Role of PKCε in RNA granule formation and protein translation

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Die Wahrheit ist eine unzerstörbare Pflanze. Man kann sie ruhig unter einen Felsen vergraben, sie stößt trotzdem durch, wenn es an der Zeit ist.

Frank Thiess

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1 Summary

Protein kinase C epsilon (PKCε) is an intracellular signaling protein activated in conditions associated with strong cellular stress such as ischemia, cancer, and inflammation. PKCε is activated within seconds and mediates rapid short-term effects but also effects lasting for hours and weeks. How acute activation transforms to long-lasting effects cannot be explained on the basis of the currently known PKCε substrates. In a PKCε substrate candidate screen, we identified several new PKCε substrates, which are known to be involved in protein translation, a process PKCε has so far not been associated with. The modulation of translation may represent a mechanism underlying the transition from acute to long-lasting effects. Thus, the aim of this work was to investigate if PKCε has a yet unrecognized role in stress responses and protein translation.

I focused on the role of PKCε in two subcellular structures, stress granules and P-bodies. Both cytoplasmic structures are vital for the modulation of protein translation in response to stress. I found that PKCε and its adaptor proteins RACK1 and RACK2 are recruited to stress granules and P-bodies in F-11, HeLa, and HEK293T cells. To investigate the function of PKCε for the formation of these RNA granules, I performed siRNA knockdown studies and blocked the activity of PKCε with pharmacological inhibitors. For data acquisition an automated “High Content Screening” microscopy approach was established, which allowed the quantification of granule formation at the single cell level. PKCε down-regulation as well as inhibition increased the number of stress granules. PKCε down-regulation also induced P-body formation, but acute PKC inhibition did not alter P-body formation. The increase of RNA-granules suggests a decrease of free and translatable mRNAs. Accordingly, I found decreased protein translation upon PKCε knockdown using a fluorescence-based method to visualize newly translated proteins (FUNCAT).

Furthermore, I found evidence that the recruitment of signaling molecules to translation modifying granules appears to be a more general phenomenon. In addition to PKCε, a member of the family of “novel PKCs”, I found classical PKCs to be involved in granule formation as well. In addition to PKCs, I also observed the RII alpha subunit of protein kinase A (PKA) to be recruited to stress granules and P-bodies in response to cell stress.

Thus, my data reveal a novel function of PKCε for the formation of RNA granules, which results in altered protein translation. My data therefore open the way to investigate, to what extend PKCε and PKA recruitment to cytoplasmic RNA granules serves a similar function for stress responses, as well as to what extend translation regulation can explain the transition from acute activation to long-lasting effects.

2 Zusammenfassung

Die Proteinkinase C epsilon (PKCε) ist ein intrazelluläres Signalprotein, welches unter starkem zellulärem Stress wie z.B. Ischemie, Krebs oder auch bei Entzündungsprozessen aktiviert wird. Die Aktivierung von PKCε geschieht innerhalb weniger Sekunden und vermittelt neben kurzfristigen Effekten auch Effekte, die Stunden oder gar Wochen anhalten. Wie genau es zu dem Übergang von akuter Aktivierung zu langanhaltenden Effekten kommt, lässt sich basierend auf den bisher bekannten PKCε-Substraten nicht erklären. In einer groß angelegten Untersuchung zur Identifizierung von PKCε-Substratkandidaten, die in unserem Labor durchgeführt wurde, fanden wir einen großen Anteil an Proteinen, die in die Proteintranslation involviert sind. Jedoch wurde PKCε im Prozess der Proteintranslation bisher nicht beschrieben. Die Modulierung der Proteinexpression könnte ein Mechanismus sein, der den Übergang von akuten zu lang anhaltenden Effekten erklärt. Daher war das Ziel dieser Arbeit, zu untersuchen, ob PKCε eine bisher unerkannte Funktion innerhalb der Stressantwort und der Proteintranslation erfüllt.

Ich konzentrierte mich auf die Rolle der PKCε in zwei subzellulären Strukturen, den sogenannten *Stress granules* und *P-bodies*. Beide zytoplasmatische Strukturen sind wesentlich an der Modulierung der Proteintranslation als Stressantwort beteiligt. Ich fand heraus, dass in F-11-, HeLa- und HEK293T-Zellen sowohl PKCε als auch ihre Adapterproteine RACK1 und RACK2 zu *Stress granules* und *P-bodies* rekrutiert werden. Um die Funktion der PKCε in diesen RNA-Strukturen zu untersuchen, reduzierte ich die zelluläre Verfügbarkeit der PKCε mittels siRNA basierter Herunterregulierung oder pharmakologischer Inhibierung. Zur Datengewinnung wurde ein automatisches „*High Content Screening*“-Mikroskopie-Konzept etabliert, das die Quantifizierung der *granules* auf Einzelzellniveau ermöglichte. Sowohl die Herunterregulierung der PKCε, als auch ihre Inhibierung erhöhte die Zahl der *Stress granules*. PKCε-Herunterregulierung erhöhte ebenfalls die Zahl der *P-bodies*, jedoch beeinflusste zumindest die akute PKC-Aktivierung die Bildung der *P-bodies* nicht. Die Erhöhung der RNA-*granules* suggeriert einen Abfall freier und translatierbarer mRNA. Dementsprechend fand ich, mit Hilfe der Durchführung einer fluoreszenzbasierten Methode zur Visualisierung neu translatierter Proteine (FUNCAT), eine Abnahme neu synthetisierter Proteine nach PKCε-Herunterregulierung.

Des Weiteren fand ich Belege dafür, dass die Rekrutierung zu translationsmodifizierenden *granules* ein generelles Phänomen zu sein scheint. Ich konnte zeigen, dass neben PKCε, einem Mitglied der „neuen“ Proteinkinase C-Familie, auch klassische PKCs bei der Entstehung der *granules* eine Rolle spielen. Über PKCs hinausgehend fand ich, dass

auch die RII alpha Untereinheit der Proteinkinase A (PKA) in Folge von Stress in *Stress granules* und *P-bodies* lokalisiert ist.

Somit decken meine Daten eine neue Funktion der PKCε innerhalb der Bildung von RNA-*granules* auf, die in einer veränderten Proteintranslation resultieren. Meine Daten eröffnen damit die Möglichkeit zu untersuchen, in welchem Maß die Rekrutierung von PKCε und PKA zu zytoplasmatischen RNA *granules* ähnliche Funktionen innerhalb der Stressantworten ausführt und in welchem Maß Translationsregulation den Übergang von akuter Aktivierung zu langanhaltenden Effekten zu erklären vermag.

3 Introduction

Protein kinase C epsilon (PKCε) is involved in a variety of cellular processes such as neurotransmitter release, ion channel regulation, mitochondrial function, alcoholism, pain, ischemia, and stroke (Prekeris, Mayhew et al. 1996, Brodie, Bogi et al. 1999, Cross, Murphy et al. 2002, Nowak, Bakajsova et al. 2004, Chou and Messing 2005, Hucho, Dina et al. 2006, Akita, Kawasaki et al. 2007, Das, Pany et al. 2009). Many of them contain aspects of cellular stress responses. Extracellular stimuli result in cellular stress response that can be very fast at the level of signal transduction (Kyriakis and Avruch 1996, Ping, Zhang et al. 1997, Cesare, Dekker et al. 1999, Kyriakis and Avruch 2001). On the other hand also slower changes mediated by the regulation of e.g. protein translation can contribute to the reaction against stress (Williams 1999, Holcik and Sonenberg 2005, Proud 2005). PKCε has been observed to mediate early responses but also long-lasting effects (Hucho, Dina et al. 2006, Barnett, Madgwick et al. 2007). If PKCε connects the fast changes of early signaling with the subsequent changes in translation is not known.

In the following, I will introduce PKC, in particular PKCε, the regulation of its function and its role in stressful situations. To facilitate the understanding of a functional role of PKCε in stress response, I will introduce common cell reactions on stress. For evaluating which tasks PKCε may have in stress response I will introduce some of its substrates and binding partners. And last but not least, as many of the substrates are involved in protein translation, I will introduce in the last part translation regulating structures, RNA granules.

3.1 Protein kinase C family

PKCε is a member of the protein kinase C (PKC) family, which is part of the ABC superfamily of protein kinases. This superfamily includes in addition to PKCs also the PKA and PKB (also known as Akt) family of protein kinases (Barnett, Madgwick et al. 2007). These kinase families share a common structural architecture. They are composed of an N-terminal regulatory region and a C-terminal kinase domain (Newton 2003). The N-terminal regulatory domain consists of a pseudo-substrate region. This autoinhibitory sequence maintains the kinase in an inactive state by sterically blocking the catalytic domain. In the catalytic domain the substrate binding site and the ATP binding domain is located (Figure 1, modified from (Newton and Johnson 1998, Barnett, Madgwick et al. 2007)). Prior to the activation of PKC, the enzyme is shifted to a primed state via phosphorylation at the activation loop by PDK1 and mTORC2 conferring optimal catalytic potential (Rosse, Linch et al. 2010). Subsequently, the binding of second messengers such as diacylglycerol, phospholipids or Ca^{2+} is essential for allosteric activation as they induce the pseudosubstrate's release from the catalytic domain (Creba, Downes et al.

1983, Newton and Johnson 1998). Upon activation, an additional mechanism regulating the intrinsic function of PKC is the translocation to and the interaction with targeting proteins such as receptors for activated C kinases (RACKs) that position the enzyme close to its regulators and substrates. Many PKCs are recruited from the cytosol to a membrane such as the plasma membrane or the Golgi (Newton 1995, Newton 2001, Takahashi, Yamagiwa et al. 2002). Moreover, translocation of activated PKCs to other intracellular sites such as cytoskeleton and nucleus has been reported (Mochly-Rosen, Khaner et al. 1991).

The PKC family consists of ten related serine-threonine kinases, divided into three groups. PKCα, β and γ are “classical” PKCs, whereas PKCε, δ, η, and θ belong to the “novel” PKC isoforms. Finally, PKCλ/ι and ζ are members of the “atypical” PKCs (Knudsen 2011).

3.1.1 Structural and functional comparison of PKC isoform groups

Classical PKCs (α, βI, βII, and γ) are activated by Ca^{2+} and diacylglycerol (DAG) in the presence of phosphatidylserine (Newton and Johnson 1998). They contain cysteine rich C1 domains that act as DAG sensors and C2 domains which bind to Ca^{2+} (Barnett, Madgwick et al. 2007)., **Error! Reference source not found.**, modified from (Barnett, Madgwick et al. 2007)). In contrast, novel PKCs (δ, ε, θ, and η) have an inverted structure of C1 and C2 domains and their activation is not dependent on calcium signals. Instead, they solely respond to DAG and phosphatidic acid. Atypical PKCs (λ/ι and ζ) lack C2 domains, typical C1 domains and a conserved Ser/Thr residue in the catalytic domain, which provides an important phosphorylation site in classical and novel PKCs (Barnett, Madgwick et al. 2007). Thus, they neither require calcium nor DAG for maximal activity (Jaken 1996). Instead, they have been suggested to be activated by other lipids such as phosphatidylinositols, arachidonic acid, and phosphatidic acid (Nakanishi, Brewer et al. 1993, Muller, Ayoub et al. 1995, Jaken 1996).

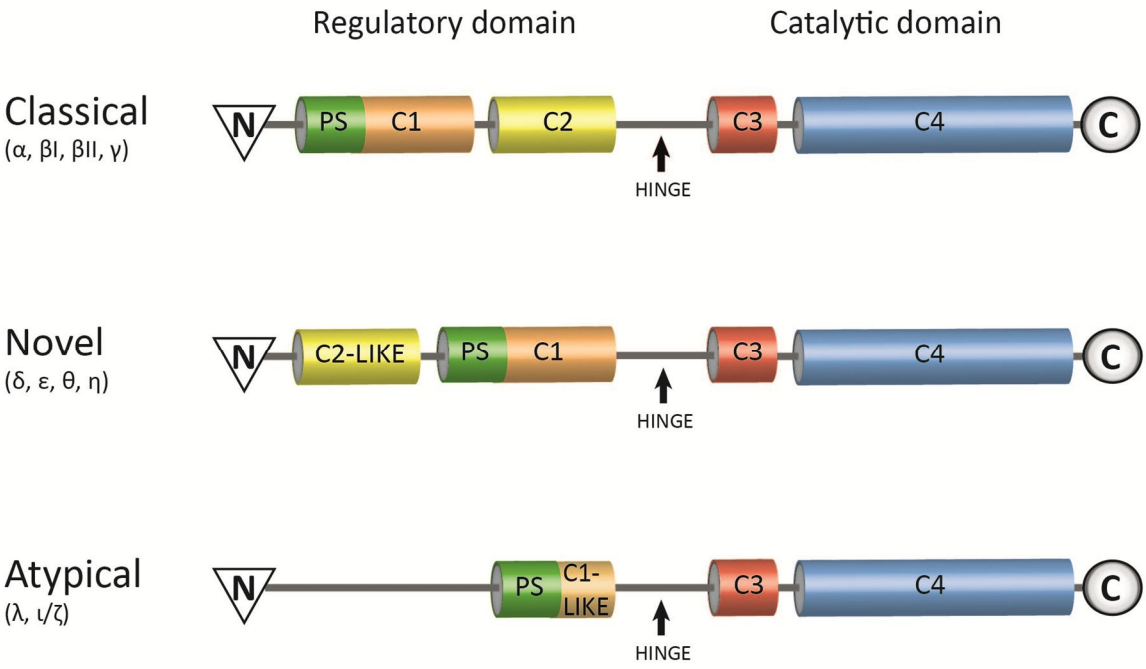

Figure 1: Domain architecture of classical (α, βI, βII, γ), novel (δ, ε, θ, η) and atypical (λ, ι/ζ) PKCs. All PKCs are composed of an N-terminal regulatory domain with an inhibitory pseudo-substrate region (PS, green) followed by a C1 region (C1-like in atypical PKCs, orange) and the hinge motif. The C1 region is composed of C1A and C1B. The C3 (red) and C4 (blue) region form the catalytic domain at the C-terminus, where the ATP- and substrate binding sites are located. While classical PKCs carry a C2 region (yellow) between C1 and the hinge motif, a C2-like (yellow) region is placed before the pseudo-substrate region in novel PKCs. Atypical PKCs lack a C2 or C2-like region (modified from Barnett, Madgwick et al. 2007).

Depending on the structure (Figure 1) and subcellular localization of each isoform, PKCs interact with a variety of targets in numerous cellular compartments such as plasma membrane, Golgi, cytoskeleton, mitochondria, and cell nuclei (Li, Lorenzo et al. 1999, Zeidman, Troller et al. 2002, Eitel, Staiger et al. 2003, Schultz, Ling et al. 2004, Spitaler and Cantrell 2004). Accordingly, PKCs are involved in numerous cellular processes such as differentiation, cell growth, apoptosis, gene expression, muscle contraction, metabolism, and endocytosis (Jaken and Parker 2000, Koivunen, Aaltonen et al. 2006).

PKCα and PKCβ are mostly expressed in bladder, muscle, heart, enteric glial, liver, pancreas, and brain and are implicated in proliferation, apoptosis, differentiation, motility, inflammation, cell growth, and metabolism (PKCβ) (Kawakami, Kawakami et al. 2002, Nakashima 2002, Van Kolen, Pullan et al. 2008). PKCγ is selectively expressed in the nervous system (Saito and Shirai 2002). Novel PKCs exhibit expression patterns and tasks similar to classical PKCs and have a functional role among others in the nervous, endocrine, exocrine, and immune system (Akita 2002, Kashiwagi, Ohba et al. 2002, Kikkawa, Matsuzaki et al. 2002, Martin and Hussaini 2005, Hayashi and Altman 2007, Van Kolen, Pullan et al. 2008).

PKCε, the kinase of interest for this work, is expressed in brain, heart, enteric, as well as pancreas and regulates the activation of nervous, endocrine, exocrine, inflammatory, and immune systems. Although PKCδ and PKCε are descended from the same family, they have sometimes opposing effects. While PKCε stimulates cell growth and differentiation, PKCδ promotes apoptosis (Duquesnes, Lezoualc'h et al. 2011). Atypical PKCs are involved in cell differentiation, motility, adhesion, and embryogenesis. They also function as NFκB activator in immune response and play a role in signaling, cell growth, protein synthesis, and gene regulation (Farese and Sajan 2010).

3.1.2 The function of PKC in stress response

Stressful stimuli initiate cellular defense mechanisms or apoptosis. The cellular response depends on the respective stressor and involves many signaling proteins. The result of cellular defense mechanisms is the maintenance or re-establishment of cellular homeostasis. Well conserved stress responses are present in all cell types of metazoans (Simmons, Fan et al. 2009). One component of cellular stress response is the activation of PKCs. PKC activation was observed after exposing the cells to stressing stimuli such as cytotoxic chemicals, physical stretch, osmotic stress, hypoxia, and inflammatory cytokines (Jiang, Zhang et al. 2011). In the following, I will give some examples of PKC involvement during hypoxia, heat, oxidative, and ischemic stress.

Especially hypoxia induced activation has been investigated in detail. The selective activation of PKC isoforms (PKCα and ε) is mediated by a pathway involving phospholipase C and intracellular calcium (Goldberg, Zhang et al. 1997). In turn, the activation of classical and novel PKC isoenzymes modulates the heat-induced stress response (Holmberg, Roos et al. 1998). Activation of PKC by 12-O-tetradecanoylphorbol 13-acetate (TPA) increased the response to heat and it was suggested that the activity of the heat shock transcription factor 1 is modulated by PKC-responsive pathways. Oxidative stress by arsenite was observed to activate calcium-dependent PKCs as a consequence of perturbed intracellular calcium homeostasis. Thus, activated PKCs could play an important role in arsenite-induced genotoxicity (Liu and Huang 1997). During ischemic stress PKCγ and ε play a key role in the control of mitochondria and gap junctions.

3.2 Protein kinase C epsilon

PKCε plays a special role in stress response. Based on structure, function, and known substrates, I will delineate a potential new function of PKCε, which I will then test experimentally in the course of this thesis.

3.2.1 Activation of PKCε

Before PKCε can be bound and activated, it needs to be phosphorylated at three conserved sites: Thr-566 in the activation loop, Ser-729 in the C-terminal hydrophobic site, and Thr-710, an autophosphorylation site (Cenni, Doppler et al. 2002, Barnett, Madgwick et al. 2007). At that time, its pseudosubstrate region inhibits PKCε by contacting the substrate binding site until activation by second messengers. Several second messengers like DAG, phosphatidylinositol 3,4,5-trisphosphate, and fatty acids shift PKCε to an active conformation by binding to the C1 domain. Dependent on the bound second messenger, PKCε translocates to the plasma membrane, cytoskeleton (DAG, tridecanoic acids), to Golgi-networks (arachidonic and linoleic acids), and other intracellular structures. Upon activation, by subcellular diffusion processes, it can encounter scaffold and adaptor proteins such as receptors for activated C kinases. Thus, substrate specificity among all PKC isoenzymes is achieved through the spatial- and temporal-targeting of PKCε to subcellular compartments (Barnett, Madgwick et al. 2007). As RACKs turned out to be interesting for this study, I will introduce these PKCε adaptor proteins in the following part.

3.2.2 The PKCε anchoring proteins RACK1 and RACK2

PKC isozymes mediate different and isozyme-specific functions. However, there is considerable structural conservation and overlap of substrates between PKC isozymes. One mechanism to assure isoform-specific functionality is the interaction of PKC isoforms to distinct docking proteins, which are located at different subcellular domains (Mochly-Rosen and Gordon 1998, Barnett, Madgwick et al. 2007). Anchoring proteins for activated PKCs were termed “receptors for activated C kinases” (RACKs). They have molecular weights between 28-33 kDa and are homologs of the β subunit of G-proteins (Mochly-Rosen, Khaner et al. 1991, Ron, Chen et al. 1994). RACKs neither function as substrate nor as inhibitor of PKC (Park, Wu et al. 2004). Instead, their function is to bring PKCs in close contact to their substrates and away from others, to stabilize the active form of PKCs and thus to increase PKC substrate phosphorylation (Mochly-Rosen 1995, Jaken and Parker 2000). It is likely that each PKC isoform has its specific RACK, although this assumption has not been confirmed for all isoforms, yet (Park, Wu et al. 2004). For the epsilon isoform of PKC two RACKs have been referred to as anchoring proteins

(Schechtman and Mochly-Rosen 2001): RACK1, which binds rather specifically with βIIPKC and interacts only with lower affinity also with PKCε (Ron, Chen et al. 1994), and RACK2 (also called β'Cop), which is a PKCε selective RACK (Csukai, Chen et al. 1997). The anchoring of PKCε to its adaptor protein occurs via the C2-like domain of PKCε and is mediated by the WD40 motif of RACKs, which is a domain involved in protein-protein interaction (Csukai, Chen et al. 1997, Akita 2002). RACKS contain seven repeats of the WD40 motif and were therefore expected to function also as adaptor proteins for several other signaling molecules apart from PKCs. Indeed, Phospholipase C γ, dynamin-1, Src tyrosine kinase, and viral proteins for example bind to RACK1 while the small ras-like GTPase, ARF, binds to RACK2 (Schechtman and Mochly-Rosen 2001). Accordingly, RACKs are involved in various cell biological processes such as vesicle recycling, apoptosis, antiviral response, and cancer by organizing signaling enzymes near to their substrates and optimizing response to normal and potentially dangerous stimuli.

