cell system being analysed, the level of expression and finally the specific assay conditions. In contrast, phospholipase C delta, which had been reported to activate ERK1/2 pathway upon over-expression [83] did function as an activator. While this activation was not very high at the assay conditions, it was consistent and significant (Fig: 3.11). Selecting a relatively weak activator as a positive control for activation helped to not only validate candidate proteins by comparing the induced effects but was also essential to keep the robustness of the assay at a high level. A strong effector should be expected to be positive even in a inferior assay system, whereas the significant and reproducible monitoring of a weak effector provides direct evidence that the chosen assay is robust enough to select even proteins that have mild effects. The control protein chosen as an inhibitor of ERK1/2 activation was dual specificity protein phosphatase 10 (DUSP10). The DUSP10 is a dual specificity protein phosphatase and de-phosphorylates MAPKs in general. Over-expression of YFP tagged DUSP10 resulted in the significant reduction or even inhibition of ERK1/2

activation (Fig: 3.11). Two other control proteins were Annexin A1 and Cystathionine gamma lyase, which both had been reported to cause sustained activation of ERK1/2 upon over expression [82],[91]. However, these proteins did not show any significant effect in the assay. This lack of ERK1/2 activation could be explained with the same reasons already discussed for the MEK2 protein. In the establishment of the assay it was important to validate that under the given conditions it was possible to detect activators and inhibitors of ERK1/2 phosphorylation. This was achieved with the PLCD4(activator) and DUSP10 (inhibitor) proteins, confirming the assay was suited to identify also novel activators and inhibitors of the ERK1/2 pathway.

4.4 Effectors of ERK1/2 activation

The screening of 200 novel uncharacterised proteins in the ERK1/2 activation assay identified 14 proteins that modulated ERK1/2 activation upon over-expression. Twelve proteins were found to be inhibitors and two were activators of the ERK1/2 pathway. A few of the candidate proteins and their potential involvement in ERK1/2 pathways are described below.

The cDNA *DKFZp779111842* encodes a protein that has been described as Melanoma inhibitory activity like protein 2 (MIA2) [92]. MIA2 shares 34 to 45% amino acid identity with MIA1. Melanoma inhibitory activity (MIA) is a 12-kDa protein that is secreted from both chondrocytes and malignant melanoma cells. MIA is described to elicit an inhibitory effect on melanoma cells in vitro [93]. It has been reported to have effects on cell growth and adhesion, and a role in melanoma metastasis and cartilage development is discussed [94]. Recently it has been reported that MIA binds to integrins, that it inhibits the ERK1/2 pathway, and that it has a role in tumour progression and spread of malignant melanomas by mediating detachment of cells from extra-cellular matrix molecules through modulating integrin activity [95]. Thus, I could confirm these recent results and that the protein can serve as an independent control, further validating the assay.

The protein encoded by the cDNA, *IRATp970A0419*, inhibited ERK1/2 activation in the assay. It was identified as Interferon alpha-inducible protein 27 (IFI27) [96], and was found to be up-regulated in psoriatic skin and some epithelial cancers [97]. Interferons, however, have been reported to induce apoptosis and to have an anti-proliferative effect on various cell systems [98]. Further, interferon alpha has been reported to inhibit the ERK1/2 pathway by a mechanism that involves cross-talk between the MAPK and the JAK/STAT pathways [99]. It has been also reported that the expression pattern of certain genes differs in cancer cells that are sensitive to

treatment as compared to the resistant cells, for e.g.; several pro-apoptotic genes were upregulated in sensitive cells when compared to the resistant cells [100]. Hence, up-regulation of IFI27 in certain cancer cells and psoriatic skin does not necessarily indicate that the cancer or increase in proliferation would be due to the up-regulation of IFI27. Rather, it could be that the expression of IFI27 is a mechanism that sensitizes cells to apoptosis.

The protein coded by the cDNA *DKFZp434J037* was identified as similar to Salt-Inducible serine/threonine kinase 2 (SNF1LK2). SNF1LK2 is a member of the AMP-activated protein kinase superfamily. SNF1 exhibits histone H3 kinase activity (phosphorylates serine residue 10) and cooperates with the histone acetyltransferase GCN5 to activate gene transcription. AMPK has been shown to differentially regulate ERK cascades by inhibiting Ras activation or by stimulating the Ras-independent pathway in response to the varying energy status of the cell [101]. These findings fit to the outcome of SNF1LK2 in the ERK1/2 activation assay as an inhibitor.

The solute carrier family 25, member 31, was identified to be a strong inhibitor of ERK1/2. This protein is coded by the cDNA *DKFZp434N1235.* It was identified to be similar to adenine nucleotide translocase 1 (ANT1). ANT1 has been found to be upregulated in several degenerative disease conditions and is associated with sensitization of cells to apoptosis induction [102]. Inhibition of ERK1/2 activity is often found in degenerative disease and has been reported to be an important mechanism for apoptosis induction. Though the mechanism by which ANT1 over-expression inhibits ERK1/2 activation is unclear, it can be assumed that changes in mitochondrial membrane potential could be induced by over-expression of ANT1, and these would trigger cellular events that lead to apoptosis, including inactivation of ERK1/2.

Over-expression of the Rho-Guanine nucleotide exchange factor 3 (GEF3) coded by the cDNA *DKFZp434F2429* inhibited ERK1/2 activation in the assay. Nucleotide exchange factors form an integral part upstream of Ras in the Ras-Raf-MEK-ERK pathway, and the expression of GEFs regulates the activation of the pathway. However, recent studies suggest that the over-expression of CNrasGEF leads to an inhibition of the ERK pathway and ultimately leads to apoptosis. The data obtained in the ERK1/2 assay support these novel findings.