3.2.3 PKCε in stressful situations

PKCε is widely expressed with especially strong expression in neurons. There, it has been found to be involved in functions such as neurotransmitter release, ion channel regulation, neurite outgrowth, and presynaptic regulation (Dumuis, Sebben et al. 1988, Akita, Ohno et al. 1994, Prekeris, Mayhew et al. , Brodie, Bogi et al. 1999, Chen, Cantrell et al. 2005, Shirai, Adachi et al. 2008). Moreover, PKCε has been reported to play a role in many signaling systems including proliferation, differentiation, gene expression, muscle contraction, apoptosis, metabolism, transport, adhesion, and motility (DeCoy, Snapper et al. 1995, Jobbagy, Olah et al. 1999, Balciunaite, Jones et al. 2000, Racke, Wang et al. 2001, Akita 2002, Ali and Sarna 2002, Mehta, Radominska-Pandya et al. 2002, Yonekawa and Akita 2008). In particular, PKCε has been also described to be central for the response to strong cellular stressors. This among others involves responses to ischemia, ethanol (Choi, Wang et al. 2002), cancer development (Cacace, Ueffing et al. 1998), and viral infections (Pfeffer, Eisenkraft et al. 1991). Both early responses and long-lasting effects have been shown to be mediated by PKCε (Hucho, Dina et al. 2006, Barnett, Madgwick et al. 2007). In the following I will give specific examples of PKCε function in some selected stressful situations.

3.2.3.1 PKCε in pain and sensitization

In the peripheral nervous system, PKCε is expressed in so-called nociceptive neurons. These neurons are specialized in detecting acute noxious stimuli. Noxious stimuli can be of chemical, thermal, or mechanical nature and are often tissue damaging (Loeser and Treede 2008). Thus, they are strong stressors. Once detected by the nociceptive neuron, the information about these stressors is forwarded to the spinal cord and eventually to the

brain where a painful feeling is created. Nociceptive neurons alter their functionality through exposure to extracellular mediators. Inflammation and nerve damage but also for example high ethanol consumption and diabetes result in sensitization of these neurons (Tominaga, Wada et al. 2001). Many of these sensitizing responses have been shown to be dependent on the activation of the PKCε-signaling cascade (Kellerer, Mushack et al. 1997, Cesare, Dekker et al. 1999, Hodge, Mehmert et al. 1999, Olive, Mehmert et al. 2000, Sweitzer, Wong et al. 2004). For example, PKCε was shown to play a key role in the transition from acute to chronic pain: Hyperalgesic responses to inflammatory mediators such as carrageenan were prolonged to three weeks and more by PKCε activation (Aley, Messing et al. 2000). Additionally, PKCε also contributes to acute epinephrine-induced hyperalgesia by enhancing tetrodotoxin-resistant (TTX-R) channel 1 (Na) in primary afferent nociceptors, important in nociceptor sensitization and in inflammatory induced hyperalgesia (Khasar, Lin et al. 1999).

*3.2.3.2 **PKCε in ischemic heart diseases***

Much effort has been undertaken to elucidate the role of PKCε in ischemic heart diseases. Preconditioning with a short ischemic phase prior to a prolonged ischemic episode reduces heart damage (Murry, Jennings et al. 1986). During the brief preconditioning event ligands of G protein-coupled receptors (GPCRs) are released and activate the corresponding G proteins. As a result DAG is generated and activates PKCε (Inagaki, Churchill et al. 2006). Downstream of PKCε activation, ERKs and Lck may assure cardio-protection by inhibition of pro-apoptotic proteins (Heidkamp, Bayer et al. 2001, Baines, Zhang et al. 2002, Ping, Song et al. 2002, Inagaki and Mochly-Rosen 2005).

*3.2.3.3 **PKCε is severely oncogenic***

PKCε is the only PKC isoform involved in tumorigenesis in several tissues. By Raf-1 activation it affects the stress-induced ras-signaling cascade, which is one of the best characterized signaling pathways in cancer biology (Cai, Smola et al. 1997, Hamilton, Liao et al. 2001, Akita 2002). In animal models it has been shown that overexpression of PKCε results in metastatic disease. In addition to proliferation, dysregulation of cellular adhesion and motility are further key components of a metastatic phenotype. The role of PKCε in these processes was extensively reviewed by Gorin and Pan (Gorin and Pan 2009). In the context of cancer development, PKCε seems to mediate long-lasting effects that promote sustained cancer growth. Lastly, the observation that PKCε is expressed in a large number of cancer types makes this PKC isoform a potential tumor biomarker and a target in the development of anti-cancer therapeutics (Gorin and Pan 2009).

*3.2.3.4 **PKCε inhibits cell death***

PKCε contributes to cancer development not only by inducing unregulated cell growth, but also by short-term followed by long-term inhibition of apoptosis (Basu and Sivaprasad 2007). It promotes for example the survival of lung cancer cells by dysregulating stress-activated caspases, which are essential for programmed cell death (Ding, Wang et al. 2002). Similarly PKCε interacts with Bax, thus promoting the survival of human prostate cancer cells (McJilton, Van Sikes et al. 2003). Not least, it is implicated in anti-apoptotic pathways by modulating Bcl-2 family members (Pardo, Arcaro et al. 2002, Gorin and Pan 2009).

*3.2.3.5 **PKCε in antiviral resistance***

Interferon alpha (IFN-alpha) treatment leads to a rapid increase in intracellular DAG and a time-dependent activation of PKCε (Pfeffer, Eisenkraft et al. 1991). However, PKCε has also virus promoting effects, as it was described by Filone et al. (Filone, Hanna et al. 2010). They observed that an infection with the Rift Valley fever virus, which cycles between an insect vector and a mammalian host, is dependent on PKCε.

Thus, as indicated above, there is ample evidence for the involvement of PKCε in the response to stressful situations mediating both, short-term and long-lasting effects. To elucidate a mechanism responsible for this contrary effectiveness in respect of time, I will discuss general cellular stress responses in the following part.

3.3 Common cell reactions on stress

In general, cells respond upon stress by activation of cell survival pathways to recover from stress if possible and if necessary initiate programmed cell death of damaged cells (Fulda, Gorman et al. 2010). The cellular stress response can be divided into fast, but short and slower, sustained responses. While fast responses occur on the level of signal transduction, slower responses modulate protein expression (Kyriakis and Avruch 1996, Williams 1999).

3.3.1 Short response

Rapid responses are important for the acute reaction after stress, promoting cell survival or inducing apoptosis. When cells are exposed to stress, signaling transduction events are usually initiated within seconds and include the activation of distinct signaling proteins such as ERK or PKCs and the stimulation of certain signaling cascades such as SAPK/p38 cascade or MAPK cascade (Kyriakis and Avruch 1996, Kyriakis and Avruch 2001, de Nadal, Ammerer et al. 2011, Runchel, Matsuzawa et al. 2011). Here,

phosphorylation is a mechanism, promoting very fast signal transduction (de Nadal, Ammerer et al. 2011).

One example for a fast reaction in a stressful situation is the response to oxidative stress. Oxidative stress results from increased concentrations of reactive oxygen species (ROS), when the amount of ROS exceeds the capability of the intracellular antioxidant systems (Runchel, Matsuzawa et al. 2011). ROS production can be induced by several pathways and a number of stimuli, including osmotic stress, UV irradiation, chemotherapeutic drugs, heavy metals, and ischemia/reperfusion (Waxman 1996, Herrling, Fuchs et al. 2003, Leonard, Harris et al. 2004, Winter-Vann and Johnson 2007, Lau, Wang et al. 2008). One of the most important signaling pathways in response to oxidative stress is the mitogen-activated protein kinase (MAPK) cascade, which is activated by numerous cellular stresses and ligand-receptor bindings (Runchel, Matsuzawa et al. 2011). H_2O_2 for example, the most stable ROS, activates several MAPK pathways including ERK1/2 and p38, all of which mediate different cellular responses that alter for example proliferation and survival (Guyton, Liu et al. 1996, Runchel, Matsuzawa et al. 2011).

3.3.2 Sustained response

Slower and more sustained responses modulate gene and protein expression, which play a role after a few minutes of stress exposure (Holcik and Sonenberg 2005). In response to stress the production of growth-related proteins is reduced while the production of stress-related proteins is enhanced (de Nadal, Ammerer et al. 2011). This is achieved by several mechanisms. One of them is the regulation of distinct translation factors such as eukaryotic translation initiation factor 4E (eIF4E) and eIF2A for example by heat shock proteins. One cytoplasmic location, where protein translation is controlled is stress granules, one type of cytoplasmic RNA granules (Wang, Flynn et al. 1998). Interestingly, PKCε has so far not been described to phosphorylate proteins, which regulate translation. However, protein translation is the mechanism of interest in terms of mediating long-lasting effects.

Above, I pointed out that the common cellular stress response is divided into short and sustained response. Also I introduced short-term and long-lasting effects of PKCε in stress response. To approach the understanding of how PKCε can mediate both effects I will elaborate on downstream substrates and binding partners of PKCε in the next part. PKCε substrates involved in translational processes would strongly support the hypothesis that PKCε mediates long-lasting effects by the regulation of protein translation.

3.4 PKCε substrates, substrate candidates, and binding partners

The substrates of a kinase determine the biological response. Thus, to know the substrates of a kinase allows insight into the function of the kinase. Accordingly, a number of substrates for PKCε have been identified using various techniques such as overlay assays, PKCε knockdown, overexpression, knockout mice and in vitro phosphorylation (Hodge, Mehmert et al. 1999, Waldron and Rozengurt 2003, Zhao, Sutherland et al. 2004, Montgomery, Rundell et al. 2005, Aziz, Manoharan et al. 2007, Kobayashi, Winslow et al. 2012). They function as kinases (e.g. Akt), transcription factors (e.g. Stat 3), ion channels (e.g. TRPV1), or play a role in cytoskeleton regulation (e.g. peripherin, actin) (Prekeris, Mayhew et al. 1996, Sugiura, Tominaga et al. 2002, Wu, Thakore et al. 2004, Akita, Kawasaki et al. 2007, Aziz, Manoharan et al. 2007, Newton and Messing 2010) (A selection of PKCε substrates is listed in Table 1). Nevertheless, substrates involved in the regulation of translation and/or RNA granule formation have not been identified until recently. My coworkers in the group of Tim Hucho used a more sensitive technique, which was in vitro phosphorylation of large number of candidate proteins coded for by an expression library. Thereby, we identified numerous novel PKCε substrate candidates (Schreier, Isensee et al., under review at MCB). Several of them are associated with the cytoskeleton (e.g. myosin-10), are part of the survival machinery (e.g. nucleolar protein 3) or mediate different cellular functions (e.g. amyloid-like protein 1 precursor). These substrate candidates extended the already known PKCε substrate list. Moreover, we found substrates strongly suggesting a new biological aspect of PKCε to be involved in. About 30 % of the identified PKCε substrates and substrate candidates are involved in aspects of protein translation (Table 1).

Finding such a large number of proteins to be regulated by PKCε and to modulate translation makes the investigation difficult. As protein translation is a process of central importance to the cell, any knockdown or mutation of a single component will be compensated by the large number of proteins involved. Thus, one might rather start to investigate, if PKCε is localized to subcellular structures involved in protein translation such as stress granules and P-bodies. Both will be introduced in the next part.

Table 1: Selected PKCε substrates and candidates. PKCε substrates function as kinases anchoring proteins, transcription factors, and ion channels or play a role in the regulation of cytoskeleton. Recently PKCε substrates and substrate candidates (#) involved in protein translation were found (bold). Clone IDs marked with an asterisk are validated PKCε substrates.

Protein	Function	Reference
RACK1, RACK2	Anchoring/ binding protein	(Besson, Wilson et al. 2002)
PDLIM5 (ENH-1)		(Maeno-Hikichi, Chang et al. 2003)
TRAM		(McGettrick, Brint et al. 2006)
Histone H1		(Zhao, Sutherland et al. 2004)
14-3-3		(Saurin, Durgan et al. 2008)
PKCε	Kinase/ stress response	(Cenni, Doppler et al. 2002)
Akt/PKB		(Wu, Thakore et al. 2004)
PKD		(Waldron and Rozengurt 2003)
Phospholipase A2*, Glutathione peroxidase 4, isoform A*		(Schreier, Isensee et al., under review at MCB)
Stat 3	Transcription	(Aziz, Manoharan et al. 2007)
Cyclin-L2*, Zinc finger protein 354A* and 232*		(Schreier, Isensee et al., under review at MCB)
$GABA_A$ γ2 subunit	Ion channel	(Qi, Song et al. 2007)
TRPV1		(Sugiura, Tominaga et al. 2002)
Peripherin	Cytoskeleton	(Sunesson, Hellman et al. 2008)
Vimentin		(Ivaska, Vuoriluoto et al. 2005)
Keratin		(Akita, Kawasaki et al. 2007)
Actin		(Prekeris, Mayhew et al. 1996)
IQGAP1		(Grohmanova, Schlaepfer et al. 2004)
CMyBPC		(Montgomery, Rundell et al. 2005)
Myosin-10, Microtubule associated proteins 1A/1B light chain 3A precursor		(Schreier, Isensee et al., under review at MCB)
Nucleolar protein 3*, Acidic leucine-rich nuclear phosphoprotein 32 family member B	Survival	
Amyloid-like protein 1 precursor*	Neurite outgrowth	
Astrocytic phosphoprotein PEA-15*, Nucleophosmin*	Apoptosis	
Ubiquitin carboxyl-terminal hydrolase 10	Degradation	
40S ribosomal protein S3a*, S6#, S8*	**Translation**	
60S ribosomal protein L3*, L6#, L18*, L23a*, L29*		
Putative RNA-binding protein 15B#		
Elongation factor 1-gamma*		
Splicing factor, arginine/serine-rich 2#, 7*, 16*		
RNA-binding protein with serine-rich domain 1*		

3.5 RNA granules

Cellular stressors silence the translation of particular mRNAs for a certain period of time (Anderson and Kedersha 2006). Those mRNAs has to be sorted somewhere in the cell for re-initiation, storage or decay. The respective mRNAs are packaged in non-membranous ribonucleoprotein (RNP) granules that are even visible with a light microscope. Different types of such RNA granules have been observed in mammalian cells, for instance germ cell granules, processing bodies (P-bodies), stress granules, and neuronal granules. Although there composition is heterogeneous, they also share some proteins (Moser and Fritzler 2010). An exchange of proteins and mRNAs between different types of RNA granules is therefore suggested. RNA granules harbor translationally arrested mRNAs. Despite the large number of studies investigating the different RNA granule types, the signaling pathways as well as molecular mechanisms for assembly and disassembly is only fragmentarily understood.

3.5.1 Stress granules

The formation of stress granules is one way cells react on stress to impede cell death and restore homeostasis. Stress granules are non-membranous cytoplasmic foci with a size of 100 nm up to 2 µm (Table 2). They appear as a consequence of defects in translation initiation, when function of eIF2, eIF4a, or eIF4G is decreased (Anderson and Kedersha 2002, Dang, Kedersha et al. 2006, Mazroui, Sukarieh et al. 2006, Beckham and Parker 2008). This mostly is caused by different environmental stresses like oxidative stress, heat stress, viral infections and more (Nover, Scharf et al. 1989, Kedersha, Gupta et al. 1999, Raaben, Groot Koerkamp et al. 2007). Due to the depleted stores of eIF2-GTP-tRNA, T-cell internal antigen 1 (TIA-1) is included into a translationally silent non-canonical pre-initiation complex lacking eIF2 and eIF5. The prion like domains of TIA-1 promote TIA-1 self-aggregation leading to an accumulation of those non-canonical pre-initiation complexes into structures named as stress granules. Thus, stress granules harbor untranslated mRNAs upon stress. During times of environmental stress, the synthesis of “housekeeping” proteins is disrupted whereas the translation of damage repair proteins is enhanced (Anderson and Kedersha 2002). Up to now, the function of stress granules is not fully understood, but it is suggested that they are able to protect RNAs from harmful conditions. During and after environmental stress, stress granules seem to be a decision point for untranslated mRNAs that could be further stored, degraded or released and translation is re-initiated (Nagy and Pogany 2012). Interestingly not only RNAs, small ribosomal subunits and translation factors are components of stress granules but also many proteins that play a role in various fields including neurological diseases, silencing, transcription, signaling, cell cycle, apoptosis and more (Table 3).

RACK1 for example, the receptor for activated C kinases (see above), was observed to be a component of stress granules upon certain stresses and thus to regulate apoptosis. By binding to the 40S ribosomal subunit in the stalled pre-initiation complex RACK1 is sequestered into stress granules upon type 1 stress like hypoxia, heatshock and arsenite (Sengupta, Nilsson et al. 2004, Arimoto, Fukuda et al. 2008). Thereby apoptosis is blocked. In contrast, upon type 2 stress like UV, cytokines or H_2O_2 RACK1 induces the stress responsive MAPK pathways leading to apoptosis (Sengupta, Nilsson et al. 2004, Arimoto, Fukuda et al. 2008). Stress granule formation is therefore a reaction of the cell on stress to avoid apoptosis.

Table 2: Structural characteristics of stress granules and P-bodies. Modified from (Moser and Fritzler 2010).

	Cytoplasmic foci	Size [nm]	Associated with ribosomes	Move-ment	Micro-tubule-dependent	Contain mRNA
Stress granules	yes	100-2000	yes (40S)	no (change shape)	yes (formation)	yes
P-bodies	yes	100-300	no	yes	yes	yes

3.5.2 P-bodies

Often stress granules are located directly next to a second RNP granule type: P-bodies. Similar to stress granules, P-bodies are cytoplasmic foci that harbor translational silenced mRNAs. In contrast to stress granules, P-bodies are even present in unstressed cells. However, upon environmental stresses the number of P-bodies is increased. Furthermore, a protein and mRNA shuttling was observed between both structures (Wilczynska, Aigueperse et al. 2005, Beckham and Parker 2008). P-bodies contain enzymes of the mRNA decay pathway, including proteins of the decapping enzyme complex like DCP1/2 (Fenger-Gron, Fillman et al. 2005). Moreover, they contain proteins of RNP assembly (Ingelfinger, Arndt-Jovin et al. 2002). In mammalian cells components of the mRNA silencing pathways are present in P-bodies, too, as well as protein translation initiation factors (Anderson and Kedersha 2006). In contrast to stress granule formation, P-body formation does not require eIF2α phosphorylation in mammalian cells (Kedersha, Stoecklin et al. 2005). The motility of P-bodies and stress granules is also different. Whereas stress granules do not move along microtubules but undergo fission and fusion processes during their formation, live-cell imaging revealed that P-bodies are mainly

anchored to and move along microtubules (Aizer, Brody et al. 2008). Nevertheless, there are also stationary P-bodies identified at the centrosome. The movement of proteins stored in P-bodies differs among P-body components. DCP1 proteins are permanently exchanged with the cytoplasm while DCP2 is a stationary P-body core component. Some important structural characteristics are summarized in Table 2.

Both, stress granules and P-bodies contain proteins involved in stress related signaling processes or disorders (Table 3), some of which were also discussed in context of PKCε. Both structures regulate protein translation and stress granules were even shown to recruit the receptor for activated C kinases, RACK1, which lead to the question to be answered if also PKCε is recruited to stress granules and P-bodies, thus regulating translation processes.

Table 3: Selected proteins involved in stress response or development of diseases, which are localized to stress granules and/or P-bodies. ND = no data.

	Proteins	Stress granules	P-bodies	Reference
Intellectual disabilities	FMRP	+	+	(Barbee, Estes et al. 2006, Kim, Dong et al. 2006)
	PQBP1	+	ND	(Kunde, Musante et al. 2011)
Heat shock	HSF1	+	ND	(Alastalo, Hellesuo et al. 2003)
Apoptosis	RACK1	+	-	(Arimoto, Fukuda et al. 2008, Otsuka, Takata et al. 2011)
	TIA-1	+	?	(Buchan and Parker 2009)
Antiviral response	APOBEC 3G	+	+	(Gallois-Montbrun, Kramer et al. 2007)
Neurodegeneration	TDP-43	+	ND	(Dewey, Cenik et al. 2012)
Survival	ADAR1; Tudor-SN	+	ND; -	(Weissbach and Scadden 2012)
Carbon and phosphorous starvation	TORC1	+	ND	(Takahara and Maeda 2012)

PKCε is shown to be involved in the response to stressful situations mediating both, short-term and long-lasting effects. Substrates of PKCε suggest that PKCε connects the fast changes with the subsequent changes through the regulation of protein translation, potentially by the localization to stress granules and P-bodies.