The protein coded by the cDNA *IMAGp998J1311548* was designated as the Translocon associated protein-delta. Translocon-associated protein (TRAP)-delta subunit is assumed to be involved in the secretion of proteins and to play a role in protein transport into the Endoplasmic reticulum. The highest concentration of TRAP-delta transcripts was observed in pancreas, where large quantities of lipases, nucleases, and proteases are synthesized and secreted. Over-

expression of this protein resulted in the inhibition of ERK1/2. The mechanism involved in this process is not clear and needs to be elucidated.

The protein coded by the cDNA *DKFZp434I0515* was identified as Radial spoke head like -1(Rshl1). Rshl1 was the first human homolog of radial spoke head protein with high homology to proteins of sea urchins and the protozoan *Chlamydomonas reinhardtii*, at the myotonic dystrophy-1 locus (chromosome19q13.3) [85]. It shares homology with radial spoke proteins p63, rsp4 and rsp6, which are a part of the radial spoke head of the cilia and flagella. In the lower organisms, these proteins are essential for normal ciliary or flagellar action. Expression of the mammalian homolog was detected in adult testis and was thus suggested to be a candidate gene for familial primary ciliary dyskinesia. However, over-expression of RshlL1 in NIH3T3 cells was reported by us to inhibit proliferation [84]. In another study, Rshl1was reported to be down-regulated in Kidney cancer [40]. In relation to these studies, Rshl1 was identified here to be an inhibitor of ERK1/2 activation, indicating the involvement of Rshl1 in cellular processes controlling the cell cycle.

While all the proteins described above are valid candidates for further characterisation of their respective roles in the ERK1/2 signalling pathway, I had to decide on one protein for follow up studies because of time constraints. I decided for the Rshl1 protein, since related data from own previous work had been available for this protein, and because the suggested involvement of that protein in cilia function made especially this protein interesting to decipher its potential function in cancer-relevant cell signalling.

4.5 Detailed functional analysis of Radial spoke head like-1 (Rshl1)

4.5.1 Localisation of Rshl1

Endogenous Rshl1 localized to the cytoplasm and nucleus in HEK-293T cells. However, the localisation was not uniform in all the cells within a population. In some cells Rshl1 was found primarily in the nucleus and in others in was found to be distributed in the cytoplasm as well as in the nucleus. Rshl1 was also found to co-localise with acetylated-tubulin, which is a marker for primary cilia and for the cytoskeleton. However, Rshl1 was absent in the cilia of some cells. Whether the ciliary localisation of Rshl1 depends on the cell cycle phase or the confluence of cells is yet to be analysed. However, the staining pattern suggests that Rshl1 is not a structural component of the cilium but that it is rather involved dynamic signalling processes that are associated with the cilium. Rshl1 might thus be a member of the intraflagellar transport

proteins (IFT) that have been shown to be important for ciliary assembly and signal transduction. Recently, PDGFR alpha was reported to also localize primarily to cilia and that it was preferentially activated during re-entry into the cell cycle. It has also been reported that MEK1/2 localize to the primary cilia in G0 cells and that these proteins are recruited in PDGFR alpha mediated signalling upon release from the G0 stage and re-entry into the cell cycle [103]. These findings emphasise the importance of cilia and cilia mediated signalling in cell cycle regulation and provide a direct and somewhat unexpected link between these processes. Localisation of Rshl1 to the nucleus and the cytoplasm indicates that it may also play a role in processes other than cilia formation. Another radial spoke protein, rsp3, has been reported to localize in the cytoplasm and nucleus of neurons and this protein is suggested to play a role in development and differentiation [104]. The localisation of Rshl1, and its relation to other radial spoke proteins are indicative for a possible direct involvement of this protein in the ERK1/2 signalling pathway.

4.5.2 Rshl1 interacts and co-localises with ERK3, MEK1 and CDK2

Using antibody arrays Rshl1 was identified to interact with MEK1, pan ERK, CDK2, p70 and caspase-7. Interaction with MEK1, ERK3 and CDK2 was then confirmed by co-immunoprecipitations. Co-localisation studies with these interaction partners were also performed so as to obtain complementary data which should then shed light on the cellular compartments where the proteins interact, and to help understand the biological context in which Rshl1 functions. Over-expressed Rshl1 indeed co-localized with MEK1, ERK1/2 and CDK2 in the cytoplasm. Though ERK1/2 was not detected in the antibody array as well as the co-immuniprecipitation studies as an interacting partner, its co-localisation with Rshl1 suggests that Rshl1 does not interact with ERK1/2 directly but could interact with ERK1/2 as a part of a larger interaction complex involving more than one protein.

Co-localisation studies of endogenous Rshl1 with ERK1/2, ERK3, MEK1 and CDK2 in non-synchronized cells revealed that Rshl1 co-localized with CDK2 in the nucleus in some cells and the cytoplasm in other. The varying localisation of Rshl1 between the cytoplasm and nucleus and the different co-localisation pattern with CDK2 suggests that Rshl1 localisation and its interactions is regulated in cell cycle phase dependant manner. Hence, co- localisation studies with endogenous Rshl1 in different phases of the cell cycle were performed. Cells blocked in the G0/G1 phase showed nuclear localisation of Rshl1 and co-localisation with CDK2 and MEK1. In cells blocked in the G2/M phase, Rshl1 localized to the cytoplasm mainly and co-localized

with ERK1/2 and CDK2. Thus co-localisation was confirmed for all proteins that had been identified to interact with Rshl1.

4.5.3 Over-expression of Rshl1 arrests cells in G0/G1 phase

The anti-proliferative role of ciliary proteins and cilia has already been described. Primary cilia of G0/G1 cells have been speculated to be sensory organelles that send signals from the environment to block cell cycle progression under appropriate conditions [105]. Moreover, it has been shown that primary cilia are expressed in most cells in the early G0/G1 phase and they have been linked to cell cycle regulation and tissue homeostasis [86, 106, 107]. For example; over-expression of a ciliary transport protein (Tg737) in hepatocytes has been shown to reduce the rate of proliferation [108]. The results from the BrdU incorporation experiments suggest that Rshl1 slows down proliferation by arresting cells, or by delaying cells from passing through the S phase. HEK-293T cells that over-expressed Rshl1 were first blocked in the G0 phase by DIF-3 treatment and then released from the arrest by removing DIF-3. The cells then failed to enter the S-phase even after four hours after the release from the arrest. Progression through the cell cycle is regulated by several factors one of which are cyclin dependent kinase inhibitors (CKI). The CKIs (including p27, $p57^{KIP2}$) have been shown to bind to cyclin-cdk complexes and are necessary for full activation of cyclin D/cdk4 activity, but are inhibitory when bound to cyclin E/CDK2 and cyclin A/CDK2 complexes [109]. The anti-proliferative role of Rshl1 can thus be attributed to the observed induction of $p57^{KIP2}$ expression in cells over-expressing YFP-Rshl1. Further, Rshl1 over-expressing cells also had higher levels of ERK3. ERK3 has been speculated to play a role in cell cycle regulation, especially via arresting cells in G0/G1. In actively dividing NIH3T3 cells, ERK3 has been reported to be regulated by rapid degradation via the ubiquitin proteasome pathway. The over-expression of a stabilized form of ERK3, which cannot be targeted by the ubiquitin-proteasome pathway, caused a G1-phase arrest in NIH3T3 cells [110]. The observed increase in the level of ERK3 expression in cells over-expressing YFP-Rshl1 and the fact that Rshl1 interacts with ERK3 suggest that Rshl1 stabilizes ERK3 in HEK-293T cells by interacting with ERK3. Rshl1 would then protect ERK3 from proteasome mediated degradation, thereby inducing the growth arrest of cells. However, the mechanism of how Rshl1 interaction stabilizes ERK3 and how ERK3 induces growth arrest is yet to be analysed.

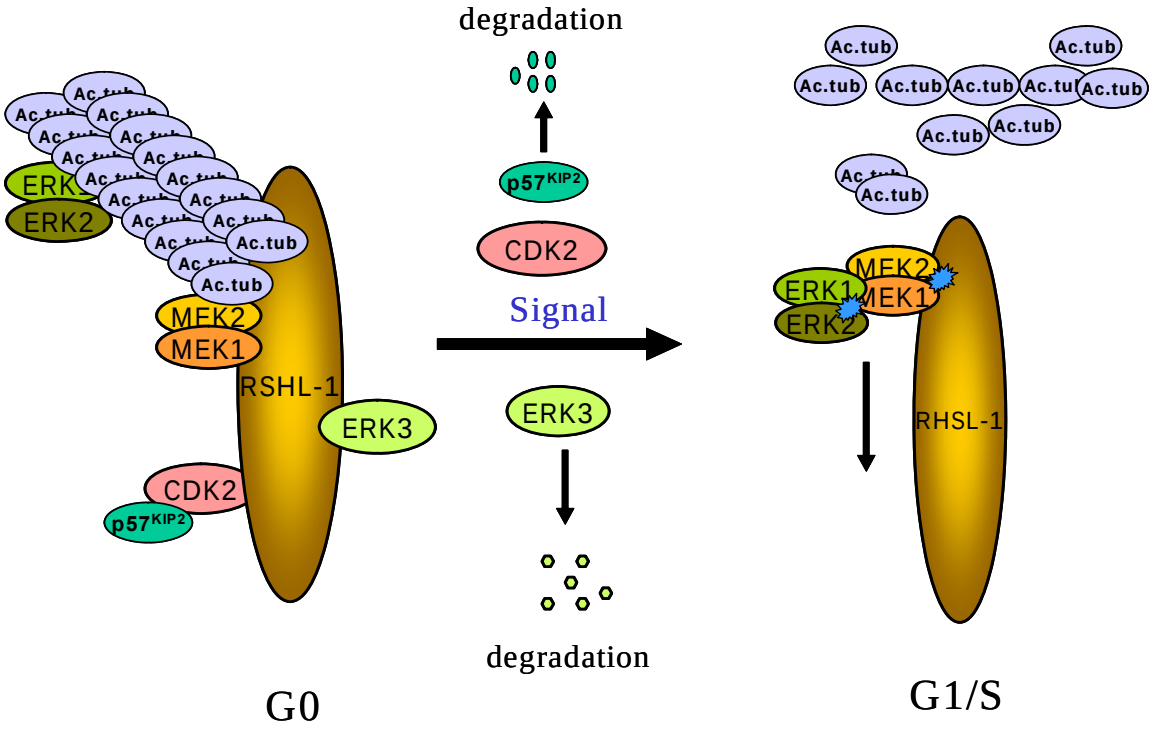


Figure 4.1: Model representing the function of endogenous Rshl1. Rshl1 localizes to the primary cilia in the G0 phase. It interacts with MEK1, CDK2 and ERK3 in this phase and sequesters MEK from ERK1/2 at the same time stabilizes ERK3. It also induces expression of CKI, $p57^{KIP2}$. Expression of $p57^{KIP2}$ along with the stabilization of ERK3 together lead to the inhibition of CDK2 activity and cell cycle arrest and homeostasis. Upon receiving an appropriate signal, Rshl1 dissociates from MEK1 and ERK3 thus allowing MEK-ERK interaction and activation of ERK1/2 pathway. Simultaneously, dissociation from ERK3 leads to its degradation by the proteasome pathway leading cells to progress through cell cycle and proliferation.