4 Aim

Cells respond to damaging stimuli to maintain functional homeostasis. In many cases, early responses are mediated by kinase-dependent signal transduction, while slower responses are mediated by regulation of protein translation. PKCε has been described to be activated acutely in situations, where cellular stress is eminent. However, PKCε also induces changes outlasting the presence of the cellular stressor. Yet, it is unknown, if PKCε itself has the potential to induce the long-lasting responses through e.g. modulation of protein translation and if thereby PKCε connects the early with slower but longer lasting responses. This question is of particular importance as PKCε in general has so far not been associated with protein translation.

In a kinase substrate identification screen performed in the laboratory of Tim Hucho, several PKCε substrate candidates were identified, which are known to be involved in various aspects of protein translation (Schreier, Isensee et al., under review at MCB). Thus, I set out to answer the following questions:

1) Is PKCε recruited to stress granules in response to cellular stress and is this a cell type independent effect?

2) Is PKCε recruited also to another type of granule involved in stress response, namely to P-bodies?

3) Are the PKCε adaptor proteins RACK1 and RACK2 recruited to stress granules and P-bodies?

4) Does PKCε activation, PKCε down-regulation or PKCε inhibition affect stress granule formation?

5) Does reduced PKCε availability change P-body formation?

6) Does PKCε itself regulate protein synthesis?

7) Are also other signaling components such as PKA recruited to stress granules and P-bodies?

To interlink stress response, PKCε activation and stress granule formation adds a novel aspect to the processes PKCε is involved in as well as to the mechanisms of translation regulation. As PKCε has been associated with the development of various diseases, my results will allow also the investigation of novel disease mechanisms and the development of therapeutic targets.

5 Materials

5.1 Animals and cell lines

5.1.1 Animals

For cell biological studies adult male Spraque-Dawley rats were purchased from Harlan Winkelmann, Borchen, Germany. Animals were housed in a controlled environment under a 12 hours light-dark cycle. Food and water were available ad libitum. Care and use of animals were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the LAGeSo, Berlin, Germany.

5.1.2 Cell lines

HeLa and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % FCS, 1 % penicillin/streptomycin (P/S), and 1.5 % glutamine.

F-11 cells were cultured in F-12 Ham medium supplemented with 15 % FBS, 1 % P/S, and 1.5 % glutamine.

All cell lines were incubated in a humidified incubator with 5 % CO_2 at 37 °C.

5.2 Antibodies

5.2.1 Primary antibodies

Antibody	Company	Cat#	Dilution
Mouse anti-PKCε	BD	610085	IF: 1:200
Rabbit anti-PKCε	Abcam	ab4140	IF: 1:200
Rabbit anti-SN134KP4	*Robert Messing*		IF: 1:1000
Mouse anti-Ataxin-2	BD	611378	IF: 1:100-1:200
Rabbit anti-Ataxin-2-Like	Bethyl Laboratories	A301-369A	IF: 1:300
Mouse anti-DCP1A	Abnova	H00055802-M06	IF: 1:1000
Rabbit anti-DDX6	Novus	NB200-191	IF: 1:500
Goat anti-TIA-1	Santa Cruz	sc-1751	IF: 1:100
Mouse anti-TIAR	BD	610352	IF: 1:300
Rabbit anti-RACK1	Genway	18-003-44199	IF: 1:300

Mouse anti-β'COP (RACK2)	Sigma	G2279	IF: 1:200
Mouse anti-β-Tubulin	Sigma	T0198	WB: 1:1000
Mouse anti-PKA RIIα	BD	612242	IF: 1:500
Rabbit anti-RIIY116	Abcam	32514	IF: 1:1000

5.2.2 Secondary antibodies

Chicken anti-rabbit Alexa 488	Invitrogen	A21441	1:1000
Chicken anti-rabbit Alexa 594	Invitrogen	A21442	1:1000
Chicken anti-mouse Alexa 488	Invitrogen	A11029	1:1000
Chicken anti-mouse Alexa 594	Invitrogen	A21201	1:1000
Goat anti-rabbit Alexa 488	Invitrogen	A11034	1:1000
Goat anti-rabbit Alexa 594	Invitrogen	A11037	1:1000
Goat anti-mouse Alexa 488	Invitrogen	A11029	1:1000
Goat anti-mouse Alexa 594	Invitrogen	A11032	1:1000

5.3 Media, sera, and supplements

Medium	Company	Cat#
Dulbecco's Modified Eagle Medium (DMEM)	Lonza	BE12-707F
F-12 Ham Nutrient Mixture	Invitrogen	31765-027
Hank's BSS	Invitrogen	14170-070
Hibernate-A-Methionine	BrainBits	HA
MEM	Invitrogen	42360-024
Neurobasal-A Medium	Invitrogen	12349-015

5.4 Buffers

Buffer	Composition
1x Blotting buffer	48 mM Tris, 39 mM Glycine, 0,04 % SDS, 20 % Methanol
5x Laemmli loading buffer	62.5 mM Tris-HCl pH 6.8; 5 % β-Mercaptoethanol; 50 % Glycerol; 2 % SDS; 0.1 % Bromphenol Blue
2x “Magic Mix”	8 M urea; 15 mM Tris-HCl pH 7.5; 8.7 % Glycerin; 1 % SDS; 0.4 ‰ Bromphenol Blue; 143 mM β-Mercaptoethanol
TBS	137 mM NaCl; 26 mM KCl; 25 mM Tris
TBST	0.1 % Tween in TBS

5.5 Drugs and chemicals

Drug/Chemical	Company	Cat#
Aceton, p.a.	Merck	1.00014.2500
Azidohomoalanine (AHA)	Invitrogen	C10102
Ammonium persulfate	Biorad	161-0700
Anisomycin	Sigma	A9789
B-27 Supplement (50x)	Invitrogen	17504
ß-Mercaptoethanol	Merck	8.05740.0250
Bisindolylmaleimide I (BIM)	Calbiochem	203291
Bovine serum albumine (BSA)	Sigma	A3912
Calcium chloride	Merck	102392
Casyton	Innovatis	43001
Chloroform, p.a.	Merck	1.02445.2500
Collagenase P	Roche	11 213 873 001
Complete protease inhibitor	Roche	1 697 498

Copper(II) sulfate ($CuSo_4$)	Sigma	451657-10G
4’,6’-Diamidino-2phenylindole dihydrochloride (DAPI)	Serva	18860.01
Bromphenol Blue	Serva	15375
Dimethylsulfoxid (DMSO)	Merck	1.02950:0500
Dulbecco’s Phosphate Buffered Saline (DPBS)	BioWhittaker	BE17-521
EDTA, p.a.	Lager, Merck	1.08418.1000
17-ß-Estradiol – water soluble	Sigma	E4389
Ethanol, p.a.	Merck	1.00983.2500
Fetal bovine serum (FBS)	Sigma	F7524
Fluoromount-G	SouthernBiotech	0100-01
L-Glutamate	Sigma	27647
L-Glutamine	Sigma	G6392
Glycerin	Merck	1.04095
Glycine, p.a.	Merck	1.04201.1000
Gö6976	Calbiochem	365250
Immersol	Zeiss	518F
Isopropanol, p.a.	Merck	1.09634.2500
Potassium chloride, p.a.	Merck	104936
Laminin	Invitrogen	23017-015
Magnesiumsulfate heptahydrate	SERVA	39773
Methanol, p.a.	Merck	1.06009.2500
N,N,N',N'-Tetramethylethylenediamine (TEMED)	Invitrogen	15524-010
Normal donkey serum	Dianova	017-000-121
Normal goat serum	Dianova	005-000-121

PageRuler Prestained Protein Ladder	Fermentas	SM0671
Paraformaldehyde	Sigma	158127
Penicillin/Streptomycin 10000 U	Lonza	BE17-512F
Phorbol 12-myristat 13-acetate (PMA)	Sigma	P 8139
Poly-L-Ornithin Hydrochloride	Sigma	P2533
Acrylamide	Roth	3029.1
DNase	Promega	M610A
Hydrochloric acid 32 %, p.a.	Merck	1.00319.1000
Hydrochloric acid, 1N	Merck	1.09057
Ro32-0432	Calbiochem	557525
Sigmacote	Sigma	SL2-100ML
Skim milk powder	Sucofin	
Sodium arsenite solution	Sigma	35000-1L-R
Sodium chloride, p.a.	Merck	106404
Sodium dodecyl sulfate (SDS)	Roth	4360.1
Sodium hydroxide 1N	Merck	1.09137
Sterile distilled water	Lonza	BE17-724F
SuperSignal West Femto Substrate	Pierce	34095
SuperSignal West Pico Substrate	Pierce	34080
Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA)	Sigma	678937
Tris(carcoxyethyl)phosphine (TCEP)	Sigma	C4706
Tris(hydroxymethyl)aminomethane, p.a. (Tris)	Merck	1.08382.0500
Triton X-100	Roth	3051.2
Trypsin	Worthington	LS 003703

Tween 20	Sigma	P9416
Urea	Merck	1.08487.1000
Xylencyanol FF	Serva	38505

5.6 SiRNAs

ON-TARGETplus SMARTpool to knockdown human PKCε (PRKCE, L-040148-00-0005) and a non-targeting control pool (D-001810-10-05) were purchased from Thermo Scientific. The PKCε-specific pool contained the following siRNAs:

siRNA	Target Sequence	Molecular Weight [g/mol]
J-004653-06, PRKCE	GUGGAGACCUCAUGUUUCA	13,429.8
J-004653-08, PRKCE	CGGAAACACCCGUACCUUA	13,444.9
J-004653-08, PRKCE	GACGUGGACUGCACAAUGA	13,444.9
J-004653-09, PRKCE	GACCAAGGACCGCCUCUUU	13,459.9

5.7 Markers

Page Ruler prestained protein ladder (Fermentas, # SM0671) was used as protein marker. Marker bands are: ~ 170, 130, 100, 70 (reference band orange), 55, 40, 35, 25, 15, and 10 (reference band green) kDa.

5.8 Laboratory equipment

Equipment	Company
Cell scraper	Techno Plastic Products
Centrifuge Rotanta 4402	Hettich Centrifuges
Centrifuge EBA 12	Hettich Centrifuges
Centrifuge EBA 12R	Hettich Centrifuges
Cleanbench CA/R6E	Cleanair
Cover slips, round, 12 mm	Fisher
Drying block, Type 556	Fisher

Falcon tubes (50 ml, 15 ml)	Falcon
Filter paper	Whatmann International
Filter tips	Biozym
FUJI Film Super RX	Helmut Roesch Ltd
Imaging plates, 96 well (µ-clear)	Greiner
Immobilon-P Transfer Membrane	Millipore
Garbage bag	Roth
Magnetic stirrer IKAMAG RCT	Ika
Multichannel pipettes (0.5 – 10 µl, 5 -100 µl)	Eppendorf
ND-1000 Spectrometer	Peqlab
pH Meter 763	Knick
Pipetboy acu Classic	Integra Bioscience
Pipettes (2 µl, 20 µl, 200 µl, 1000 µl)	Gilson
Pipette tips	Gilson
Plastic cell culture 24-well plate	Biochrom
Plastic cell culture 6-well plate	Biochrom
Power Supplier	Biorad
QIAshredder (250)	Qiagen
Reaction tubes 1.5 ml	Roth
Reaction tubes 2 ml	Eppendorf
Rongeur	FST
Scalpel	FST
Scissors	FST
SDS Mini-Protean 3 Cell System	Biorad
Serological pipettes	Corning
Slides	Roth
Steri-Cycle CO_2 incubator 381	Thermo Scientific
Thermomixer 5436	Eppendorf
Tissues	Kimtech

Trans-Blot Semi-Dry Transfer Cell	Biorad
Vortex Genie 1	Scientific Industries

5.9 Microscopes

Microscope	Company
LSM510 (Equipped with 2.5x, 10x, 20x, as well as 40x, 63x, and 100x)	Zeiss
LSM 700 (Equipped with 2.5x, 10x, 20x, as well as 40x, 63x, and 100x)	Zeiss
ArrayScan VTI (Automated immunofluorescence microscope, Equipped with 10x, 20x, 40x objectives)	Cellomics
Zeiss Axiovert 100 (Equipped with 5x, 10x, 20x, and 40x objectives)	Zeiss
Zeiss Axioskop (Equipped with 5x, 10x, 20x, 40x and 63x objectives)	Zeiss

5.10 Software

Software for image acquisition and analysis

Cellomics vHCS Scan, version 7.6.2.1

Cellomics View

Image J, NIH

LSM Image Examiner, version 4.2, Zeiss

Zen, Zeiss

Software for statistical analysis

GraphPad Prism

Microsoft Excel

6 Methods

6.1 Primary rat DRG cultures

6.1.1 Preparation of glass cover slips

Cover slips were immersed in chloroform:methanol (2:1 v/v) for 20 min, dried at 50 °C, and incubated in 20 % sulfuric acid for 20 min at room temperature (RT). Sulfuric acid was removed by washing the cover slips in dH_2O. The cover slips were then incubated in 0.1 N sodium hydroxide at RT for 5 min. To remove sodium hydroxide cover slips were washed once more with dH_2O and stored in pure ethanol. Finally, the cover slips were flamed and coated with laminin (5 µg/ml)/polyornithin (0.1 mg/ml) for 2 hours (h) at RT.

6.1.2 Dissection of DRG neurons

To prepare DRG neurons one rat was killed by slow CO_2 intoxication. After cervical dislocation, the back hair was removed with an electric shaver. The skin along the backbone was sliced off with the help of a scalpel. The spine was exposed by removing muscles and connective tissue using a rongeur. The exposed spinal processes of the vertebras and adjacent tissue was removed. The spinal ganglia of the first lumbal vertebra (L1) were exposed and removed by cutting the nerve processes. Preparation of spinal ganglia L2 to L6 was performed the same way and temporarily stored in MEM-medium.

6.1.3 Digestion, dissociation, and culture of DRG neurons

Spinal ganglia were desheathed and digested in 5 ml MEM + 100 µl Collagenase P (0.5 U) for 1 h at 37 °C, transferred into 2.5 ml Hank's BSS supplemented with EDTA (f.c. = 0.025 %) and trypsin (f.c. = 470 U/ml) for 8 min at 37 °C. Digestion was stopped by adding 10 mM $MgSO_4$. The DRGs were transferred into 1.25 ml MEM with a silicone coated and fire polished pasteur pipette and triturated by carefully pipetting 10 times up and down. After sedimentation of undissociated tissue, the supernatant was collected in a separate clean tube. Then 1.25 ml MEM was added to the undissociated tissue and triturated again. The procedure was repeated four times. For the last two times, a smaller opening of the pipette was chosen. The collected supernatant was pooled and centrifuged at 100 x g for 5 min at RT. The cell pellet was washed with 1 ml Neurobasal A medium supplemented with 2 % (v/v) B27, 0.5 mM L-glutamine, 25 µM L-glutamate and penicillin/streptomycin 100 U/ml. Cells were centrifuged again (100 x g, 5 min, RT) and supernatant was removed. The cell pellet was resuspended in 1 ml Neurobasal A/B27 medium by triturating 10 times with a wide opening Pasteur pipette. The cell suspension was diluted in NeurobasalA/B27 medium and neurons were plated in poly-L-ornithine (0.1 mg/ml) / laminin (5 µg/ml)-pre-coated 6 well plates (TTP, Trasadingen, Switzerland), or 24

well plates (TPP, with glass cover slips). The culture volume was 2 ml and 250 µl for 6 well and 24 well plates, respectively. Cells were incubated over night at 37 °C and 5 % CO_2 to adhere.

6.2 Culture of cell lines

HeLa and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, (Eagle 1955)) containing 10% FBS, 1% penicillin/streptomycin and 1.5 % glutamine. F-11 cells were cultured in F-12 Ham medium (Invitrogen, (Ham 1965)) supplemented with 15% FBS, 1% penicillin/streptomycin and 1.5% glutamine.

Cell lines were incubated in a humidified incubator with 5% CO_2 at 37 °C. Dependent on the experiment, cells were seeded in 6 (TPP), 24 (TPP with glass cover slips) or 96 (µ-clear, Greiner) well plates. The culture volume for 96 well plates was 100 µl.

6.3 Stimulation

To induce stress granule formation, cells were stimulated with 0.1 mM or 0.5 mM sodium arsenite in PBS for 1 h (unless otherwise indicated) at 37 °C. For stimulation, half of the medium was removed, mixed with arsenite, and added back to the cells. For fixation, the medium was removed and 4 % PFA in PBS was added to the cells for 10 min incubation at RT. Afterwards the cells were washed three times with PBS.

To investigate the impact of PKC activation on stress granule formation, F-11 cells were incubated with 1 µM PMA or 10 nM 17-β-estradiol alone or as a double stimulation in combination with 0.2 mM arsenite for 15, 20, 30, and 60 min. The cells were fixed by replacing the medium with 4 % paraformaldehyde (PFA) in PBS for 10 min at RT and washed three times with PBS. Stock solution of PMA was prepared in 100 % DMSO (f.c. of DMSO on cells was 0.2 %). 17-β-estradiol was dissolved in PBS.

For PKC inhibitor studies, HeLa cells were pre-treated for 15 min with the general PKC inhibitor BIM (0.05, 0.1, 0.5, 1, 5 µM), or the selective PKCα and β inhibitor Gö6976. The selective PKCε inhibitor Ro32-0432 and the solvent control DMSO was applied 30 min before sodium arsenite stimulation was performed. Stock solutions of BIM and Ro32-0432 were prepared in PBS. Gö6976 was dissolved in 100 % DMSO (f.c. of DMSO on cells was 0.2 %).

6.4 Immunocytochemistry of DRG cultures, F-11, HEK293T, and HeLa cells

Blocking and permeabilization of fixed cells was performed by incubation in 2 % goat serum, 1 % BSA, 0.1 % Triton X-100, and 0.05 % Tween 20 in PBs for 1 h at RT. The cells were then incubated with primary antibodies diluted in 1 % BSA in PBS for 1 h at RT or overnight at 4 °C. After three washes with PBS (10 min, RT) cells were incubated with secondary Alexa dye-coupled antibodies (1:1000) for 1 h at RT. In 96 well plates the DAPI staining was performed together with the secondary antibody staining. After three final washes (30 min, RT), the plates were sealed and stored at 4 °C until scanning. Cover slips were mounted with Fluoromount/DAPI 1:20000.

For stress granule staining mouse anti-Ataxin-2 1:200, rabbit anti-Ataxin-2L 1:300, goat anti-TIA-1 1:100, or goat anti-TIAR 1:200 was used. P-bodies were stained with mouse anti-DCP1a 1:1000 or rabbit anti DDX6 1:500. PKCε was stained with mouse anti-PKCε 1:200 or rabbit anti-PKCεSN134KP4 1:500. RACK1 and RACK2 (β'COP) were stained with rabbit anti-RACK1 1:300 and mouse anti-β'COP 1:200. As secondary antibodies chicken anti-rabbit Alexa 488, chicken anti-mouse Alexa 488, or/and chicken anti-mouse Alexa 594 (all 1:1000) was used. PKA subunit RII alpha was stained with mouse anti-PKA RII alpha.

6.5 Quantification of PKCε colocalization with stress granules and P-bodies

An immunofluorescence microscope (Axioskop, Zeiss) was used to study if PKCε was located to stress granules and P-bodies. Single stainings were used as control to avoid false positive results resulting from bleed-through. To quantify the incidence of colocalization, cells were selected in the DAPI channel, where neither PKCε nor stress granules nor P-bodies were visible. Then I switched to a channel, which shows the stress granule or P-body staining. If the cell contained at least three stress granules or P-bodies it was considered to be a stress granule or P-body positive cell. Next, I switched to the channel, where the PKCε staining was visible and checked for PKCε granules colocalizing with stress granules or P-bodies. Cells were stated to be colocalization positive in case of two or more stress granules or P-bodies showing colocalization with PKCε.

6.6 Confocal imaging

For colocalization studies the LSM700 (Zeiss, 63x objective) and Zen software (Zeiss) were used. Images were taken in best signal mode. By checking the intensity profile for

each channel, oversaturated pictures were avoided and single stainings were used as control. This was especially important for all kinds of granules to avoid spillover between channels. Due to the small size of P-bodies, contrast and brightness of relevant pictures were changed manually to facilitate the identification for the reader. Image processing was performed with Image J.