Results obtained from this study suggest that Rshl1 localizes to the primary cilia in the G0 phase. It interacts with MEK, ERK3 and CDK2 and sequesters MEK from ERK1/2 thus inhibiting the ERK1/2 pathway. It stabilizes ERK3 by interacting with it and thus protecting it from degradation by the ubiquitin proteasome pathway which in turn brings about cell cycle arrest by a mechanism which is not yet understood. Rshl1 also induces expression of $p57^{KIP2}$ which delays cell cycle progression via the inhibition of CDK activity. Upon receiving an appropriate signal, Rshl1 dissociates from ERK3 and MEK1, leading to the degradation of ERK3 and association of MEK with ERK1/2, respectively. These events trigger the activation of the ERK1/2 pathway and other events like degradation of $p57^{KIP2}$, that release the cells from cell cycle arrest allowing them to pass through the cell cycle and proliferate. In normal cells and tissues, Rshl1 expression induces cell cycle arrest, helps to maintain homeostasis, and regulates cell proliferation depending on the signals received. However, in cancer cells, where Rshl1 has been reported to be down regulated, homeostasis is not maintained and cells undergo

uncontrolled passage through cell cycle phases and proliferation. These results in combination with previous knowledge from the literature suggest a direct involvement of Rshl1 in cell cycle control of normal and cancer cells.

In summary, I have shown that Rshl1 localizes to the primary cilia. It interacts and co-localizes with ERK1/2, MEK1 and CDK2 in a cell cycle phase dependant manner. Localisation of Rshl1 to primary cilia and interaction with members of the ERK/MEK pathway and CDK2 further strengthen the possibility of Rshl1 involvement in cilia mediated signalling pathway that regulates cell cycle. I additionally showed that over-expression of Rshl1 blocks cell cycle progression and arrests cells in G0/G1 phase mediated by ERK3 and $p57^{KIP2}$. Apart from its interaction and involvement in the above mentioned cellular processes the fact that it is down regulated in Kidney cancer adds to the significance of Rshl1 and its role as a potential tumor suppressor.

5 Outlook

It is apparent that the techniques used to assess the impact of a modifier of signal transduction need to take the underlying biological complexity of signalling networks into consideration. Single readouts of protein function (in my case, ERK1/2 activity), whether based on global activity or on transcriptional response, give important hints regarding the function of unknown proteins under study. However, taking into consideration the enormous subtlety and complexity of cell signalling, some loss of information regarding the activity of signalling proteins and the role of novel players in these pathways is unavoidable. Nevertheless, such questions are of growing importance in order to understand the complexity. Together, with the results from complementary assays, as in our case, a proliferation assay and an apoptosis assay, the significance of the information regarding the function of novel uncharacterised proteins with relation to cell cycle regulation and disease is undeniable. I screened 200 novel proteins and identified 7 novel modulators of ERK1/2 pathway which often showed clear involvement also in either cell proliferation or apoptosis. Results obtained from the detailed functional analysis of one such candidate (Rshl1) that was identified as an inhibitor of ERK1/2 activation and proliferation, clearly showed the strength and efficiency of this approach and emphasises the importance of such screens in the field of functional genomics. Having screened a number of different proteins, a much smaller number of candidates has been identified. During my thesis I had been able to concentrate on the detailed functional characterisation of only one of these

candidates. However, also the other proteins, as well as more proteins being identified in the ongoing screen, deserve further characterisation. Rshl1 is likely not a good candidate for therapy, as a knock-down of Rshl1 function is associated with cancer progression. However, a continuation of the screen will in the future likely allow to identify activators of the ERK1/2 signalling pathway, and the detailed functional characterisation of such proteins will determine their potential to serve as drug targets in the fight of cancer.

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Meher Majety

7 Abbreviations

°C	degrees centigrade
µg	micrograms
µl	micro litre
µM	micro molar
µm	micro meter
a.a.	amino acids
Ab	antibody
Abs	absorbance
Amp	ampicillin
APC	allophycocyanin
APS	ammonium per sulfate
ATP	adenosine triphosphate
BCA	bicinchonic acid
bp	base pairs
BrdU	bromo-deoxy uridine
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CFP	cyan fluorescent protein
Co-IP	co-immunoprecipitation
DAPI	4', 6-Diamidino-2-phenylindole
ddH_2O	double distilled water
dNTP	deoxynucleotide triphosphate
DMEM	dulbecco´s modified Eagle’s medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbant assay
FACS	fluorescence actovated cell sorter
FBS	fetal bovine serum
Fw	forward

gm	grams
GFP	green fluorescent protein
GTP	guanosine triphosphate
hr(s)	hour(s)
HRP	horse radish peroxide
IP	immunoprecipitation
Kan	kanamycin
kb	kilo bases
kDa	kilodalton
kV	kilovolts
LB	Luria Bertani
M	molar
mg	milligrams
min	minute(s)
ml	milliliter
mM	millimolar
ng	nanograms
nm	nanometer
O.D	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pM	pico molar
PMSF	phenylmethylsulfonyl fluoride
PS	penicillin / streptomycin
PVDF	polyvinylidene fluoride
RFU	relative fluorescence units
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
Rv	reverse
SDS	sodium dodecyl sulfate

SDS-PAGE	SDS-polyacrylamide gel electrophoresis
siRNA	small interfering ribo nucleic acid
TAE	tris/Acetate/EDTA-buffer
TEMED	N, N, N´, N´-Tetramethylethylenediamide
v	volt
WB	western blotting
YFP	yellow fluorescent protein