6.7 Down-regulation of PKCε

Using siRNAs is one method of down-regulating proteins. SiRNAs were ordered from Thermo Scientific. ON-TARGEt plus non-targeting Pool (D-001810-10-05) as control pool and ON-TARGEt plus SMART pool Human PRKCE (L-04014800-00-0005) were used to perform PKCε knockdown in HeLa cells. Transfections were performed with Lipofectamine 2000 according to the manufacturer's instructions. Briefly, 2-3 h after seeding the cells, oligo-Lipofectamine 2000 complexes were prepared. Therefore siRNAs were diluted in OptiMEM (+Glutamax) and gently mixed. Lipofectamine 2000 was diluted in OptiMEM (+Glutamax), was gently mixed, and incubated for 5 min at RT. The diluted oligomer was combined with the diluted Lipofectamine 2000. This solution was gently mixed and incubated for 20 min at RT. The oligomer Lipofectamine 2000 complexes were added to the cells. Cells were incubated at 37 °C in a CO_2 for 48 h. All siRNAs were used in 50 nM final concentration.

After 48 h cells were stimulated with sodium arsenite to induce stress and/or treated with pharmacological PKC inhibitors or activators to investigate the impact of PKCε on RNA granule formation.

6.8 SDS-PAGE and Western immunoblotting

For knockdown control, HeLa cells were seeded in 6 well plates and PKCε knockdown was performed as described. The medium was removed and for cell lysis 120 µl lysis buffer ("Magic Mix", 8 M Urea, 15 M Tris/HCl pH 7.5, 8.7 % Glycerin, 1 % SDS, 0.4 % Bromophenol Blue + 143 mM ß-Mercaptoethanol) was added. The cells were scraped off and put into QIAshredder columns. These were centrifuged for 2 min at 12000 rpm in an EBA 12 centrifuge (rotor 1412) to shredder the DNA. Protein concentration was measured with a NanoDrop spectrometer. To separate proteins according to their molecular weight, 10 µg (in 20 µl) of denatured protein samples were loaded per lane on a 10 % SDS polyacrylamide gel. Empty lanes were filled with the same amount of lysis buffer (without ß-Mercaptoethanol). SDS-PAGE was performed at 100 V for about 1 h until the blue colored migration front had left the end of the gel. Proteins were then transferred to a

PVDF membrane. Therefore PVDF membranes were activated in 100 % methanol for 5 min and rinsed with water. Afterwards they were incubated in 1x blotting buffer (48 mM Tris, 39 mM glycine, 0.04 % SDS, 20 % methanol). Gels with separated proteins were shortly incubated in 1x blotting buffer. The blot was assembled as following: three layers of Whatman filter papers soaked in 1x blotting buffer, activated PVDF membrane with the gel placed on top, and finally again three layers of soaked Whatman papers. Proteins were transferred to PVDF membranes applying 2.5 mA/cm^2 for 40 min. After blotting, PVDF membranes were blocked with 5 % skim milk in TBST for 1 h at RT followed by primary antibody incubation in TBST over night at 4 °C. For detection of PKCε the rabbit anti-PKCε SN134KP4 serum (1:1000) was used. As loading control I probed the blot membrane with mouse anti-β-Tubulin (1:1000). Membranes were washed three times 10 minutes in TBST and incubated with anti-rabbit-HRP (1:1000) and anti-mouse-HRP (1:1000), respectively. Blot membranes were developed with Super Signal West Femto trying to avoid oversaturated signals by choosing optimal exposure times.

6.9 Automated immunofluorescence microscopy

For data acquisition from large scale experiments performed in 96 well plates I used the automated high content immunofluorescence microscope Cellomics Array Scan VTI and the scan software Cellomics vHCS Scan. In the software ArrayScan VTI (700 series) version 7.6.2.1 “colocalization V4” was used as bio application. HeLa cell nuclei were identified automatically according to the following object identification parameters:

- size: 63.4 – 2535.0 μm^2
- ratio of perimeter squared to 4π area: 1 – 2
- length-to-width ratio: 1 – 3
- average intensity: 50 – 1500
- total intensity: $5x10^4$ – $2x10^7$
- intensity variation: 10 – 500

For stress granule and P-body identification a circle of 8.45 μm^2 was defined around the nuclei, which approximately describes the whole cell. Adjacent cells with overlapping regions of interest are separated according to the mean distance between both nuclei. In this area stress granules and P-bodies were recognized according to the following parameters:

- size: 0.8 – 11.6 μm^2
- ratio of perimeter squared to 4π area: 1 – 2.5
- length-to-width ratio: 1 – 2.5
- average intensity: 100 – 5000
- total intensity: 0 – $1x10^7$
- intensity variation: 0 – 32767

6.10 Fluorescence non-canonical amino acid tagging (FUNCAT)

To visualize newly synthesized proteins, I used the fluorescence based method FUNCAT (Dieterich, Hodas et al. 2010). HeLa cells were starved in methionine-free Hibernate A medium for 30 min. Then they were treated with the methionine surrogate azidohomoalanine (AHA, 2 mM, 1 h), which is incorporated into proteins instead of methionine (Figure 2 A, BI). After fixation, cells were blocked and permeabilized for 1 h in B-Block containing 10 % NDS, 2 % BSA, 0.1 % Triton X-100 in PBS pH 7.4. Afterwards AHA and the fluorescence dye 5-TAMRA were clicked together by a click-reaction (Figure 2 A, BII). Therefore cells were incubated with 5-TAMRA (1:1000), TBTA (200 µM), TCEP (400 µM) and $CuSO_4$ (200 µM) in PBS pH 7.8 over night with gentle agitation. The next day, cells were washed three times for 10 min with FUNCAT wash buffer (0.5 mM EDTA, 1 % Tween-20 in PBS pH 7.6), followed by two times washing for 10 min with PBS pH 7.4. Cells were incubated with rabbit anti-PKCε SN134KP4 1:1000 in B-Block for 1 h at RT. After washing, the secondary goat anti-rabbit Alexa 488 antibody was incubated for 1 h at RT. Coverslips were mounted with Fluoromount/DAPI 1:20000. Thus, FUNCAT allows the visualization of newly synthesized proteins with an immunofluorescence microscope without using radioactivity based methods. As an assay control the translation inhibitor anisomycin (40 µM) was treated together with AHA. To quantify the mean signal intensity of the cells, I used confocal pictures of 100 cells per condition (randomly selected in the DAPI channel) and manually defined the region of interest by drawing circles around each cell using ImageJ. Mean intensity of these regions of interest was measured and compared. Three independent experiments were performed in duplicates.

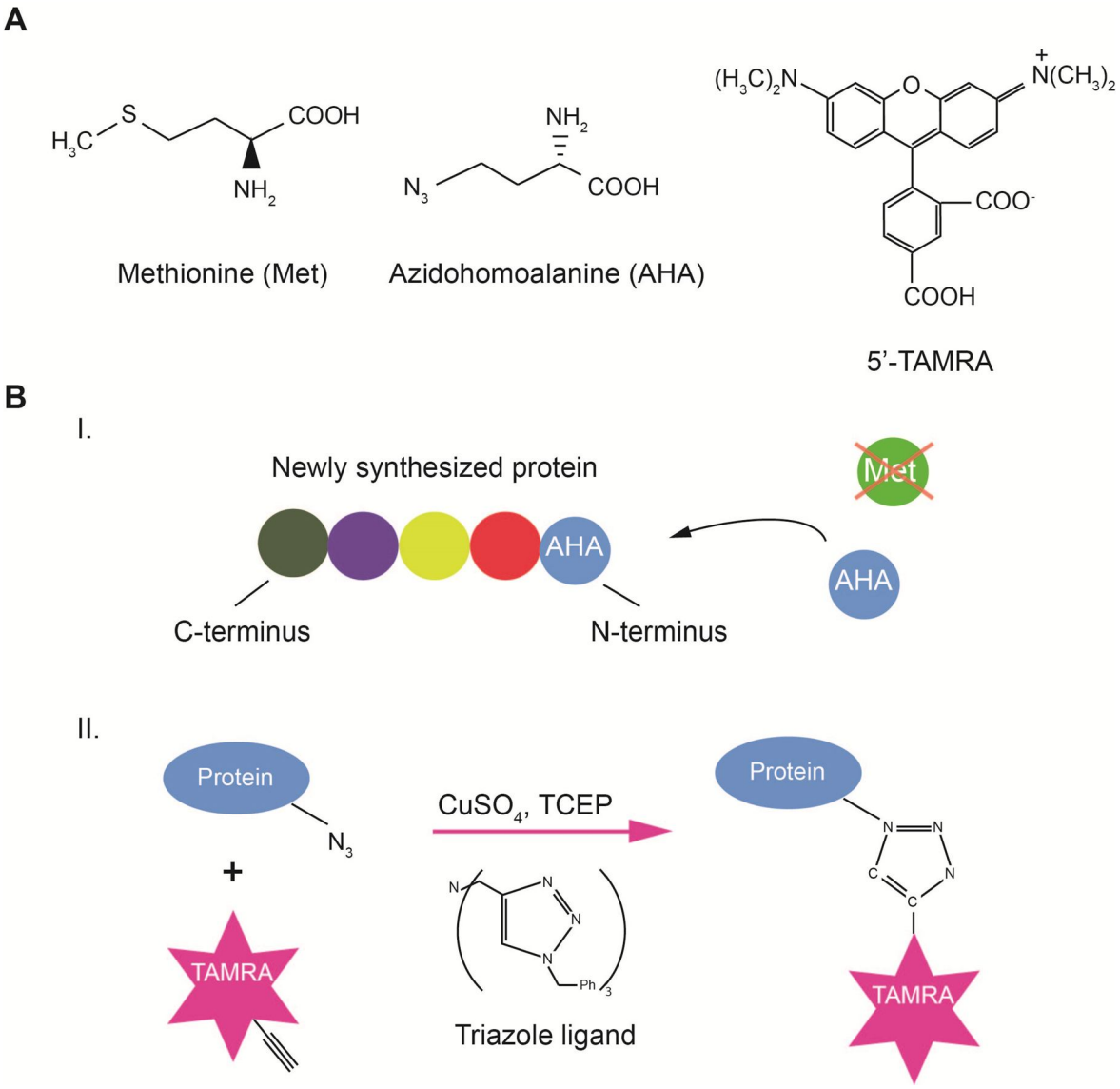

Figure 2: The procedure of FUNCAT. A) Chemical structure of methionine (Met), the methionine analog azidohomoalanine (AHA), and the fluorescence dye 5'-TAMRA. **B)** I. As a result of starving the cells in a methionine free medium (Hibernate A –Met), the methionine analog AHA was incorporated into newly synthesized proteins instead of methionine. II. AHA containing proteins were coupled to the immunofluorescence dye 5'-TAMRA via "click-chemistry". Therefore 5'-TAMRA was incubated with the cells in presence of a triazole ligand, $CuSO_4$, and TCEP (modified from Dieterich, Hodas et al. 2010).

6.11 Statistical analysis

All experiments were performed at least three times on separate days. Statistical analysis of the data was performed with One-way ANOVA and Tukey's post test using GraphPad Prism. Errors are indicated as ± SEM (unless otherwise indicated). $P < 0.05$ was considered as statistically significant.

7 Results

In a screen performed by coworkers in the laboratory, a large number of novel substrates of PKCε were identified, which are known to be involved in protein translation. To establish that PKCε is involved in protein translation, I first tested, if under cellular stress PKCε is localized in subcellular structures of protein translation such as stress granules and P-bodies (7.1 and 7.2). Then, I aimed to support a functional role for PKCε in both structures by testing also for colocalization with the PKCε anchoring proteins RACK1 and 2 (7.3), by investigating the influence of changed PKCε availability on stress granule and P-body formation (7.4 and 7.5), and by testing, whether PKCε itself is involved in the modulation of protein translation (7.6). In the last part, I widened the focus and investigated, if under cellular stress also other signaling enzymes than PKCε such as the protein kinase A (PKA) are recruited to stress granule and P-bodies (7.7).

7.1 Test for colocalization of stress granules and PKCε in various cell types

Protein translation is modulated among others by the formation of stress granules. Thus, I tested whether PKCε is recruited to stress granules under conditions of cellular stress.

First, I aimed to induce stress granules in different cell types. Then I analyzed if the stress granules colocalized with PKCε.

7.1.1 Arsenite treatment induces stress granules in F-11 cells

Since PKCε is strongly expressed in neurons, I asked if it is possible to induce stress granules in F-11 cells, a neuronal cell line. F-11 cells are a fusion product of mouse neuroblastoma cells and rat embryonic dorsal root ganglia (DRGs) neurons and was used as a neuronal model e.g. by Goswami et al. (Platika, Boulos et al. 1985, Goswami, Rademacher et al. 2010). To induce stress granule formation, I followed the experimental procedure shown in Figure 3, unless otherwise described.

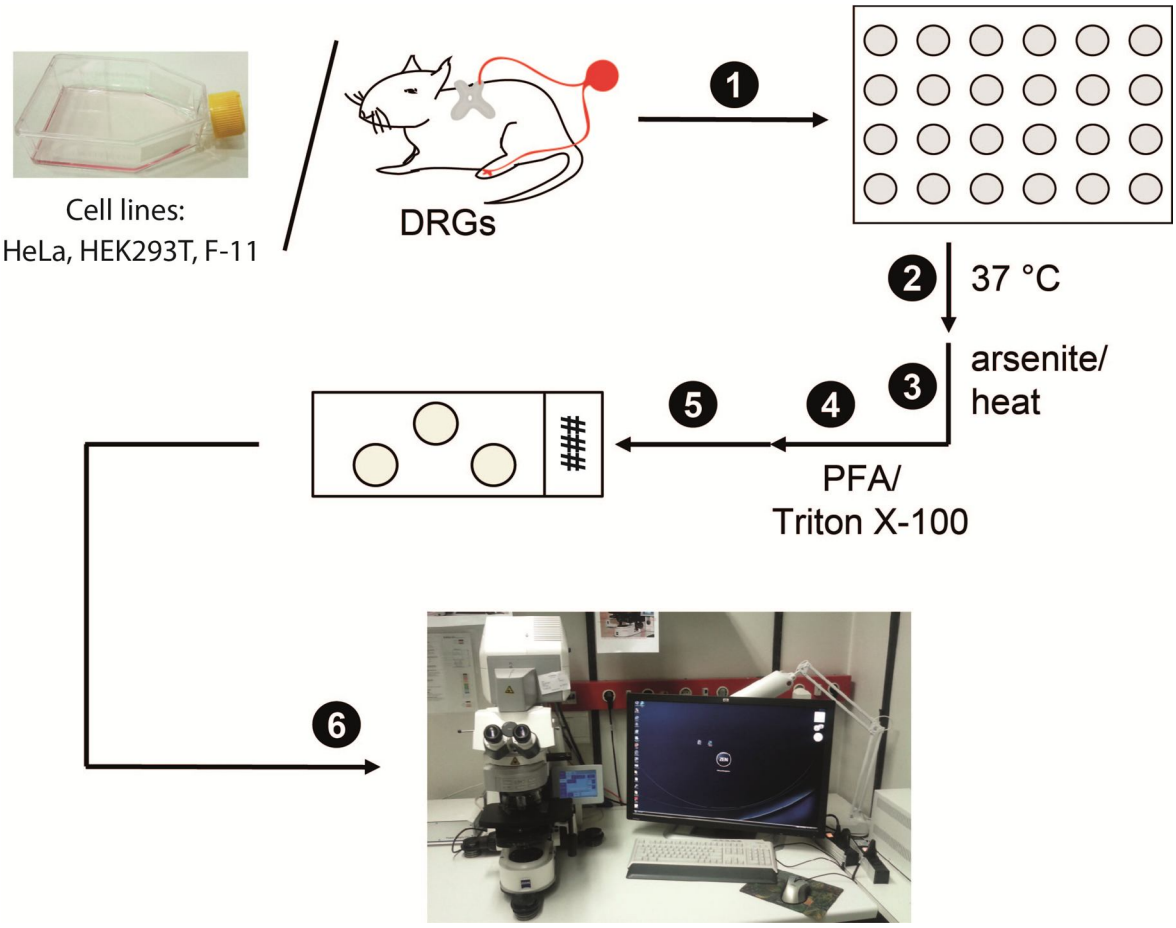

Figure 3: The experimental procedure of cell analysis from culturing to confocal images. 1. DRG neurons, F-11, HEK293T, or HeLa cells were seeded onto cover slips in 24 well plates. 2. Cell cultures were cultured either overnight (cell lines) or for three or five days (DRG neurons). 3. Cells were stressed with arsenite (various concentrations) or heat (44 °C). 4. Cells were fixed with PFA and permeabilized with Triton X-100. 5. Unspecific antibody binding was blocked with normal goat/donkey serum and cultures were stained with different antibodies detecting stress granules and PKCε. Afterwards they were mounted on slides. 6. Cultures were analyzed using confocal imaging.

Cells were treated with 0.2 mM arsenite for 10, 15, 20, 30, and 60 min. After arsenite stress F-11 cells were fixed, permeabilized, blocked to avoid unspecific antibody bindings, and stained with the stress granule marker Ataxin-2 (ATXN2). Cells were analyzed using confocal microscopy (Figure 4 A).

Consistent with published data, unstimulated cells did not develop stress granules (Kedersha, Cho et al. 2000). A clear trend of arsenite induced stress granule formation was visible already at 15 min though statistically not significant yet (3 ± 1 % of the cells contained stress granules, n = 4 independent experiments, each in duplicates with 100 analyzed cells per condition, $P > 0.05$, Figure 4 B). After 20 min I found 14 % stress granule positive cells (14 ± 1 %, $P < 0.001$). This value increased strongly after 30 min of arsenite treatment (59 % ± 3 %, $P < 0.001$). After 1 h of arsenite treatment around 77 % were counted as stress granule positive cells (77 ± 5 %, $P < 0.001$).

But not only the percentage of cells showing stress granules changed. Dependent on the duration of the arsenite treatment, the size and the subcellular arrangement of stress granules varied. Confocal images show that increased duration of arsenite treatment lead to clearer, larger stress granules, which were arranged in a circular shape around the nucleus in the cytoplasm of the cells (Figure 4 A).

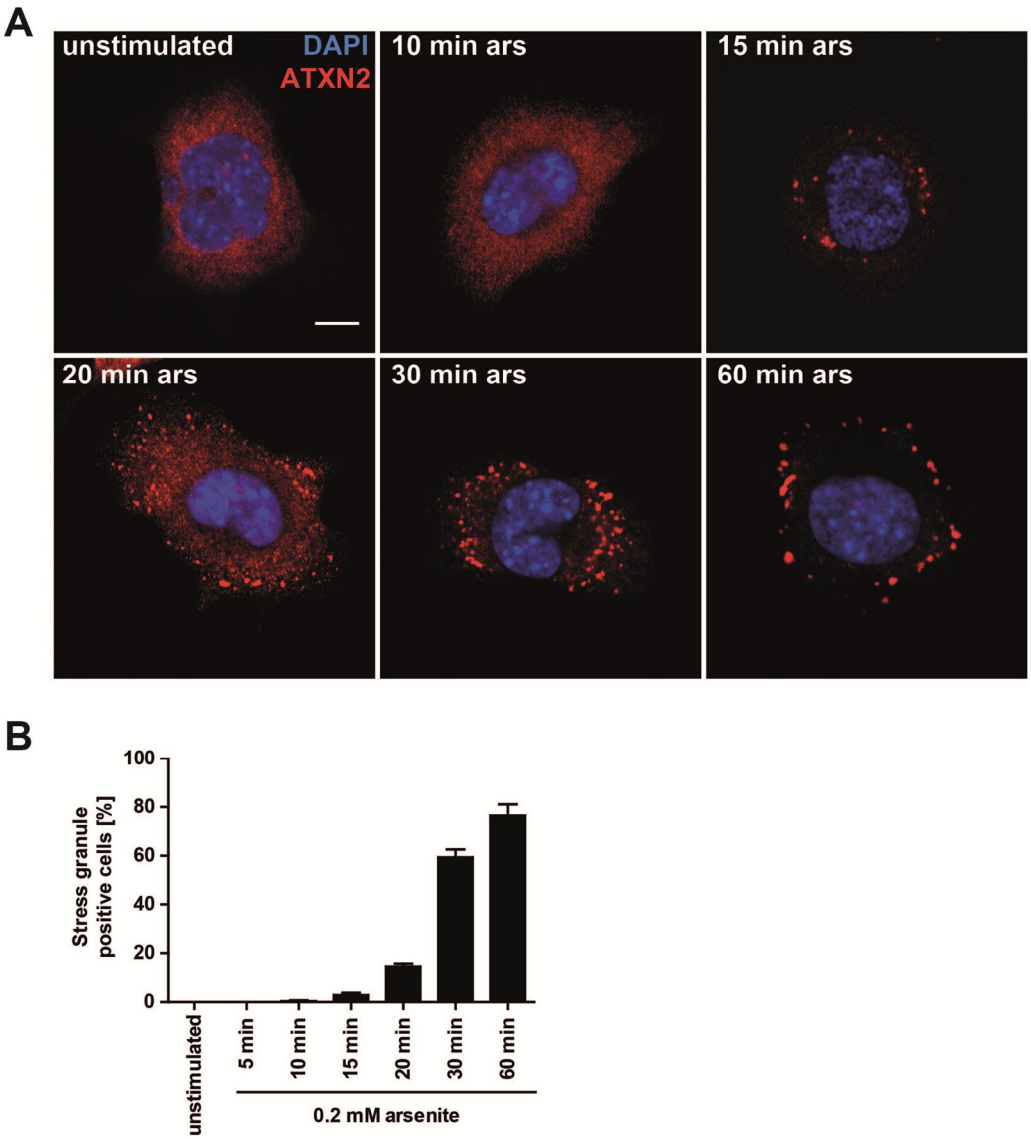

Figure 4: Stress granule formation after arsenite treatment in F-11 cells. A) Confocal images of F-11 cells immunofluorescence labelled with the stress granule marker Ataxin-2 (red). Stress granule formation did not occur in unstressed cells. The longer arsenite was applied, the larger the stress granules appeared. The cell nucleus was stained with DAPI. Scale bar is 10 µm. **B)** Quantification was performed via counting by eye. 100 cells per coverslip were analyzed. If they contained more than four granules they were considered to be stress granule positive. Unstimulated cells lacked stress granules (0 %). Arsenite treatment for 5 min did not induce stress granule formation (0 %), while 10 min arsenite resulted in 0.3 % stress granule positive cells. The number of stress granule positive cells increased with extension of arsenite treatment. 15 min arsenite treatment produced 3 ± 1 % stress granule positive cells, 20 min 15 ± 1 %, 30 min 59 ± 3 %, and 60 min 77 ± 5 %. Results are expressed as ± SEM from four independent experiments with duplicate wells counting 100 cells per duplicate.