8 Supplements

List of proteins screened in the ERK1/2 activation assay

CloneID	AccNO	RZPDID	Symbol	GeneName
akxq28A228	BC006540	IMAGp958A228	BRCC3	BRCA1/BRCA2-containing complex, subunit 3
akxq28ak055600	AK055600	NULL	NULL	NULL
akxq28c1710741	BC022064	IMAGp998C1710741	FATE1	fetal and adult testis expressed 1
akxq28c171466	BC022305	IMAGp958C171466	CLIC2	chloride intracellular channel 2
akxq28D0812213	BM541828	IMAGp998D0812213	LOC203547	hypothetical protein LOC203547
akxq28d201286	BC032121	IMAGp958D201286	FAM58A	family with sequence similarity 58, member A
akxq28D211790	BC015744	IMAGp958D211790	XX-FW81657	DNA segment on chromosome X (unique) 9879 expressed sequence
akxq28e054515	AI218223	IMAGp998E054515	CTAG1B	cancer/testis antigen 1B
akxq28e16117	BC002606	IMAGp998E16117	PDZD4	PDZ domain containing 4
akxq28f031904	AA425725	IMAGp998F031904	STK23	serine/threonine kinase 23
akxq28f10122	BC002416	IMAGp958F10122	BGN	biglycan
akxq28G021845	BC025298	IMAGp958G021845	IL9R	interleukin 9 receptor
akxq28g159617	BC008203	IMAGp998G159617	CXorf12	chromosome X open reading frame 12
akxq28g17175	R59087	IMAGp998G17175	NULL	NULL
akxq28g228444	BC001561	IMAGp998G228444	DNASE1L1	deoxyribonuclease I-like 1
akxq28i0610741	BC040301	IMAGp998I0610741	PASD1	PAS domain containing 1
akxq28i1411448	BI824481	IMAGp998I1411448	AVPR2	arginine vasopressin receptor 2 (nephrogenic diabetes insipidus)
akxq28i14160	BC000255	IMAGp958I14160	FUNDC2	FUN14 domain containing 2
akxq28i19182	BC000738	IMAGp958I19182	EMD	emerin (Emery-Dreifuss muscular dystrophy)
akxq28i205	BC000308	IMAGp958I205	ARD1A	ARD1 homolog A, N-acetyltransferase (S. cerevisiae)
akxq28i231435	BC012114	IMAGp958I231435	IKBKG	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma
akxq28j056793	AW517432	IMAGp998J056793	NULL	NULL
akxq28j1311548	BC032351	IMAGp998J1311548	SSR4	signal sequence receptor, delta (translocon-associated protein delta)
akxq28j234	BC002392	IMAGp958J234	MPP1	membrane protein, palmitoylated 1, 55kDa
akxq28k05855	BC006170	IMAGp958K05855	IDS	iduronate 2-sulfatase (Hunter syndrome)
akxq28l16384	BC025269	IMAGp958L16384	TMLHE	trimethyllysine hydroxylase, epsilon
akxq28L23832	BC011612	IMAGp958L23832	MECP2	methyl CpG binding protein 2 (Rett syndrome)
akxq28m09186	BC007631	IMAGp958M09186	PNMA6A	paraneoplastic antigen like 6A
akxq28n1210739	BC025382	IMAGp998N1210739	TKTL1	transketolase-like 1
akxq28n167159	BC003358	IMAGp998N167159	RPL10	ribosomal protein L10
akxq28o048419	BC000933	IMAGp998O048419	IDH3G	isocitrate dehydrogenase 3 (NAD+) gamma
akxq28o05362	H40393	IMAGp998O05362	CXorf52	chromosome X open reading frame 52
akxq28o054208	AI038709	IMAGp998O054208	SPRY3	sprouty homolog 3 (Drosophila)

akxq28o22185	BC000724	IMAGp958O22185	ATP6AP1	ATPase, H+ transporting, lysosomal accessory protein 1
akxq28u66048	BG430930	IMAGp958J081364	NULL	NULL
hamy2_11n4	AL136544	DKFZp761N0411	SMC6L1	SMC6 structural maintenance of chromosomes 6-like 1 (yeast)
hamy2_16n19	NULL	DKFZp761N1916	NULL	NULL
hamy2_1f24	AL136562	DKFZp761F241	CCDC3	coiled-coil domain containing 3
hamy2_1h17	AL137502	DKFZp761H171	RRAGD	Ras-related GTP binding D
hamy2_24k15	AL136581	DKFZp761K1524	C14orf135	chromosome 14 open reading frame 135
hamy2_2h17	AL157429	DKFZp761H172	BANP	BTG3 associated nuclear protein
hamy2_7j19	AL583909	DKFZp761J197	KIAA1539	KIAA1539
hfbr1_10j21	AL834397	DKFZp547J2110	TRNT1	tRNA nucleotidyl transferase, CCA-adding, 1
hfbr1_15b14	AL834131	DKFZp547B1415	FLJ36888	hypothetical protein FLJ36888
hfbr1_15f6	AL834132	DKFZp547F0615	C10orf83	chromosome 10 open reading frame 83
hfbr1_3f21	AL512743	DKFZp547F213	SYT13	synaptotagmin XIII
hfbr1_4l13	AL162061	DKFZp547L134	NUP62	nucleoporin 62kDa
hfbr1_5c24	AL834449	DKFZp547C245	RTN4R	reticulon 4 receptor
hfbr1_6j22	AL834457	DKFZp547J226	SYNPR	synaptoporin
hfbr1_6l10	AL512715	DKFZp547L106	ARHGEF2	rho/rac guanine nucleotide exchange factor (GEF) 2
hfbr2_16p15	AL110128	DKFZp564P1516	PPT2	palmitoyl-protein thioesterase 2
hfbr2_2h1	AL136633	DKFZp564H012	MRPL18	mitochondrial ribosomal protein L18
hfbr2_2h10	AL136634	DKFZp564H102	SUHW4	suppressor of hairy wing homolog 4 (Drosophila)
hfbr2_3b16	AL050268	DKFZp564B163	RAB1A	RAB1A, member RAS oncogene family
hfbr2_3c10	AL050269	DKFZp564C103	NAT9	N-acetyltransferase 9
hfbr2_3m17	AL136644	DKFZp564M173	PHPT1	phosphohistidine phosphatase 1
hfbr2_62f10	AL713790	DKFZp564F1062	SLC30A8	solute carrier family 30 (zinc transporter), member 8
hfbr2_6i20	AL136665	DKFZp564I206	MRPL15	mitochondrial ribosomal protein L15
hfbr2_72b11	AL136669	DKFZp564B1172	ICF45	interphase cyctoplasmic foci protein 45
hfbr2_72b18	AL136670	DKFZp564B1872	POLI	polymerase (DNA directed) iota
hfbr2_72n12	AL136676	DKFZp564N1272	GABARAPL1	GABA(A) receptor-associated protein like 1
hfbr2_78c24	AL136680	DKFZp564C2478	GBP3	guanylate binding protein 3
hfbr2_78k24	AL136690	DKFZp564K2478	USP18	ubiquitin specific peptidase 18
hfbr2_7a24	AL713701	DKFZp564A247	C21orf7	chromosome 21 open reading frame 7
hfbr2_7b16	AL110297	DKFZp564B167	BRP44	brain protein 44
hfbr2_7c4	AL133600	DKFZp564C047	STAM2	signal transducing adaptor molecule (SH3 domain and ITAM motif) 2
hfbr2_7j4	AL136694	DKFZp564J047	C1orf49	chromosome 1 open reading frame 49
hfbr2_7k24	AL110233	DKFZp564K247	HIGD1A	HIG1 domain family, member 1A
hfkd2_24a15	AL136704	DKFZp566A1524	FAM49A	family with sequence similarity 49, member A
hfkd2_24e23	AL136708	DKFZp566E2324	C6orf59	chromosome 6 open reading frame 59
hfkd2_24n20	AL136709	DKFZp566N2024	ABI3	ABI gene family, member 3
hfkd2_3i13	AL136711	DKFZp566I133	VMP1	transmembrane protein 49
hfkd2_46k19	AL136721	DKFZp566K1946	PCBD2	pterin-4 alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha

				(TCF1) 2
hfkd2_46m10	AL136723	DKFZp566M1046	C11orf56	chromosome 11 open reading frame 56
hfkd2_4j16	AL117566	DKFZp566J164	UBE1C	ubiquitin-activating enzyme E1C (UBA3 homolog, yeast)
hlcc1_18i9	AL832636	DKFZp451I0918	TXLNA	taxilin alpha
hlcc1_1m20	NULL	DKFZp451M201	NULL	NULL
hlcc1_3e22	AL831980	DKFZp451E223	NRAP	nebulin-related anchoring protein
hlcc2_1m1	NULL	DKFZp313M011	NULL	NULL
hlcc3_116l20	BX538218	DKFZp686L20116	RP11-308B5	similar to hypothetical protein MGC17347
hlcc3_148h13	NULL	DKFZp686H13148	NULL	NULL
hlcc3_159g12r1s1	NULL	NULL	NULL	NULL
hlcc3_171f5	BX647330	DKFZp686F05171	IFI27	interferon, alpha-inducible protein 27
hlcc3_223m12	NULL	DKFZp686M12223	NULL	NULL
hlcc3_261i1r1s1	NULL	NULL	NULL	NULL
hlcc3_51c2	NULL	DKFZp686C0251	NULL	NULL
hlcc3_52b8	BX537406	DKFZp686B0852	TRIAD3	TRIAD3 protein
hlcc3_52f6	BX537408	DKFZp686F0652	RAB5B	RAB5B, member RAS oncogene family
hlcc3_52k13	BX537967	DKFZp686K1352	TCF12	transcription factor 12 (HTF4, helix-loop-helix transcription factors 4)
hlcc3_59e24	BX537969	DKFZp686E2459	RBM10	RNA binding motif protein 10
hlcc3_59o14	BX537412	DKFZp686O1459	DYNC1I2	dynein, cytoplasmic 1, intermediate chain 2
hlcc3_59p19	BX537413	DKFZp686P1959	STRA6	stimulated by retinoic acid gene 6 homolog (mouse)
hlcc3_74g21	NULL	DKFZp686G2174	NULL	NULL
hlcc3_82o22	BX537382	DKFZp686O2282	SLC38A3	solute carrier family 38, member 3
hlcc3_86a15	BX537971	DKFZp686A1586	LOC493856	similar to RIKEN cDNA 1500009M05 gene
hlcc3_86f23	BX537972	DKFZp686F2386	LYSMD3	LysM, putative peptidoglycan-binding, domain containing 3
hlcc3_98b23	NULL	DKFZp686B2398	NULL	NULL
hlcc4_5d1r1s1	NULL	NULL	NULL	NULL
hmel2_11h17	AL359618	DKFZp762H1711	CHCHD8	coiled-coil-helix-coiled-coil-helix domain containing 8
hmel2_12e11	AL162047	DKFZp762E1112	NCOA4	nuclear receptor coactivator 4
hmel2_12j1	AL713689	DKFZp762J0112	KIAA1524	KIAA1524
hncc1_42m18	BX648228	NULL	MIA2	melanoma inhibitory activity 2
htes3_10n10	AL136733	DKFZp434N1010	UBAP1	ubiquitin associated protein 1
htes3_10n4	AL136734	DKFZp434N0410	RAB18	RAB18, member RAS oncogene family
htes3_13c10	AL136747	DKFZp434C1013	CSTF2T	cleavage stimulation factor, 3' pre-RNA, subunit 2, 64kDa, tau variant
htes3_14g5	AL136750	DKFZp434G0514	LYAR	hypothetical protein FLJ20425
htes3_15c5	AL133046	DKFZp434C0515	SSX2IP	synovial sarcoma, X breakpoint 2 interacting protein
htes3_15h1	AL136760	DKFZp434H0115	TTC25	tetratricopeptide repeat domain 25
htes3_15i5	AL136761	DKFZp434I0515	Rshl1	Radial spokehead-like 1
htes3_17b4	AL133067	DKFZp434B0417	PDZRN4	PDZ domain containing RING finger 4
htes3_17i21	AL136777	DKFZp434I2117	FAM57B	family with sequence similarity 57, member B