To corroborate the identification of stress granules by the only recently identified Ataxin-2 (Nonhoff, Ralser et al. 2007), I co-stained arsenite stressed F-11 cells (0.5 mM, 1 h) with a more commonly used marker of stress granules, TIA-1, and Ataxin-2 (Figure 5). Indeed, Ataxin-2 labeled granules colocalized with TIA-1 labeled stress granules.

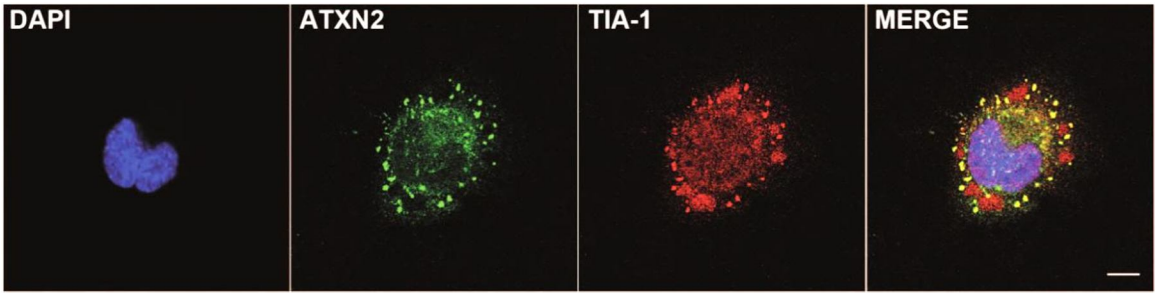

Figure 5: Stress granules in F-11 cells stained with Ataxin-2 and TIA-1. F-11 cells were stressed with 0.5 mM arsenite for 1 h. After PFA fixation, permeabilization and blocking cells were labeled with the two stress granule markers Ataxin-2 (green) and TIA-1 (red). Ataxin-2 stained stress granules colocalized with TIA-1 stained stress granules. The nucleus was stained with DAPI (blue). Scale bar is 10 µm.

7.1.2 Heat treatment induces stress granules in F-11 cells

Stress granule formation also occurs upon heat stress (Bond 2006). Thus, alternatively, F-11 cells were subjected to heat stress of 44°C for 60 min. If F-11 cells were subjected to temperature stress (44 °C) for 30 min and 60 min instead of arsenite stress, both the morphology of stress granules and the kinetics of formation were different (Figure 6). Heat induced stress granules were smaller and less round compared to arsenite induced stress granules. I found 80 % stress granule positive cells already after 30 min of heat stress. The number of stress granule positive cells did not increase anymore even after 60 min of heat stress, which indicates that a plateau of stress granule formation was reached already after 30 min of heat exposure (82 ± 3 % and 82 ± 3 % for 30 and 60 min heat stress, respectively, n = 4 independent experiments in duplicates with 100 analyzed cells per condition, $P < 0.001$).

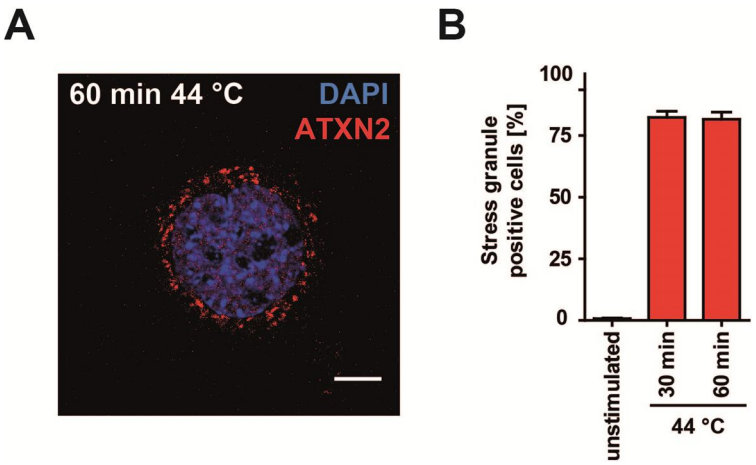

Figure 6: Stress granule formation upon heat treatment in F-11 cells. A) Confocal images of stress granule formation (ATXN2, red) upon heat treatment showed a similar arrangement of stress granules in the cytoplasm compared to arsenite induced stress granules. Only the shape of heat stress granules was smaller and by the majority more oval. Cell nucleus was stained with DAPI. Scale bar is 10 µm. B) Quantification by eye with 100 cells analyzed per condition. The maximum number of stress granule positive cells was already reached after 30 min heat treatment and was not increased further even after 60 min heat stress (82 ± 3 % and 82 ± 3 % for 30 and 60 min heat treatment, respectively). Results are expressed as ± SEM from four independent experiments with duplicate wells counting 100 cells per duplicate.

7.1.3 PKCε colocalizes with stress granules in F-11 cells upon arsenite treatment

Next, I addressed the question if F-11 cells exhibit colocalization between PKCε and stress granules. In unstimulated F-11 cells, the PKCε staining was distributed all over the cytoplasm with less intensity in the nucleus (Figure 7 A, upper panel). In some unstressed F-11 cells I observed faint granular structures labeled by the PKCε antibody. I found no stress granules by Ataxin-2 staining. If the cells were stressed with arsenite (0.5 mM, 1 h), and co-stained for Ataxin-2 and PKCε, the cytoplasmic PKCε staining changed drastically. I observed clear granular structures that colocalized with the stress granule marker Ataxin-2 (Figure 7 A, lower panel and B). In addition, I observed smaller PKCε granules not colocalizing with stress granules. As shown in section 1.2, these small PKCε-positive granules, which I observed also in unstressed cells, could be identified as a different type of RNA granules.

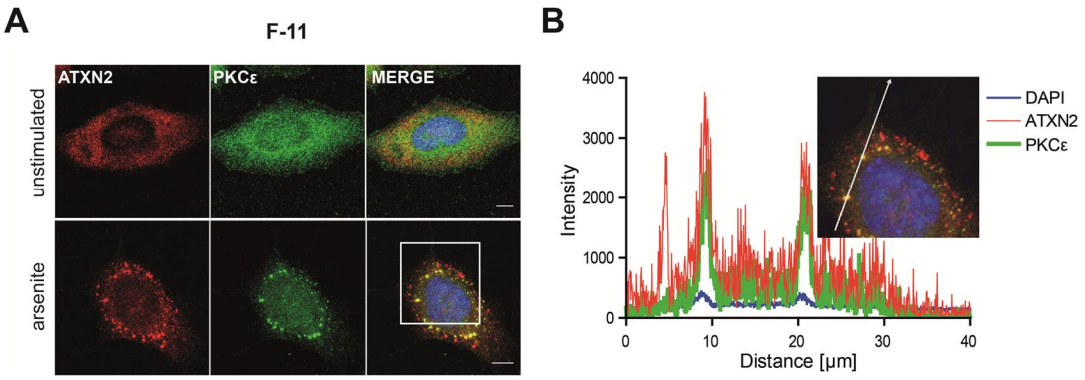

Figure 7: PKCε colocalizes with stress granules in arsenite stimulated F-11 cells. A) Confocal images of unstimulated or 0.5 mM arsenite (1 h) treated F-11 cells. Stress granules were stained with ATXN2 (red) and seemed to form an inner and outer "circle" in the cytoplasm. PKCε (green) was mainly distributed all over the cytoplasm accumulating at different sized foci in unstimulated (upper panels) as well as arsenite treated cells (lower panels). Large PKCε foci in arsenite treated cells colocalized with the inner "circle" of stress granules. Nuclei were stained with DAPI (blue). Scale bar is 10 µm. B) The intensity profile of the enlarged section (marked with a square in A) of arsenite treated F-11 cells illustrate a colocalization between PKCε and stress granules.

7.1.4 PKCε colocalizes with stress granules in F-11 cells upon heat treatment

I tested for a recruitment of PKCε to heat induced stress granules by subjecting the cells to treatment with 44 °C for 1 h. Thereby I found that indeed PKCε is recruited to stress granules after heat stress (Figure 8). This extends the observation of the PKCε recruitment to stress granules upon arsenite stress.

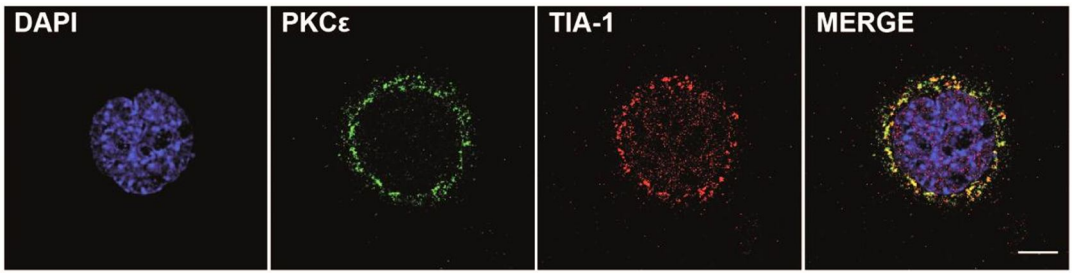

Figure 8: PKCε is recruited to stress granules upon heat stress in F-11 cells. Confocal images of F-11 cells which were subjected to 44 °C for 1 h. Stress granules were stained with TIA-1 (red). PKCε (green) is accumulated at different foci that colocalized with stress granules. Nuclei were stained with DAPI (blue). Scale bar is 10 µm.

7.1.5 Quantification of the PKCε colocalization with stress granules upon arsenite stress is similar to heat stress

To quantify the colocalization between PKCε granules and stress granules after heat and arsenite treatment, I counted 100 cells per coverslip with regard to two criteria. First, I analyzed, if the cell was stress granule positive or not. Here I found around 85 % stress granule positive cells for arsenite and around 77 % stress granule positive cells for heat

treatment (Figure 9 A). Second, in case of a stress granule positive cell, I analyzed whether there was colocalization of PKCε granules and stress granules in the cell. If I found two stress granules in the same cell to colocalize with PKCε granules, I counted the cell as a "colocalization positive" cell. Thereby I found both, for arsenite and heat stress, 100 % of stress granule cells to colocalize with PKCε in stress granule structures (Figure 9 B). Data are based on three independent experiments each performed in duplicates.

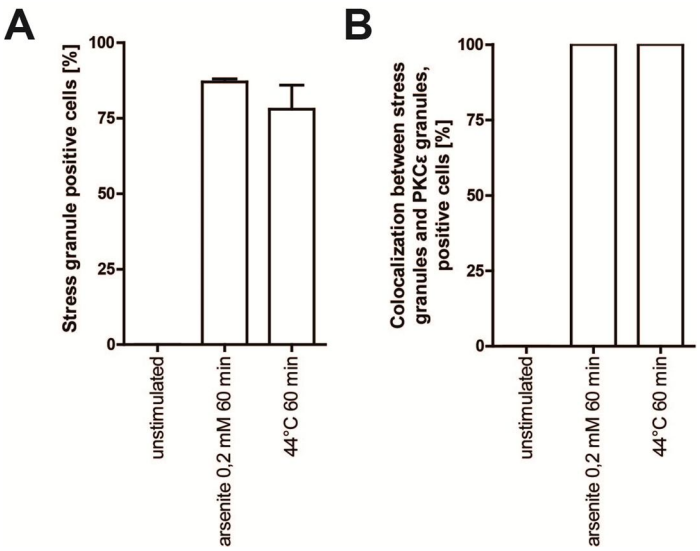

Figure 9: Quantification of colocalization between PKCε and stress granules. 100 cells per culture were counted by eye and analyzed concerning two criteria: 1. whether the cell was stress granule positive and 2. if a stress granule positive cell showed colocalization between stress granules and PKCε granules for at least two stress granules. **A)** Upon arsenite stress ~85 % stress granule positive cells were found, upon heat stress ~77 % stress granule positive cells. $P < 0.001$. **B)** 100% of stress granule positive cells upon arsenite and heat treatment showed colocalization between stress granules and PKCε granules for at least two stress granules. $P < 0.001$. Three independent experiments were performed in duplicates. Error bars indicate the SEM.

Both, arsenite and heat stressors led to similar results, but arsenite induced stress granules provided slight advantages for analysis due to their better identifiable shape. Thus, for all following experiments I decided to focus on arsenite stress.

7.1.6 Arsenite induces stress granule formation in neurites

Since F-11 cells are derived from neurons, they also form long and thin neurites which branch off from the cell bodies or connect the cells. By analyzing stress granule formation and colocalization with PKCε, I found also in neurites stress granules containing PKCε (Figure 10). However, these results require additional and more detailed investigation by quantification.

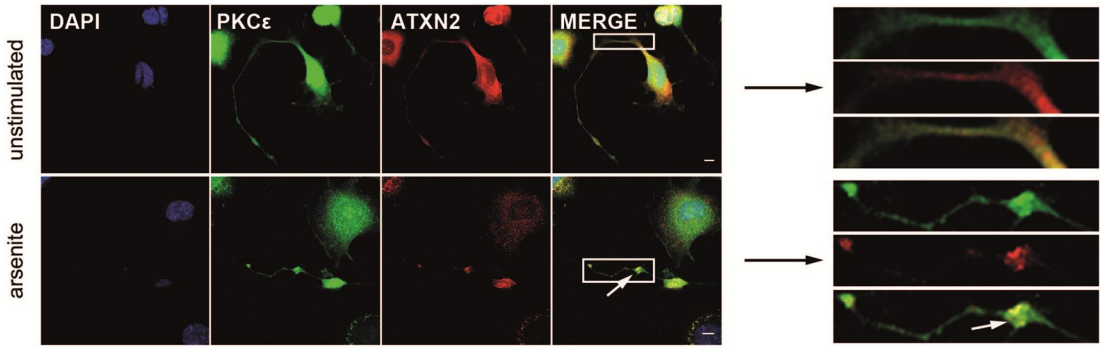

Figure 10: Stress granule formation in neurites and colocalization with PKCε. F-11 cells were either left unstimulated or were stressed with 0.5 mM arsenite for 1 h. They were stained for PKCε (green) and the stress granule marker Ataxin-2 (red). In unstimulated cells neurites were stress granule free (upper panel, magnification of the region marked with a white rectangle right-hand) whereas the neurites of arsenite stressed cells contained stress granules that colocalize with PKCε. Nucleus was stained with DAPI. Scale bar is 10 µm.

7.1.7 Stress granule induction in DRG neurons

PKCε is recruited to stress granules in the neuronal F-11 cell line. However, a neuronal cell line does not perfectly reflect ongoing events in neurons. As the laboratory of Tim Hucho is mostly investigating phenomena in primary neurons, I examined if stress granules are also inducible in DRG neurons, which detect a diversity of stimuli, and if PKCε is localized to them as well.

In unstressed DRG cultures the PKCε staining was distributed all over the cytoplasm (Figure 11 A). In contrast to F-11 cells, the Ataxin-2 staining revealed bright areas nearby the membrane already in unstimulated cells. Stress granule formation upon 0.5 mM arsenite treatment for 1 h did not occur in cell cultures maintained for one and two days as first trial experiments have shown. Thus, I tested if stress granules are inducible in cultures maintained for three and five days. I found few Ataxin-2 labeled granules in only few of the DRG neurons. Of these few granules only some were colocalized with PKCε labeled granules (Figure 11 B).

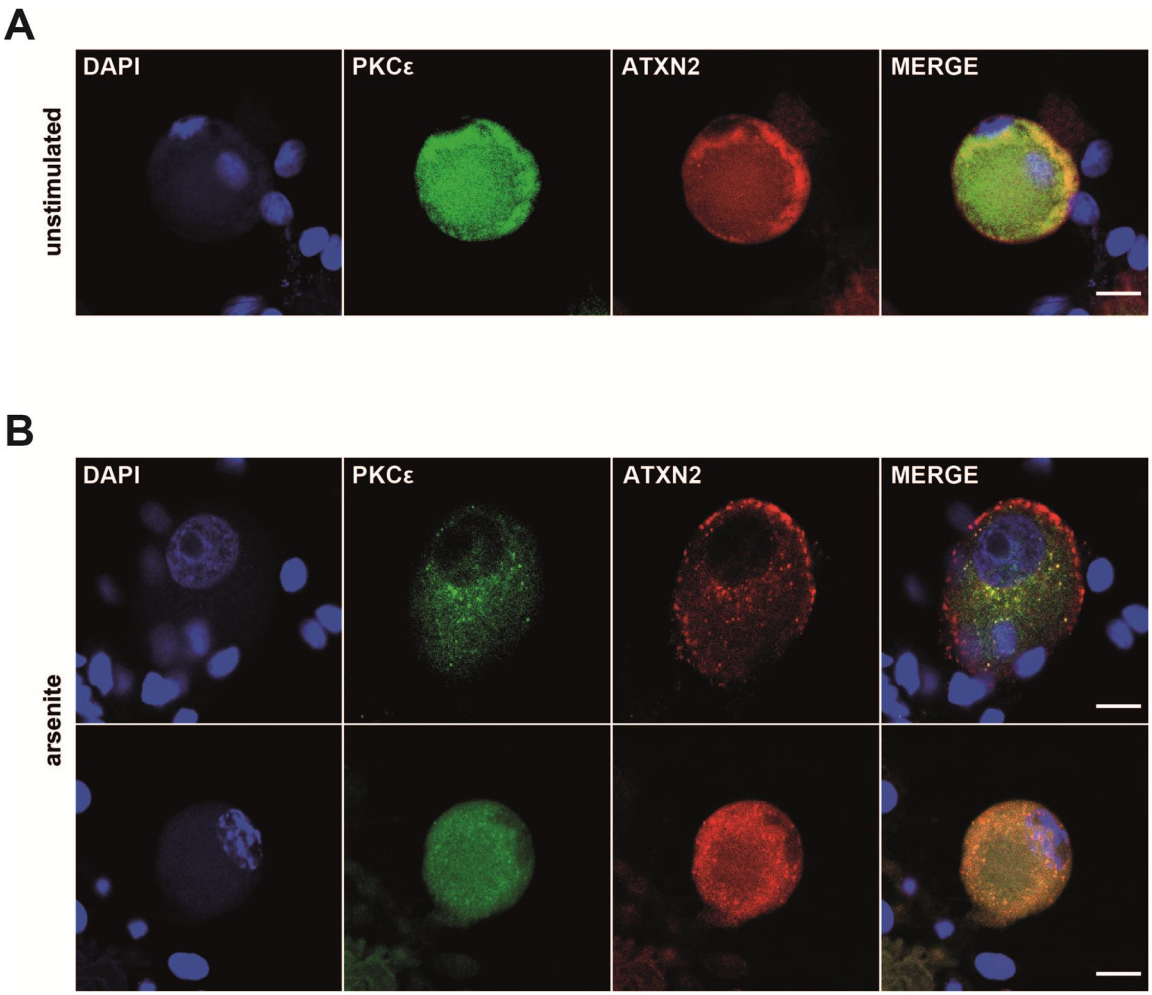

Figure 11: Induction of stress granules in DRG neurons. A) Confocal images of an unstimulated DRG neuron after three days in culture. PKCε staining was distributed all over the cytoplasm (green), no stress granules were visible (ATXN2, red). Glia cells were attached to the neuron (DAPI, bright blue nuclei). Scale bar is 10 µm. **B)** Confocal images of 0.5 mM arsenite (60 min) treated DRG neurons after three days in culture showed granular structures in the cytoplasm of PKCε stained cells (green). These granules sometimes colocalized with the granules observed with the stress granule marker Ataxin-2 (ATXN2, red). Additionally Ataxin-2 staining showed granular structures nearby the cell membrane (upper panel). These granules were not colocalized with PKCε. Nuclei were stained with DAPI (blue). Glia cells were attached on top of and below to the DRG neuron and in close vicinity (small nuclei). The cytoplasm of glia cells was not visible as they are in a different focus plain compared to the DRG neurons. Scale bar is 10 µm.

However, this observation was highly variable among the replicates. I therefore concluded that it is difficult to observe stress granule formation in cultured DRG neurons. Evaluation was especially challenged by the strong proliferation of glia, which moved toward and on top of neurons. As stress granule formation was strong in glia cells this masked neuronal phenomena. Moreover, influence on stress granule formation by signaling events in glia cells could not be excluded. Because of the observed inconsistent results and adverse culture conditions I decided not to proceed with the analysis of stress granules in DRG neurons.

7.1.8 PKCε colocalizes with stress granules in HEK293T cells

Next, I asked if the observed colocalization between stress granules and PKCε in F-11 cells is restricted to F-11 cells or if PKCε is translocated to stress granules also in other cell lines such as HEK293T, a human embryonic kidney cell line with some neuron-like properties (Shaw, Morse et al. 2002).

As F-11 cells, unstimulated HEK293T cells lacked stress granules. Thus, Ataxin-2 staining was almost not visible. The PKCε staining in unstimulated cells was similar to F-11 cells. PKCε is distributed all over the cytoplasm accumulating in several small granules (Figure 12 A, upper panel).

To induce stress granules I stressed HEK293T cells with 0.5 mM arsenite for 1 h and stained for PKCε and the stress granule marker Ataxin-2. Analyzing the samples with a confocal microscope I observed colocalization of stress granules and PKCε granules in arsenite stressed HEK293T cells (Figure 12 A, lower panel). The colocalization is illustrated with an intensity profile of the line scan shown in the magnification (Figure 12 B).

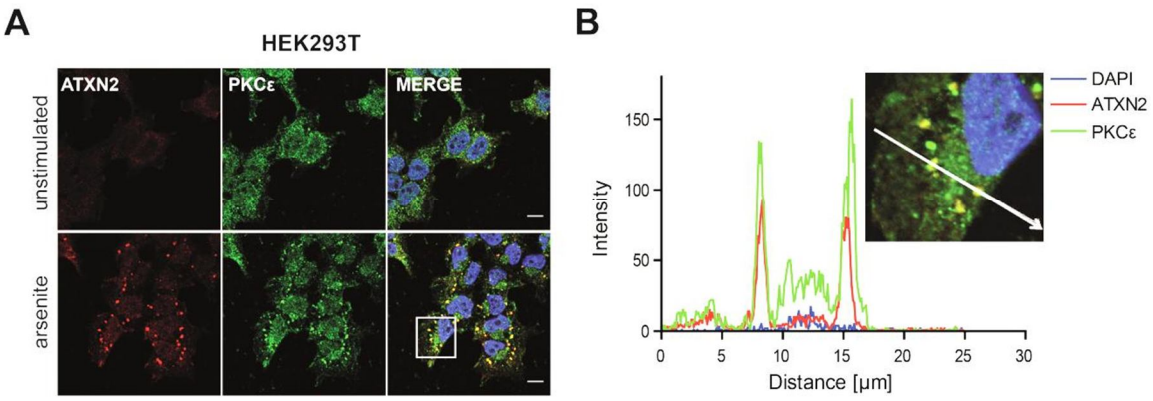

Figure 12: PKCε colocalizes with stress granules in arsenite stimulated HEK293T cells. A) Confocal images of unstimulated or 0.5 mM arsenite (1 h) treated HEK293T cells. Stress granules were stained with ATXN2 (red). PKCε (green) was mainly distributed all over the cytoplasm accumulating different sized foci in unstimulated (upper panels) as well as arsenite treated cells (lower panels). Large PKCε foci in arsenite treated cells colocalized with stress granules. Nuclei were stained with DAPI (blue). Scale bar is 10 µm. **B)** The intensity profile of the enlarged region of arsenite treated HEK293T cells illustrate a colocalization between PKCε and stress granules.

7.1.9 PKCε colocalizes with stress granules in HeLa cells

The HeLa cell line is among the most commonly used human cell line in life science research. It was derived from a lethal cervical carcinoma. Also in stress granule research, HeLa cells were used extensively (Souquere, Mollet et al. 2009). I examined if PKCε is recruited to stress granules also in the non-neuronal HeLa cell line. The experimental procedure I followed is shown in Figure 3. For stress granule induction the cells were

stressed with 0.5 mM arsenite for 1 h and stained for the stress granule marker Ataxin-2 and PKCε.

In unstimulated HeLa cells, the PKCε staining was similar to F-11 and HEK293T cells. It was distributed all over the cytoplasm and with less intensity in the nucleus and accumulated in some cells to small granules. If the cells were stressed with arsenite, the cytoplasmic PKCε staining changed evidently. I observed clearly granular structures colocalizing with Ataxin-2 stained stress granules (Figure 13 A). Colocalization between PKCε granules and stress granules is indicated with the intensity profile of the line scan in the magnification (Figure 13 A, lower panel and B). Since HeLa cells showed the largest and most clearly contoured granules, I performed most of the following experiments in HeLa cells.

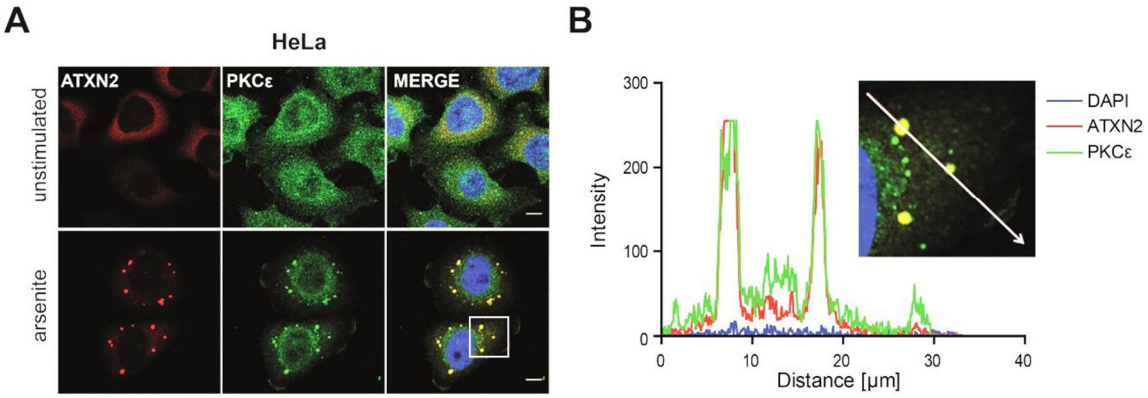

Figure 13: PKCε colocalizes with stress granules in arsenite stimulated HeLa cells. A) Confocal images of unstimulated or 0.5 mM arsenite (1 h) treated HeLa cells. Stress granules were stained with Ataxin-2 (red). PKCε (green) was mainly distributed all over the cytoplasm accumulating different sized foci in unstimulated (upper panels) as well as arsenite treated cells (lower panels). Large PKCε foci in arsenite treated cells colocalized with stress granules. Nuclei were stained with DAPI (blue). Scale bar is 10 µm. B) The intensity profile of the enlarged section marked with a rectangle in A) of arsenite treated HeLa cells illustrate a colocalization between PKCε and stress granules.

7.1.10 Summary part I

In the first part of my thesis I asked, if PKCε is localized to translation modulating stress granules. I investigated stress granule formation in the neuronal F-11 cell line upon arsenite and heat treatment. I found that the shape of the granules and the kinetics of their formation differ dependent on the stimulus. I observed that PKCε is recruited to stress granules upon both, arsenite and heat stress in F-11 cells. Quantifying the colocalization of stress granules with PKCε granules, I found that all stress granule positive cells showed colocalization of stress granules with PKCε. I also observed stress granule formation in neurites, but quantification of colocalization with PKCε requires further research. Stress granule induction in primary DRG neurons turned out to be difficult and was therefore

neglected. To investigate if PKCε translocates to stress granules also in other cell lines, I established stress granule induction in HEK293T and HeLa cells as it was published in literature, and investigated the colocalization with PKCε. Colocalization between PKCε granules and stress granules was also observed in HEK293T and HeLa cells. Since HeLa cells showed the clearest granules in terms of brightness and shape, they turned out to be the best cells for the subsequent studies.

7.2 PKCε is recruited to P-bodies in HeLa cells

In the PKCε colocalization studies with F-11 cells reported above, I observed PKCε granules that were present already in unstressed cells. Additionally, I found PKCε granules that were not colocalized with stress granules in stressed cells but were located in close proximity to stress granules or even partially overlapping (Figure 14). Thus, I asked now of what nature these other PKCε granule could be.

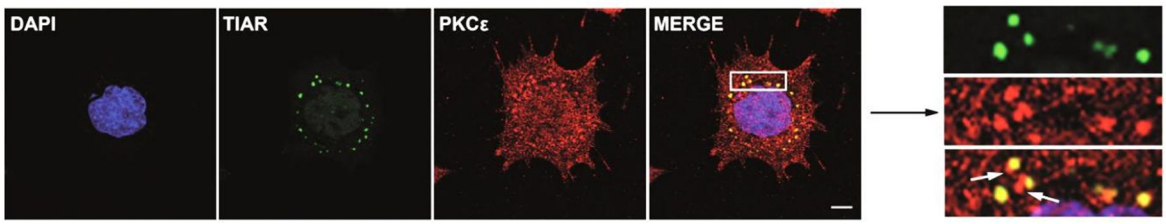

Figure 14: PKCε granules not colocalizing with stress granules are often located adjacent to them. F-11 cells were stressed with 0.5 mM arsenite (1 h) and stained for PKCε (red) and the stress granule marker TIAR (green). If stress granules and PKCε granules are not colocalized, they are often adjacent to stress granules or partially overlapping, which is indicated with the two white arrows in the magnification of the rectangle on the right hand side. Nucleus was stained with DAPI, scale bar is 10 µm.

P-bodies are another RNA granule type. Often they are localized in close vicinity to stress granules and probably interact functionally with them by changing material (Figure 15 and (Kedersha, Stoecklin et al. 2005, Wilczynska, Aigueperse et al. 2005)). Another feature of P-bodies is that they are also present in unstressed cells (Figure 15, upper line). As also the PKCε granules, which did not colocalize with stress granules, were present in unstressed cells, and as these granules were close to stress granules in stressed cells, they could be P-bodies.

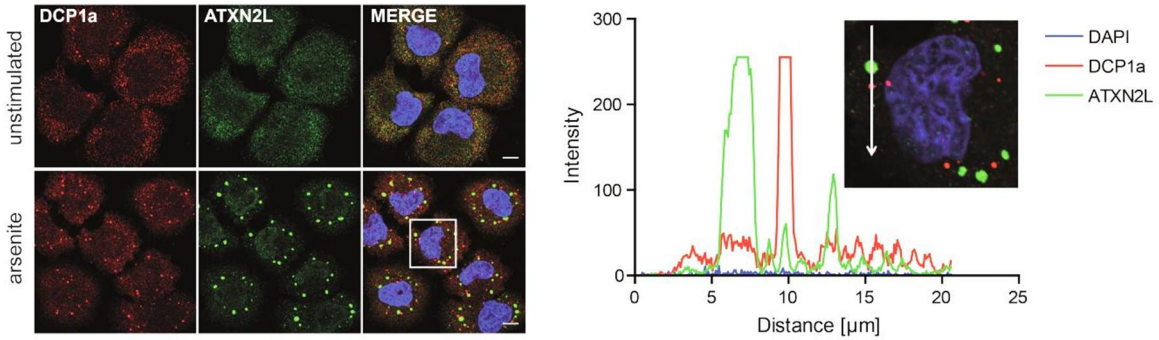

Figure 15: Stress granules and P-bodies are often located in close vicinity. Unstimulated or arsenite stressed (0.5 mM, 1h) HeLa cells were stained for the P-body marker DCP1a (red) or the stress granule marker Ataxin-2-Like (ATXN2L, green). Stress granules and P-bodies are often observed in close vicinity to each other, which is illustrated with the intensity profile of the line scan in the magnification on the right hand side. Nuclei were stained with DAPI, scale bar is 10 µm.

Thus, I examined now if PKCε is localized to P-bodies in stressed and unstressed cells. Therefore I stained unstressed and arsenite stressed HeLa cells with PKCε and the P-body marker DCP1a. Indeed, PKCε granules colocalized with P-bodies in stressed as well as unstressed cells (Figure 16).

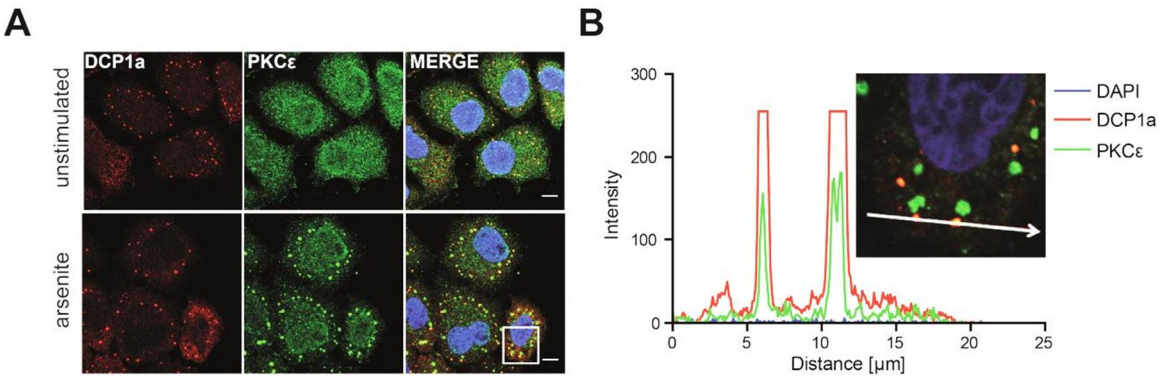

Figure 16: PKCε colocalizes with P-bodies in stressed and unstressed HeLa cells. A) Confocal images of 0.5 mM arsenite (60 min) stressed HeLa cells showed a colocalization between DCP1a stained P bodies (red) and PKCε (lower panel, green). **B)** Intensity profile showing colocalization of P-bodies and PKCε. Colocalization was even observed in unstressed HeLa cells (A, upper panel). Nuclei were stained with DAPI (blue). Scale bar is 10 µm.

To quantify the extend of colocalization between PKCε granules and P-bodies I counted in 10 cells per condition how many P-bodies were colocalized with PKCε granules. This I performed in unstressed and stressed cells. About 75 % (74.6 ± 5) of P-bodies per cell in unstimulated and ~ 84 % (83.5 ± 4) of P-bodies in arsenite stressed HeLa cells colocalized with PKCε granules (Figure 17 A). This was similar to the observation for stress granules: ~ 89 % (89.4 ± 3) of the stress granules colocalized with PKCε granules upon arsenite treatment (Figure 17 B). Thus, most of P-bodies and stress granules contain PKCε.

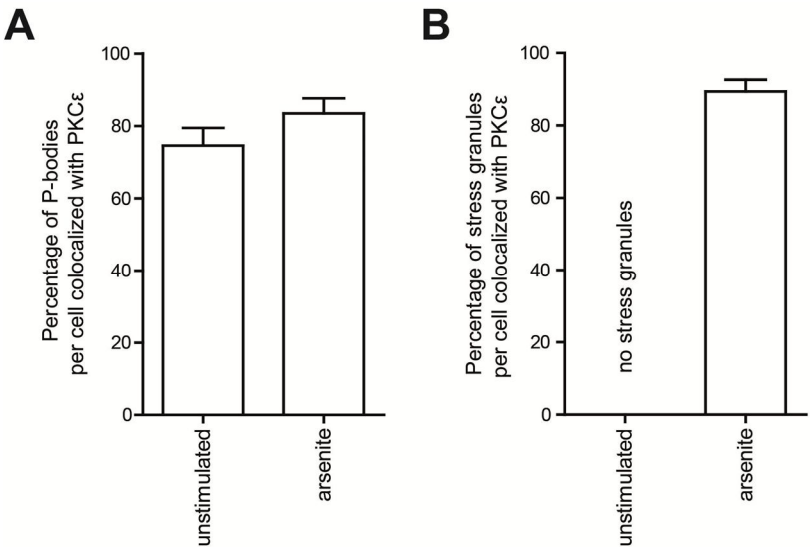

Figure 17: Colocalization of PKCε granules with P-bodies and stress granules, respectively. A) 10 cells were analyzed per condition (unstimulated and stimulated) for colocalization between P-bodies and PKCε granules. For each P-body the cell contained, colocalization with PKCε was verified by eye. In unstimulated HeLa cells 75 ± 5 % of P-bodies per cell colocalized with PKCε granules. In arsenite stressed HeLa cells 84 ± 4 % of P-bodies per cell colocalized with PKCε granules. **B)** Performed as in A). In unstimulated cells no stress granules exist. In arsenite treated cells the colocalization between stress granules and PKCε granules was with 89 ± 3 % similar to the colocalization observed between P-bodies and PKCε in arsenite treated cells. Error bars indicate the SEM.

7.2.1 Summary part II

In the second part of the thesis I identified P-bodies as additional RNA granule type, PKCε is recruited to. In contrast to stress granules, which only appear upon environmental stress, P-bodies exist also in unstressed cells. I found PKCε to be localized to these granules with and without stress. The colocalization of P-bodies and PKCε upon arsenite treatment is similar to the colocalization of PKCε and stress granules. These findings increasingly suggested a role for PKCε in RNA granules and/or in the modulation of protein translation.

7.3 The PKCε binding partners RACK1 and RACK2 are localized to stress granules and P-bodies

To support the localization of PKCε in stress granules and P-bodies, I asked next, if also binding partners of importance for activated PKCε are localized in these RNA granules. If indeed activated PKCε is translocated to stress granules, then also the receptors for activated C kinases (RACKs) should be localized there as well. This idea was supported by the recent finding of RACK1 in stress granules (Arimoto, Fukuda et al. 2008), which was approved during this thesis (Figure 18 A). Although RACK1 preferentially binds βIIPKC it can also anchor PKCε and other signaling proteins with less specificity (Ron, Chen et al.

1994). Beyond the observation of its localization to stress granules, a functional role of RACK1 for stress granule dynamics and/or processes has not been reported. In addition, if also other RACKs are localized to stress granules was so far not known.

Since RACK2 has the highest specificity among the RACKs to anchor PKCε I tested whether RACK2 is localized to stress granules. I stained arsenite stressed HeLa cells (0.5 mM, 1 h) with the stress granule marker Ataxin-2-Like and RACK2. The Ataxin-2-Like antibody was used as a stress granule marker, since the antibodies RACK2 and Ataxin-2 were derived from the same species and could not have been incubated together. Indeed, RACK2 was localized to stress granules (Figure 18 B).

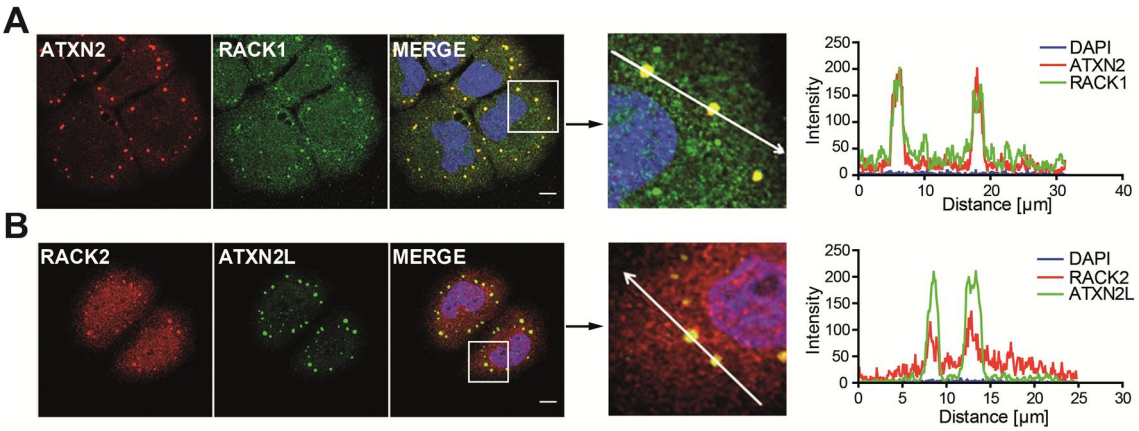

Figure 18: RACK1 and RACK2 are components of stress granules. Confocal images of 0.5 mM arsenite (1 h) treated HeLa cells. **A)** The receptor for activated C kinases 1 (RACK1, green) was recruited to stress granules (Ataxin-2, red). The colocalization is indicated with the intensity profile of the line scan of the enlarged region (right). **B)** The PKCε-selective RACK2 (red) colocalized with stress granules (Ataxin-2-like, green). The colocalization is indicated with the intensity profile of the line scan of the enlarged region (right). Nuclei were stained with DAPI (blue). Scale bar is 10 µm.