htes3_17j6	AL136778	DKFZp434J0617	RKHD3	ring finger and KH domain containing 3
htes3_17n18	AL136781	DKFZp434N1817	C3orf20	chromosome 3 open reading frame 20
htes3_19a13	AL136786	DKFZp434A1319	C16orf48	chromosome 16 open reading frame 48
htes3_1c15	AL080168	DKFZp434C151	ATG4B	ATG4 autophagy related 4 homolog B (S. cerevisiae)
htes3_1k11	AL136796	DKFZp434K111	KLHL25	kelch-like 25 (Drosophila)
htes3_1k15	AL080177	DKFZp434K151	UBL3	ubiquitin-like 3
htes3_1l15	AL117487	DKFZp434L151	TADA3L	transcriptional adaptor 3 (NGG1 homolog, yeast)-like
htes3_20k2	AL136801	DKFZp434K0220	TRPV1	transient receptor potential cation channel, subfamily V, member 1
htes3_22n13	AL713710	DKFZp434N1322	MKL1	megakaryoblastic leukemia (translocation) 1
htes3_23a19	AL136813	DKFZp434A1923	CPSF3L	cleavage and polyadenylation specific factor 3-like
htes3_26g22	AL136819	DKFZp434G2226	KIF18A	kinesin family member 18A
htes3_29f24	AL136832	DKFZp434F2429	ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3
htes3_2d11	AL080197	DKFZp434D112	LOC51233	hypothetical protein LOC51233
htes3_2d15	AL136835	DKFZp434D152	TOLLIP	toll interacting protein
htes3_2i17	AL834386	DKFZp434I172	C3orf20	chromosome 3 open reading frame 20
htes3_30a5	AL136842	DKFZp434A0530	CDC42EP3	CDC42 effector protein (Rho GTPase binding) 3
htes3_35a11	AL122068	DKFZp434A1135	RAD17	RAD17 homolog (S. pombe)
htes3_35g6	AL136853	DKFZp434G0635	BTBD1	BTB (POZ) domain containing 1
htes3_35n12	AL136857	DKFZp434N1235	SLC25A31	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 31
htes3_35n9	AL713761	DKFZp434N0935	CES2	carboxylesterase 2 (intestine, liver)
htes3_35p17	AL136859	DKFZp434P1735	ARMC4	armadillo repeat containing 4
htes3_3a7	AL080126	DKFZp434A073	KIAA0683	KIAA0683 gene product
htes3_4d6	AL110225	DKFZp434D064	DBN1	drebrin 1
htes3_4f5	AL136863	DKFZp434F054	WDR24	WD repeat domain 24
htes3_4h6	AL136864	DKFZp434H064	KLC2	kinesin light chain 2
htes3_4i11	AL080154	DKFZp434I114	C20orf28	chromosome 20 open reading frame 28
htes3_50n23	AL713673	DKFZp434N2350	C12orf25	chromosome 12 open reading frame 25
htes3_5c13	AL136874	DKFZp434C135	RNF32	ring finger protein 32
htes3_7j3	AL136891	DKFZp434J037	NUAK2	NUAK family, SNF1-like kinase, 2
htes3_7j8	AL136892	DKFZp434J087	FLJ20323	hypothetical protein FLJ20323
htes3_8g5	AL136899	DKFZp434G058	FBXO21	F-box protein 21
hute1_17k7	AL136910	DKFZp586K0717	FIP1L1	FIP1 like 1 (S. cerevisiae)
hute1_18i19	AL136911	DKFZp586I1918	LIMA1	LIM domain and actin binding 1
hute1_18i4	AL136912	DKFZp586I0418	ATG10	ATG10 autophagy related 10 homolog (S. cerevisiae)
hute1_19k9	AL050283	DKFZp586K0919	SENP3	SUMO1/sentrin/SMT3 specific peptidase 3
hute1_21p24	AL110267	DKFZp586P2421	OGN	osteoglycin (osteoinductive factor, mimecan)
hute1_23e13	AL136936	DKFZp586E1323	HSPB8	heat shock 22kDa protein 8
hute1_23h7	AL080212	DKFZp586H0723	RAP1B	RAP1B, member of RAS oncogene family
hute1_24b4	AL136940	DKFZp586B0424	HSPC049	HSPC049 protein