I asked now if RACK1 and RACK2 are also potentially anchoring proteins for PKCε in P-bodies. A recently performed knockdown-study of RACK1 did not alter the localization and expression of P-bodies (Otsuka, Takata et al. 2011). This showed that RACK1 has no influence on the formation of P-bodies, but whether it is recruited to P-bodies was so far not published. When I stained arsenite stressed HeLa cells (0.5 mM, 1 h) for the P-body marker DCP1a and RACK1 I observed a clear colocalization between P-bodies and RACK1 granules (Figure 19 A). To investigate whether the PKCε specific RACK2 is likewise located to P-bodies I stained arsenite stressed HeLa cells (0.5 mM, 1 h) for the P-body marker DDX6 and RACK2. I observed also RACK2 to be localized to P-bodies (Figure 19 B).

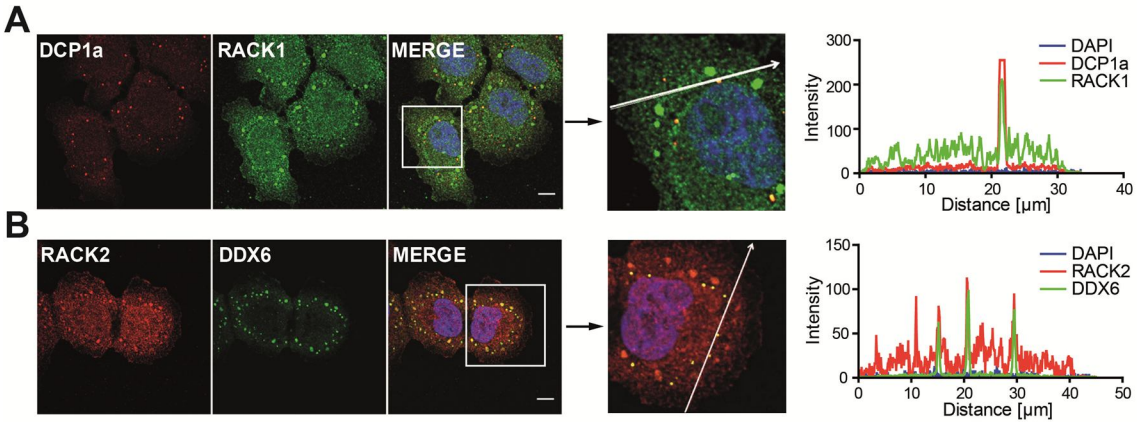

Figure 19: RACK1 and RACK2 colocalize with P-bodies. Confocal images of 0.5 mM arsenite (1 h) treated HeLa cells. **A**) The receptor for activated C kinases I (RACK1, green) colocalized with P-bodies (DCP1a, red). The colocalization is indicated with the intensity profile of the line scan of the enlarged region (right). **B)** The PKCε-selective RACK2 (red) colocalized with P-bodies (DDX6, green). The colocalization is indicated with the intensity profile of the line scan of the enlarged region (right). Nuclei were stained with DAPI (blue). Scale bar is 10 µm.

7.3.1 Summary part III

In this part, I supported my observed colocalization of PKCε with stress granules and P-bodies by investigating the localization of the PKCε binding partners RACK1 and RACK2 in these RNA granules. RACK2 has a high specificity for PKCε anchoring whereas RACK1 preferentially binds βIIPKCs but interacts with less affinity also with the ε-isoform of PKCs. The receptors for activated C kinases, RACK1 and RACK2, were located both to stress granules and P-bodies. Thus, PKCε is potentially active when localized to stress granules and P-bodies.

7.4 PKCε modulates the formation of stress granules

So far, I have shown that PKCε is recruited to stress granules during cellular stress. Now I examined if PKCε is functionally relevant for the formation of stress granules.

7.4.1 Acute activation of PKCε does not affect stress granule formation

The localization of PKCε to stress granules could indicate an involvement of PKCε in stress granule formation. Therefore I tested if activation of PKCε alters the process of stress granule formation. I tested two different activators: Estrogen (E2) since it was shown to trigger a translocation of PKCε, and PMA as a general activator of protein kinases (Hucho, Dina et al. 2006). The workflow complies with Figure 3 with the extension that 0.2 mM arsenite stress not only was applied alone for 15, 20, 30, and 60 min, but also in combination with estrogen or PMA (Figure 20). Arsenite, estrogen, and PMA treatment and unstimulated cultures were used as controls. F-11 cells were stimulated for 15, 20,

30, and 60 min. After staining, I analyzed 100 cells per condition concerning to be stress granule positive or not. The experiment was performed three times in duplicates.

Treatment with 10 nM estrogen (Figure 20 A) or 1 µM PMA (Figure 20 B) resulted in the same number of stress granule positive cells as obtained for unstimulated control cultures. Hence, neither estrogen- nor PMA-induced PKCε activation is able to induce stress granule formation.

Also, when estrogen and arsenite were applied together for 15, 20, 30 or 60 min, I found no significant change in the percentage of stress granule positive cells compared to arsenite treatment alone (Figure 20 A). The same was true for PMA applied together with arsenite (Figure 20 B). Thus, activation of PKCε does not alter stress granule formation.

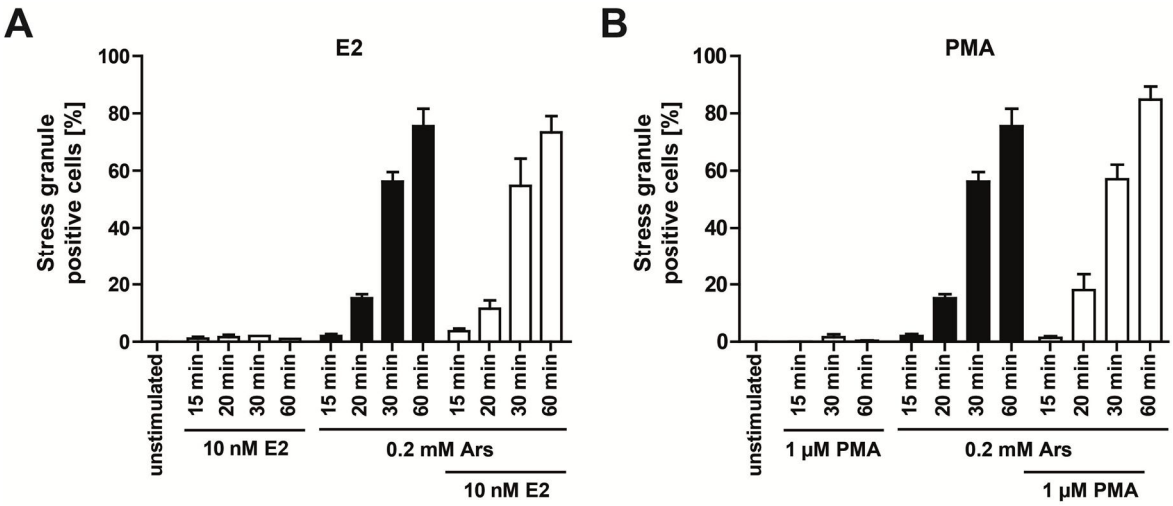

Figure 20: Stress granule formation is not affected by PKC activators. A) F-11 cells were treated with 0.2 mM arsenite in combination with 10 nM estrogen (E2) for 15, 20, 30, and 60 min. Stress granule positive cells were counted by eye as described before and compared to arsenite treatment alone. E2 treatment alone and unstimulated cells were used as controls. E2 treatment alone did not alter the number of stress granule positive cells in a significant manner compared to unstimulated cells. There was no significant change of E2/arsenite treatment compared to arsenite treatment alone. **B)** F-11 cells were treated with 0.2 mM arsenite together with 1 µM PMA for 15, 20, 30, and 60 min. PMA treatment alone and unstimulated cells were used as controls. PMA treatment alone did not alter the number of stress granule positive cells. There was no significant change of PMA/arsenite treatment compared to arsenite treatment alone. The experiment was performed three times in duplicates. Error bars indicate the SEM.

7.4.2 PKCε knockdown enhances stress granule formation

Next, I investigated whether down-regulation of PKCε resulted in a change in stress granule numbers.

For down-regulation of PKCε I transfected HeLa cells with siRNAs targeting PKCε transcripts. Three negative controls were included: 1) A culture without additional components, 2) a culture as transfection control containing lipofectamine, and 3) a culture

containing a pool of non-targeting siRNAs. First I verified the PKCε knockdown by immunoblotting. Cells were lysed and proteins were separated according to their size using SDS-PAGE. Western Blot analysis revealed that PKCε-specific siRNAs reduced the quantity of detectable PKCε by about 50 % compared to the control pool (Figure 21 A, B).

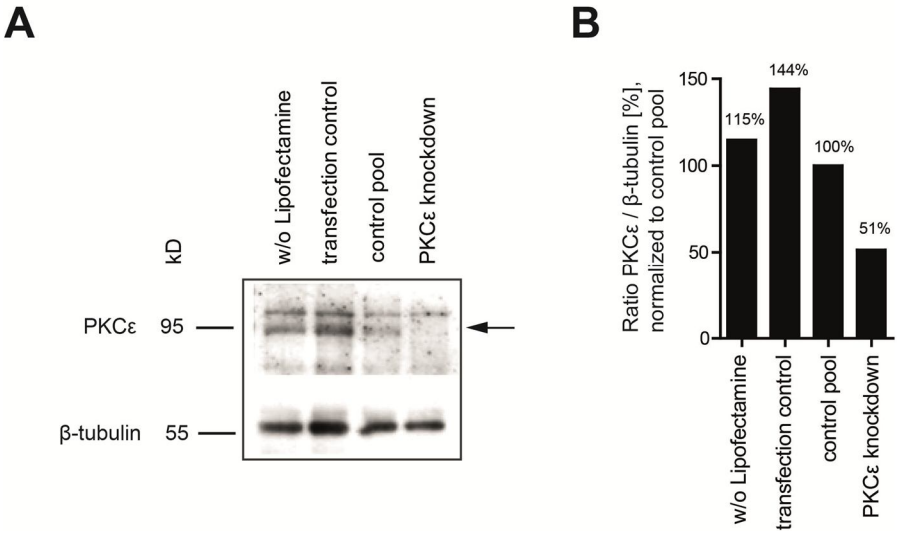

Figure 21: Knockdown of PKCε reduces the amount of intracellular PKCε by 51 %. A) Western blot of PKCε knockdown in HeLa cells. Samples with or without lipofectamin were used as transfection controls. β-tubulin was used as loading control. **B**) The specific PKCε band at 94 kDa was clearly reduced upon PKCε knockdown by 51% in comparison to the control pool.

To study the impact of reduced PKCε levels on RNA granule formation, siRNA-transfected HeLa cells were stressed with arsenite and stress granule formation was analyzed. To evaluate 100 cells and decide whether each of these cells can be accounted as stress granule positive or negative is a valid approach for experiments, where strong all or nothing changes can be expected. However, this method is not sufficient to detect small alterations in e.g. the absolute number of stress granules per cell. Therefore, together with Jörg Isensee in our group we established a more sensitive quantification method. Quantification was accomplished with an automated “High Content Screening” (HCS) microscope. Briefly, HCS-microscopes take digital images of cell cultures, identify the objects of interest, and quantify aspects of these objects. In our case, we optimized the conditions for the detection of whole cells as well as of stress granules and evaluated for the number of stress granules per cell (for details see Materials and Methods).

Knockdown experiments, long-term cell culture in 96 well plates, and analysis by HCS microscopy resulted in most robust data using HeLa cells. This was due to the relatively homogenous shape of the HeLa cells and due to their robustness against siRNAs compared to F-11 cells. Knockdown experiments lead to more cell loss in F-11 cells than in HeLa cells. Thus, the following experiments were performed with HeLa cells. The

workflow of the following experiments is shown in Figure 22. Briefly, HeLa cells were cultured in 96 well plates and stressed with 0.5 mM arsenite for 30 min upon PKCε knockdown. The cells were fixed with PFA and immunofluorescently labeled for Ataxin-2 (stress granule identification) and DAPI (cell nuclei identification) and analyzed by the HCS-microscope. Cell identification via the identification of cell nuclei was the most robust method for this study as due to the flat shape of HeLa cells, all cell nuclei were located in the same focus layer.

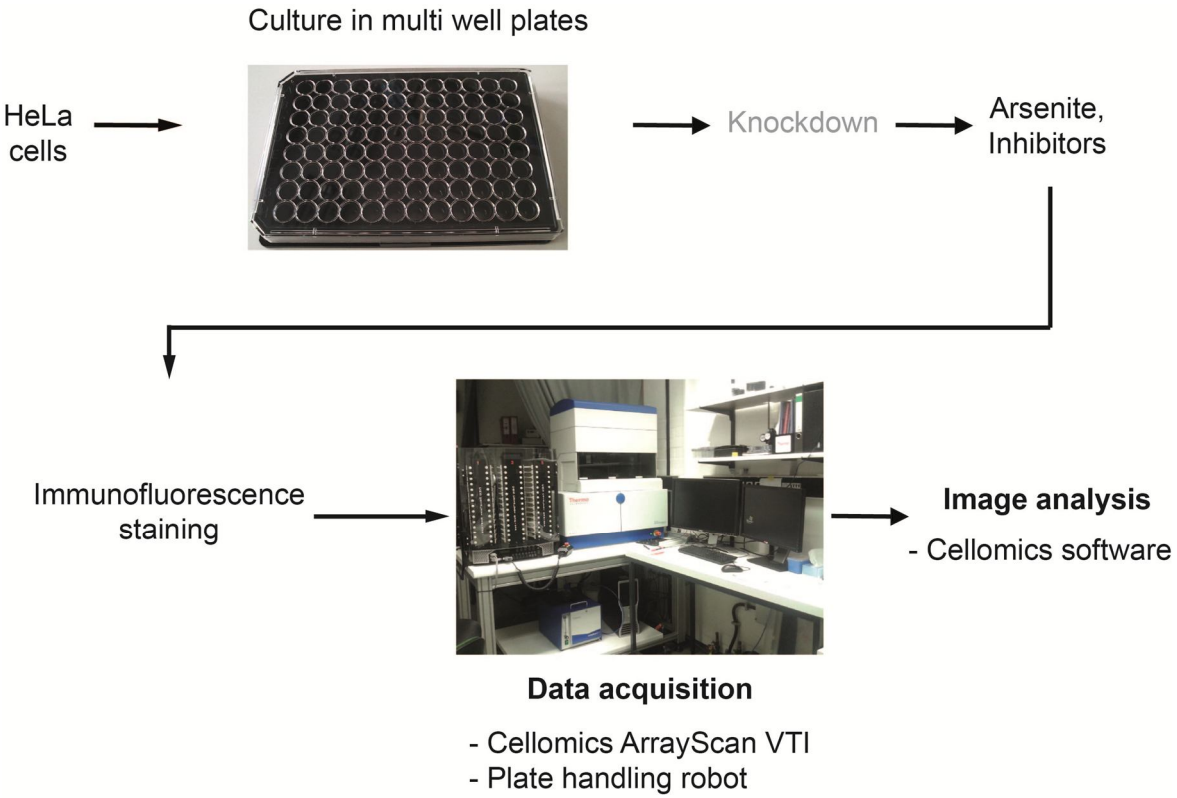

Figure 22: Workflow generating data using high content microscopy. HeLa cells were cultured in 96 well plates. The cells were optional treated with siRNAs followed by arsenite treatment or use of inhibitors. After fixation, permeabilization and blocking they were stained with antibodies and the plates were sealed with a plastic foil for the microscope. Data acquisition was performed with the Cellomics Array Scan VTI. Data and images were analyzed with Cellomics High Content Screening software.

Settings for best nuclei quantification were further optimized in the scan software based on bio application colocalization V4. We established nuclei identification by adjusting object size, shape, and intensity parameters as described in the method section (Figure 23 A). Stress granules were identified using the same parameters in a predefined area around the nucleus, which identified in good approximation the cytoplasm (Figure 23 B). Again, the adjustment of object size, shape, and intensity parameters was of critical importance. If the parameters were set to stringent, small stress granules could not have been detected, which results in loss of assay sensitivity. On the other hand, the number of artifacts increased drastically if the parameters were not set stringent enough. Thus,

optimal adjustment is of enormous importance for the quality of the assay. The cells were scanned and results such as stress granules per cell, number of cells, and stress granule intensity were analyzed. Statistical analysis was performed using prism software.

The use of an HCS microscope has several advantages: Once the settings for granule quantification are optimized, they are applied consistently to all cells. Variability due to subjective experimenter decisions is thereby excluded. In addition, compared to the quantification method I used before, not only 100 cells were analyzed but several thousands, which leads to a higher statistical robustness of the results. Moreover, I now analyzed the number of stress granules per cell instead of stress granule positive cells, which allows detecting even marginal changes. Also, during a scan much more information is gained than only the number of stress granules per cell. For example information about total intensity and size of each single detected granule but also total cell numbers, culture density and cell size can be evaluated as well. This allowed also a stringent control for comparability of culture and staining conditions.

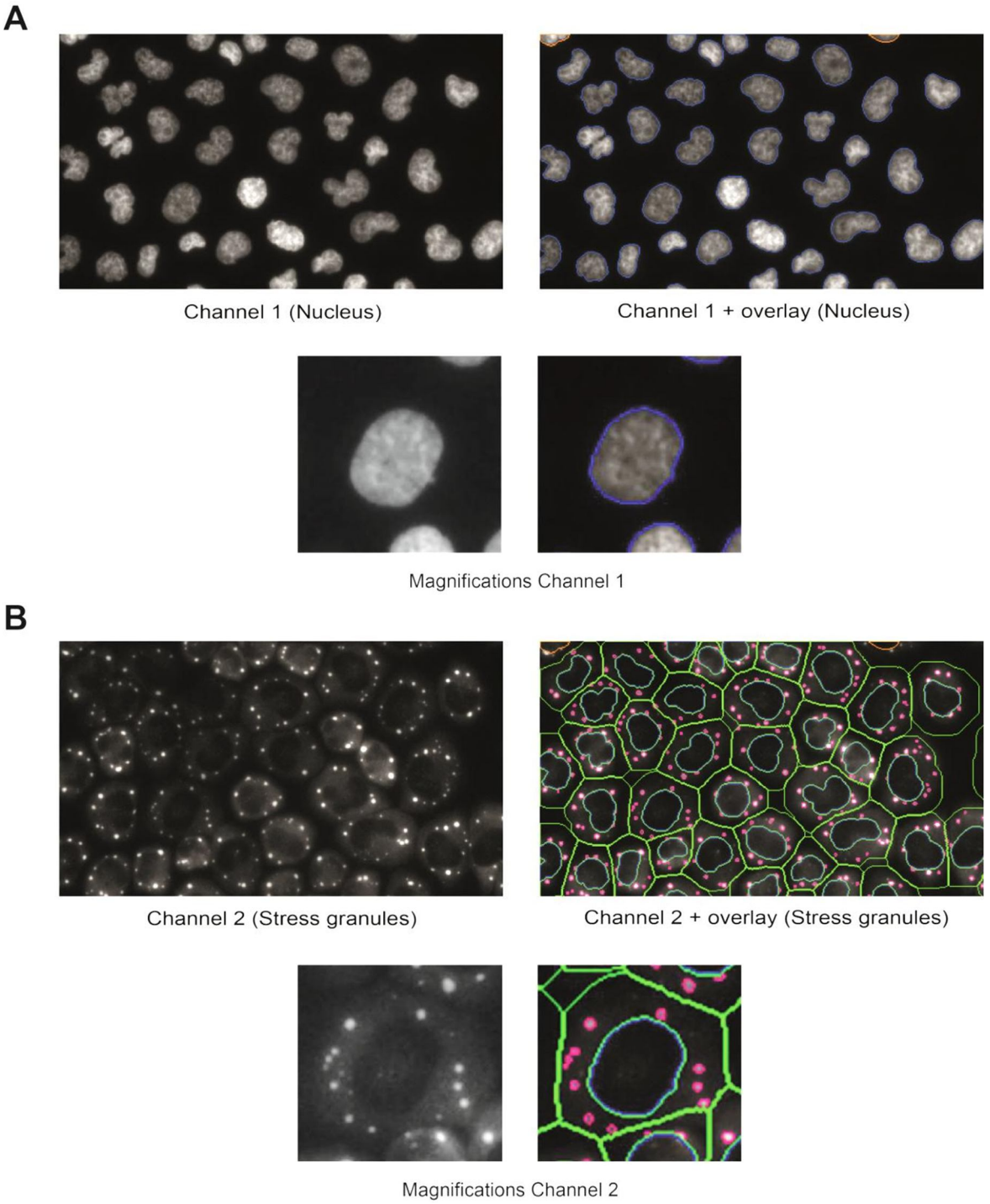

Figure 23: Stress granule identification with Cellomics software. A) Section of view field showing HeLa cell nuclei without (left) and with blue overlay after identification (right). Orange circles are nuclei eliminated by the software due to non-matching secondary selection parameters such as the variance of pixel intensity. **B)** Sections of view fields showing stress granules in HeLa cells without (left) and identified stress granules with overlay (right). Identified stress granules are displayed by red circles, cell nucleus by blue circles and cell shape in green. Orange circles indicate cells excluded by secondary parameters such as the variance of pixel intensity.