hute1_24j6	AL136944	DKFZp586J0624	SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1
IMAGp958C20216	BC004352	IMAGp958C20216	KIF22	kinesin family member 22
IMAGp958D0156	BC015149	IMAGp958D0156	MAP4	microtubule-associated protein 4
IMAGp958D231833	BC042168	IMAGp958D231833	CTSZ	cathepsin Z
IMAGp958E161161	BC011656	IMAGp958E161161	CDC27	cell division cycle 27
IMAGp958G021430	BC018739	IMAGp958G021430	BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)
IMAGp958I1955	BC008718	IMAGp958I1955	BIRC5	baculoviral IAP repeat-containing 5 (survivin)
IMAGp958O04299	BC018634	IMAGp958O04299	MAP1LC3B	microtubule-associated protein 1 light chain 3 beta
IMAGp958O182624	BC062691	IMAGp958O182624	HSPC159	HSPC159 protein
IMAGp998A1112724	BC036801	IMAGp998A1112724	LOC387856	similar to expressed sequence AI836003
IMAGp998D0110102	BC017713	IMAGp998D0110102	CDC23	CDC23 (cell division cycle 23, yeast, homolog)
IMAGp998d2012760	BM554036	IMAGp998D2012760	NUP62	nucleoporin 62kDa
IMAGp998E1010430	BC013757	IMAGp998E1010430	LRRC58	leucine rich repeat containing 58
IMAGp998H159957	BC021901	IMAGp998H159957	RAB21	RAB21, member RAS oncogene family
IMAGp998J0312216	BC035925	IMAGp998J0312216	C1orf31	chromosome 1 open reading frame 31
IMAGp998K0111687	BC057767	IMAGp998K0111687	C20orf132	chromosome 20 open reading frame 132
IRALp962A2027	BC010090	IRALp962A2027	ACTR1B	ARP1 actin-related protein 1 homolog B, centractin beta (yeast)
IRALp962G143	BC007318	IRALp962G143	MAPRE2	microtubule-associated protein, RP/EB family, member 2
IRATp970A0419	BC015492	IRATp970A0419	IFI27	interferon, alpha-inducible protein 27
IRATp970A0916	BC018190	IRATp970A0916	MT1X	metallothionein 1X
IRATp970B025	BC000870	IRATp970B025	FLJ20516	timeless-interacting protein
IRATp970C094	BC006794	IRATp970C094	IFITM3	interferon induced transmembrane protein 3 (1-8U)
IRATp970D076	BC008640	IRATp970D076	ICA1	islet cell autoantigen 1, 69kDa
IRATp970E119	BC009685	IRATp970E119	CAV1	caveolin 1, caveolae protein, 22kDa
IRATp970E1212	BC015044	IRATp970E1212	MOAP1	modulator of apoptosis 1
IRATp970E129	BC009698	IRATp970E129	APOC1	apolipoprotein C-I
IRATp970F0215	BC012841	IRATp970F0215	XBP1	X-box binding protein 1
IRAUp969A115	BC001105	IRAUp969A115	DBNDD2	dysbindin (dystrobrevin binding protein 1) domain containing 2
IRAUp969B0615	BC000785	IRAUp969B0615	SCAND1	SCAN domain containing 1
IRAUp969B0732	BC006245	IRAUp969B0732	FGF18	fibroblast growth factor 18
IRAUp969B1123	BC003513	IRAUp969B1123	CXCL14	chemokine (C-X-C motif) ligand 14
IRAUp969B1159	BC009830	IRAUp969B1159	RDH14	retinol dehydrogenase 14 (all-trans/9-cis/11-cis)
IRAUp969C0366	BC011551	IRAUp969C0366	ZC3HC1	zinc finger, C3HC-type containing 1
IRAUp969C1021	BC002778	IRAUp969C1021	MYLC2PL	myosin light chain 2, precursor lymphocyte-specific
IRAUp969C1049	BC013401	IRAUp969C1049	PHB	prohibitin
IRAUp969D0269	BC014310	IRAUp969D0269	RPL10L	ribosomal protein L10-like
IRAUp969D0336	BC007936	IRAUp969D0336	GAP43	growth associated protein 43
IRAUp969E0545	BC005916	IRAUp969E0545	PTN	pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)

IRAUp969F0711	BC000198	IRAUp969F0711	SF3B5	splicing factor 3b, subunit 5, 10kDa
IRAUp969G0266	BC012340	IRAUp969G0266	C12orf5	chromosome 12 open reading frame 5
IRAUp969G0523	BC004309	IRAUp969G0523	RAB4A	RAB4A, member RAS oncogene family
IRAUp969G0648	BC009471	IRAUp969G0648	AMACR	alpha-methylacyl-CoA racemase
IRAUp969G065	BC001693	IRAUp969G065	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1)
IRAUp969G086	BC002503	IRAUp969G086	SAT	spermidine/spermine N1-acetyltransferase
IRAUp969G0861	BC011757	IRAUp969G0861	GADD45A	growth arrest and DNA-damage-inducible, alpha
IRAUp969G0939	BC008361	IRAUp969G0939	FBXO7	F-box protein 7
IRAUp969H0369	BC019230	IRAUp969H0369	AKR1C3	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)
IRAUp969H1045	BC005919	IRAUp969H1045	PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)
IRAUp969H1218	BC004993	IRAUp969H1218	ANXA5	annexin A5

9 Own publications

Lux A, Beil C, **Majety M**, Barron S, Gallione CJ, Kuhn HM, Berg JN, Kioschis P, Marchuk DA, Hafner M. Human retroviral gag- and gag-pol-like proteins interact with the transforming growth factor-beta receptor activin receptor-like kinase 1. J Biol Chem. 2005 Mar4;280(9): 8482-93. Epub 2004 Dec 16.

Arlt D, Huber W, Liebel U, Schmidt C, **Majety M**, Sauermann M, Rosenfelder H, Bechtel S, Mehrle A, Bannasch D, Schupp I, Seiler M, Simpson JC, Hahne F, Moosmayer P, Ruschhaupt M, Guilleaume B, Wellenreuther R, Pepperkok R, Sultmann H, Poustka A, Wiemann S. Functional profiling: from microarrays via cell-based assays to novel tumor relevant modulators of the cell cycle. Cancer Res. 2005 Sep 1;65(17):7733-42.

Gandhari, M, Arens N, **Majety M**, Dorn-Beineke A, Hildenbrand R. Urokinase-type plasminogen activator induces proliferation in breast cancer cells. Int J Oncol. 2006 Jun;28(6):1463-70.

Sauermann, M., Sahin, Ö., Sültmann, H., Hahne, F., Blaskiewicz, S., **Majety, M.,** Füzesi, L., Poustka, A., Wiemann, S., Arlt, D. (2006). Vacuole membrane protein -1 (VMP-1) is a cell-cell contact protein and acts as a metastasis suppressor. **Submitted**

Hahne, F., Arlt, D., Sauermann, M., **Majety, M**., Poustka, A., Wiemann, S., Huber, W. (2006) Analysis of high throughput flow cytometry data. **Submitted**

Majety, M., Guilleaume, B., Sauermann, M., Poustka, A., Wiemann, S., Arlt, D. (2006). Radial spoke head like -1 induces cell cycle arrest upon over-expression. **In preparation**

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