I found that the number of stress granules per cell was increased upon PKCε knockdown by 14 % in arsenite treated cells compared to the cells treated with the non-targeting pool (114 ± 2 % vs. 100 ± 0 %, $p < 0.001$, n = 3 experiments, Figure 24 A). The mean of the total area of stress granules as well as average intensity remained unaffected by PKCε knockdown (Figure 24 B, C). Down-regulation of PKCε hence increases the formation of stress granules.

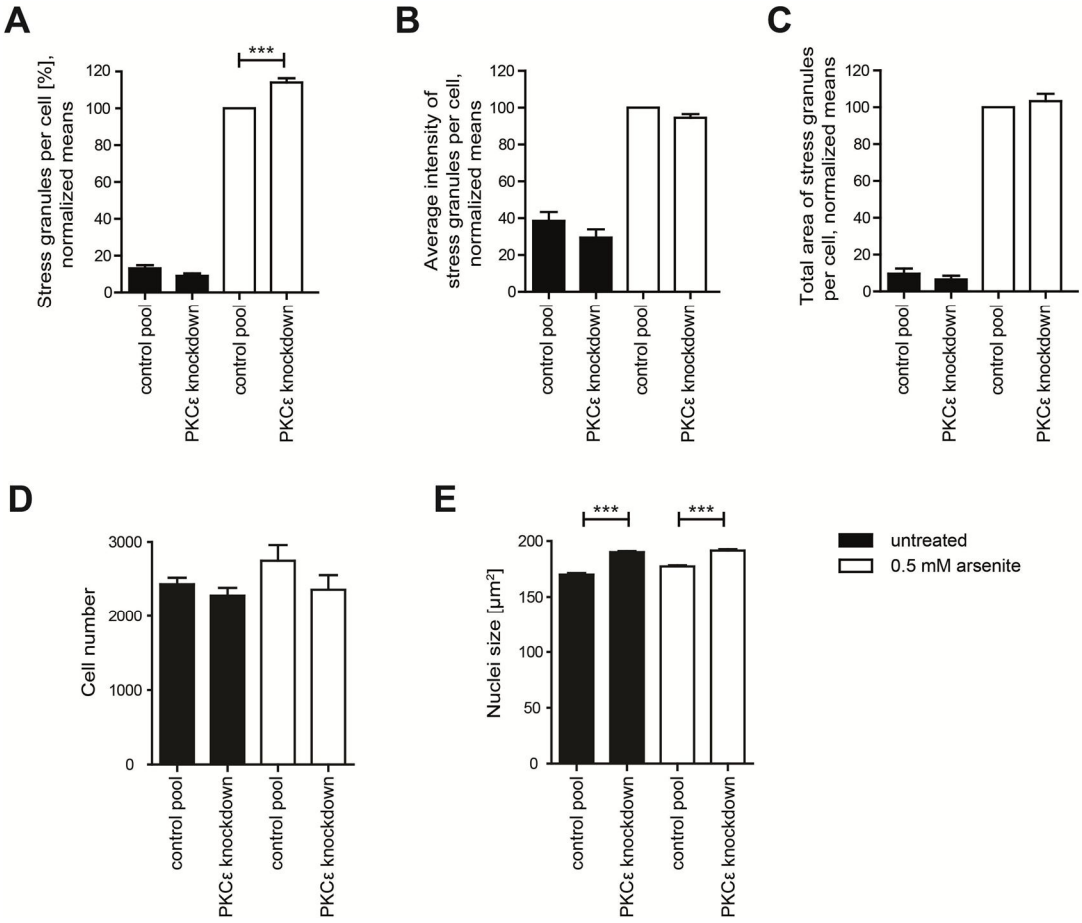

Figure 24: PKCε knockdown increases stress granule formation. A) Upon arsenite treatment the percentage of stress granules per cell was about 14 % higher compared to the control pool (114 ± 2 % vs. 100 ± 0 %, $p < 0.001$). Without arsenite treatment only a very low number of stress granules exist. **B, C)** PKCε knockdown did neither affect the average intensity of stress granules per cell (B) nor the total area of stress granules per cell (C). **D)** The transfection of siRNAs had no impact on cell survival. **E)** The nuclear size was significantly increased upon PKCε knockdown. Data were normalized to the control pool of arsenite treated cells. Three experiments were performed in triplicates with ~2000-5000 cells per well. Error bars indicate the SEM.

Although the PKCε knockdown effect on stress granules was highly significant, it was still small. Thus, additional control experiments such as the influence of transfection on cell survival were advisable to exclude artifacts. I tested therefore for the comparability of the culture conditions. The transfection of siRNAs had no impact on cell survival, which allows comparison of the cultures. (Figure 24 D). The nuclear size was significantly increased upon

PKCε knockdown (Figure 24 E) potentially also indicating a change in the transcription/translation process.

7.4.3 PKC inhibitors increase stress granule formation

To confirm the results from PKCε knockdown experiments, I tested for the impact of PKC inhibitors on stress granule formation in a pharmacological approach. Three different PKC inhibitors were used: BIM, a competitive inhibitor for the ATP binding site of general PKCs, the classical PKC inhibitor Gö6976, and Ro32-0432, a PKC inhibitor with preference for the ε isoform. The cells were pre-treated with the appropriate inhibitor for the recommended time followed by 0.5 mM arsenite stimulation. In contrast to the knockdown experiments, this did not affect the fold change of stress granules per cell compared to arsenite treatment in a statistically significant manner (Figure 25).

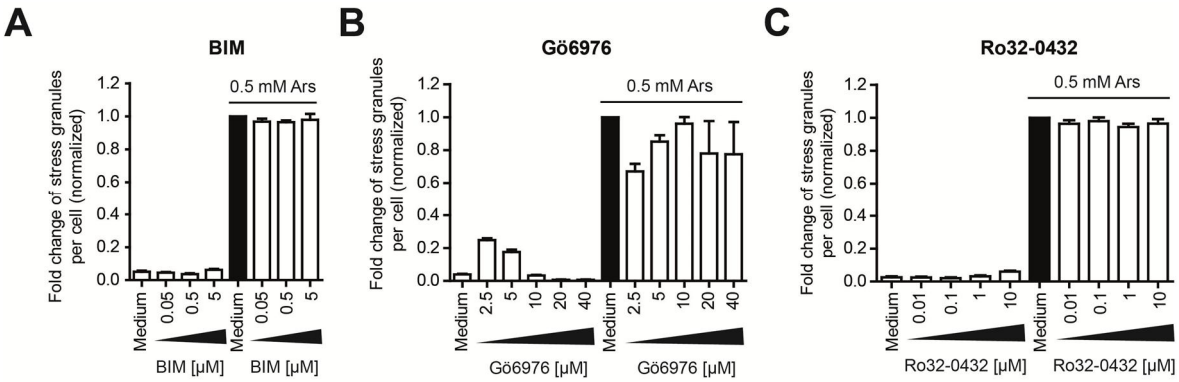

Figure 25: PKC inhibitors do not affect stress granule formation if applied with 0.5 mM arsenite. A) HeLa cells were 15 min pre-treated with the general PKC inhibitor BIM (0.005, 0.5, 5 µM) and stressed with 0.5 mM arsenite for 30 min, during which BIM was still present. The combination of BIM treatment and 0.5 mM arsenite stress did not affect stress granule formation compared to 0.5 mM arsenite stress applied alone. **B)** HeLa cells were 15 min pre-treated with the classical PKC inhibitor Gö6976 (2.5, 5, 10, 20, 40 µM) and stressed with 0.5 mM arsenite for 30 min, during which Gö6976 was still present. No effect of the inhibitor treatment with arsenite stress compared to arsenite stress alone could be detected. **C)** HeLa cell were 30 min pre-treated with the PKCε specific inhibitor Ro32-0432 (0.001, 0.1, 1, 10 µM) and stressed with 0.5 mM arsenite, during which Ro32-0432 was still present. There was no effect on stress granule formation compared to arsenite treatment alone detectable. Data were normalized for each inhibitor to 0.5 mM arsenite treatment alone. The experiment was performed three times in triplicates with ~ 2000 – 5000 cells per well. Error bars indicate the SEM.

One possible explanation could be the different incubation times of pharmacological inhibitors and siRNAs. Cells were treated with inhibitors only up to a maximum of 1 h, but cells were subjected to siRNAs for 48 h. The effect achieved with inhibitors is therefore not sufficient to affect the stress granule formation in response to very strong stressors such as 0.5 mM arsenite. Nevertheless, potentially, a lower arsenite dose could allow the detection of more subtle changes in stress granule formation as thereby a saturation of

stress granule formation is avoided. Thus, I performed a dose-response experiment with arsenite (0.3, 0.2, 0.1 mM) together with the classical inhibitor BIM (0.5, 5 µM). I found that 0.1 mM arsenite treated together with BIM led to the strongest increasing effect on stress granule formation (Figure 26).

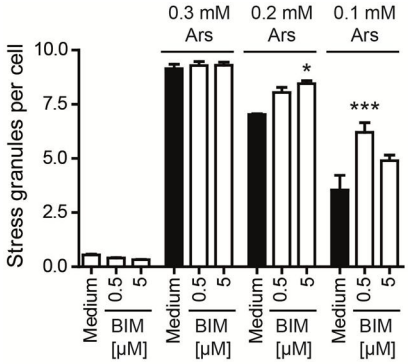

Figure 26: Arsenite-dose-response experiment shows a BIM-dependent increase in stress granule formation. HeLa cells were pre-treated with BIM (0.5, 5 µM) and 0.3, 0.2 or 0.1 mM arsenite was applied additionally for 30 min. BIM did not affect the formation of stress granules if applied together with 0.3 mM arsenite, but it altered the number of stress granules per cell if treated with 0.2 (5 µM BIM, * $P < 0.05$) or 0.1 mM arsenite (0.5 µM BIM, *** $P < 0.001$). The experiment was performed in triplicates with ~ 2000 – 5000 cells per well. Error bars indicate the SEM.

Therefore I repeated the inhibitor dose-response experiments with an arsenite dose of 0.1 mM. First I pre-treated the cells with the common PKC inhibitor BIM for 15 min followed by 0.1 mM arsenite stimulation for 60 min. Now, corroborating the results of the knockdown experiments, I observed a dose-dependent significant increase in stress granule formation upon PKC inhibition (Figure 27 A). Pre-treatment with 1 µM and 5 µM BIM significantly increased the number of stress granules 1.7 ± 0.2 or 2.2 ± 0.3-fold, respectively (**$P < 0.01$, ***$P < 0.001$).

Not only the number of stress granules per cell increased but also the number of cells without stress granules decreased (Figure 27 E). This indicates that the induction of stress granule formation occurs predominantly in cells without or with low numbers of granules. For instance I observed for cells lacking granules a probability density of ~ 0.16 in control cells without inhibitor. Compared to 0.05 (1 µM BIM) and 0.04 (5 µM BIM) in case of BIM pre-treatment. This is a reduction by ~ 69 % for 1 µM BIM and 75 % for 5 µM BIM (Figure 27 E). Also, this explained why PKC inhibition in combination with the high arsenite dose of 0.5 mM failed. There, the number of cells without or with low numbers of stress granules was probably very low already.

Similar results I obtained with the classical PKC inhibitor Gö6976. Pre-treatment with Gö6976 significantly induced the formation of stress granules at 10, 20, and 40 µM after

arsenite treatment (Figure 27 B). The increase of the fold change of stress granules per cell was 74 % (10 µM, P** < 0.01), 97 % (20 µM, ***P < 0.001), and 115 % (40 µM, ***P < 0.001). As Gö6976 inhibits predominantly the classical PKC isoforms α and β, this indicates beyond PKCε also the involvement of classical PKCs in stress granule formation. However, in part this effect is based on an inhibitory role of solvent DMSO at least for 40 µM Gö6976 (Figure 27 D). Similar to BIM inhibitor, stress granules formation occurred predominantly in cells without or with a low number of stress granules as the probability density distributions indicate a shift towards higher numbers of stress granules per cell (Figure 27 F). With a probability density of ~ 0.23 the cells did not have any stress granule in the control cells compared to ~ 0.16 (2.5 and 5 µM), ~ 0.1 (10 µM), 0.05 (20 µM, and 0.02 (40 µM) for Gö6976 pre-treated cells.

Inhibition of PKCε with the specific inhibitor Ro32-0432 resulted in increased numbers of stress granules at inhibitor concentrations > 10 µM. There, cells contained 1.58 ± 0.2 -fold increased number of stress granules per cell (***P < 0.001; Fig. 27 C). With ~ 0.11 (10 µM Ro32-0432) the probability density of cells without stress granules was 1.5 times reduced compared to the control cells (0.17; Figure 27 G).

Thus, blocking of PKCs in general and blocking of PKCε in particular increased the formation of stress granules. These findings corroborate the results obtained by knockdown of PKCε. However, the results also indicate that stress granule formation is modified not only by PKCε alone but by multiple PKC isoforms.

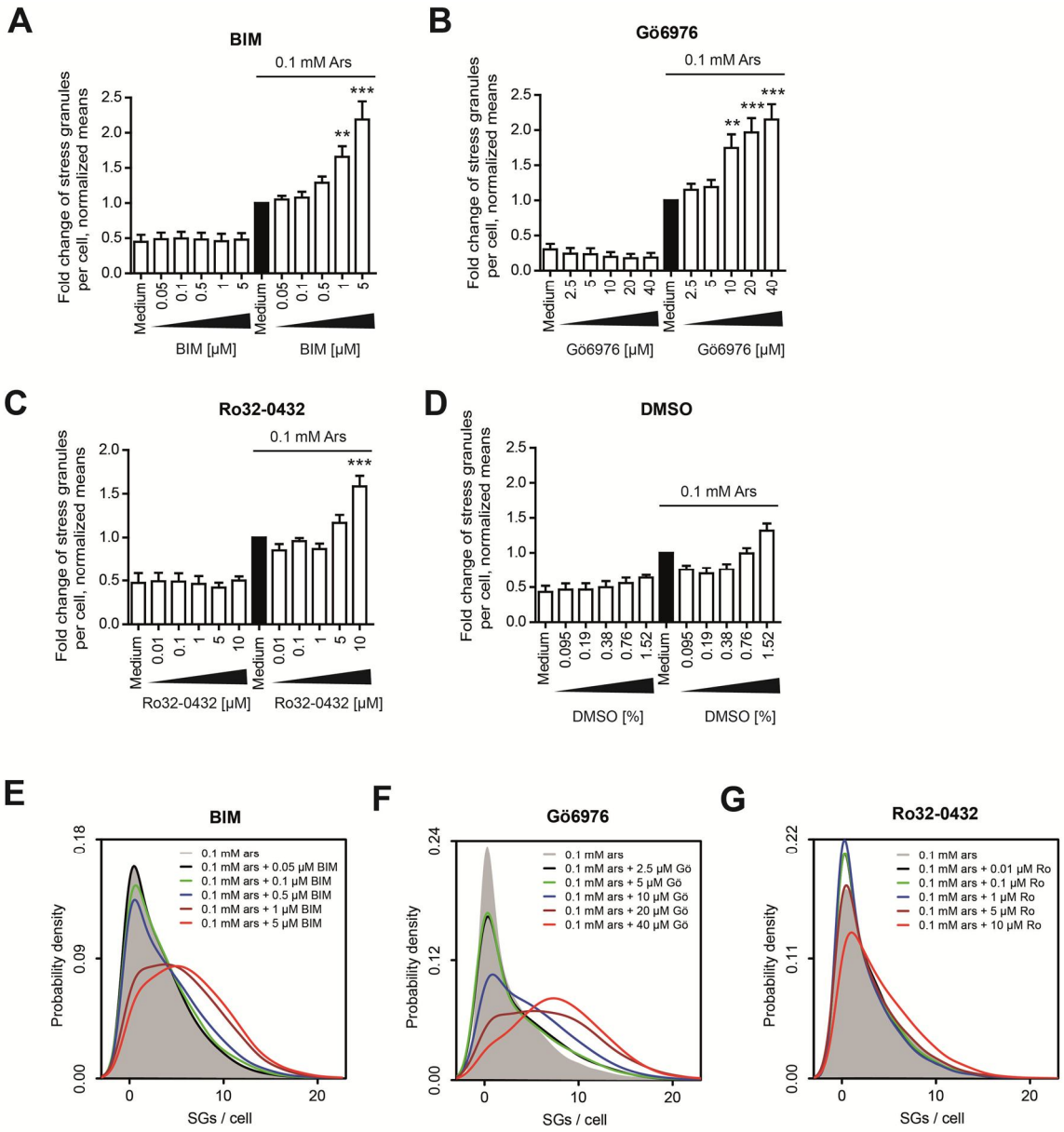

Figure 27: Inhibition of PKCs increases stress granule formation. A) Dose response pre-treatment with the general PKC inhibitor BIM before 0.1 mM arsenite treatment for 60 min led to a highly significant increase of the fold change of stress granules per cell for 1 µM (66 %, **P < 0.01) and 5 µM (119 %, ***P < 0.001). Data were normalized to medium + arsenite control without inhibitor. **B)** With the selective PKCα and β inhibitor Gö6976 a significant increase of stress granules per cell was observed for 10 µM (74 %, P** < 0.01), 20 µM (97 %, ***P < 0.001), and 40 µM (115 %, ***P < 0.001). **C)** Pre-treatment of 10 µM Ro32-0432, a PKCε-selective inhibitor, induced a significant increase of stress granules per cell by 58 % (***P < 0.001). **D)** Solvent control belongs to Gö6976, which is dissolved in DMSO. No significant change was detectable after DMSO pre-treatment. **E)** Probability density of stress granules per cell upon BIM pre-treatment. Compared to the medium control more stress granules per cell existed in BIM pre-treated HeLa cells. Furthermore the number of cells without or with only a few stress granules decreased with increasing inhibitor concentrations. **F)** Probability density of stress granules per cell upon Gö6976 pre-treatment. Similar results were observed compared to BIM inhibitor. **G)** Probability density of stress granules per cell upon Ro32-0432. Three experiments were performed in triplicates with ~2000-5000 cells per well.

7.4.4 Summary part IV

In this part of my thesis I investigated the impact of PKCε on stress granule formation. I found that an activation of PKCε with estrogen or PMA did not alter the formation of stress granules. Nevertheless, establishing a HCS microscopy approach, which allows analysis on the single cell level, I observed significantly enhanced stress granule formation per cell upon PKCε knockdown. Also, HCS microscopy provides additional information such as granule size and intensity. I found that stress granule intensity and size were not affected whereas the nuclear size of the cells was significantly increased by PKCε knockdown. Pharmacological PKC inhibition with general, classical, and novel PKC inhibitors increased the number of stress granules per cell as well. Moreover, my data indicated that stress granule formation occurred predominantly in cells without or with a low number of stress granules. All together, these findings suggested that stress granule formation is modulated by PKC isoforms including PKCε.

7.5 Reduction of PKCε availability influences P-body formation

PKCε knockdown and inhibition affected stress granule formation. PKCε was also found to be localized to P-bodies, which are known to have in part overlapping functions as stress granules including translational control. Thus, I tested now whether PKCε knockdown or inhibition also alters the formation of P-bodies.

7.5.1 PKCε knockdown enhances P-body formation

P-bodies were stained with an antibody against the P-body component DCP1a. For P-body identification we established an HCS approach based on stress granule identification criteria. Since P-bodies have a smaller size than stress granules the HCS parameters for P-body identification were adjusted, while the nuclei-based cell identification parameters were not changed (Figure 28 A, B). To avoid the measurement of artifacts instead of P-bodies, we set the parameters more stringent (see Methods section). In a tradeoff between sensitivity and specificity, some P-bodies of a very small size could not been included in the quantification. Analyzing the data, I found that the number of P-bodies per cell showed an increase of 22 % upon PKCε knockdown compared to the control cells (122 ± 2 % vs. 100 ± 0 %, $p < 0.001$, n = 3 experiments, Figure 28 C). Down-regulation of PKCε increased the number of P-bodies even in unstressed HeLa cells by 11 %, although this increase was not significant (64 ± 5 % vs. 53 ± 3 %, $p > 0.05$, n = 3 experiments, Figure 28 C, black bars). As observed for stress granules, the average intensity did not change upon PKCε knockdown. However, I found a significant increase of 24 % in the total area of P-bodies per cell compared to the control pool (124 ± 3 % vs. 100 ± 0 %, $p < 0.01$; Figure 28 D, E).

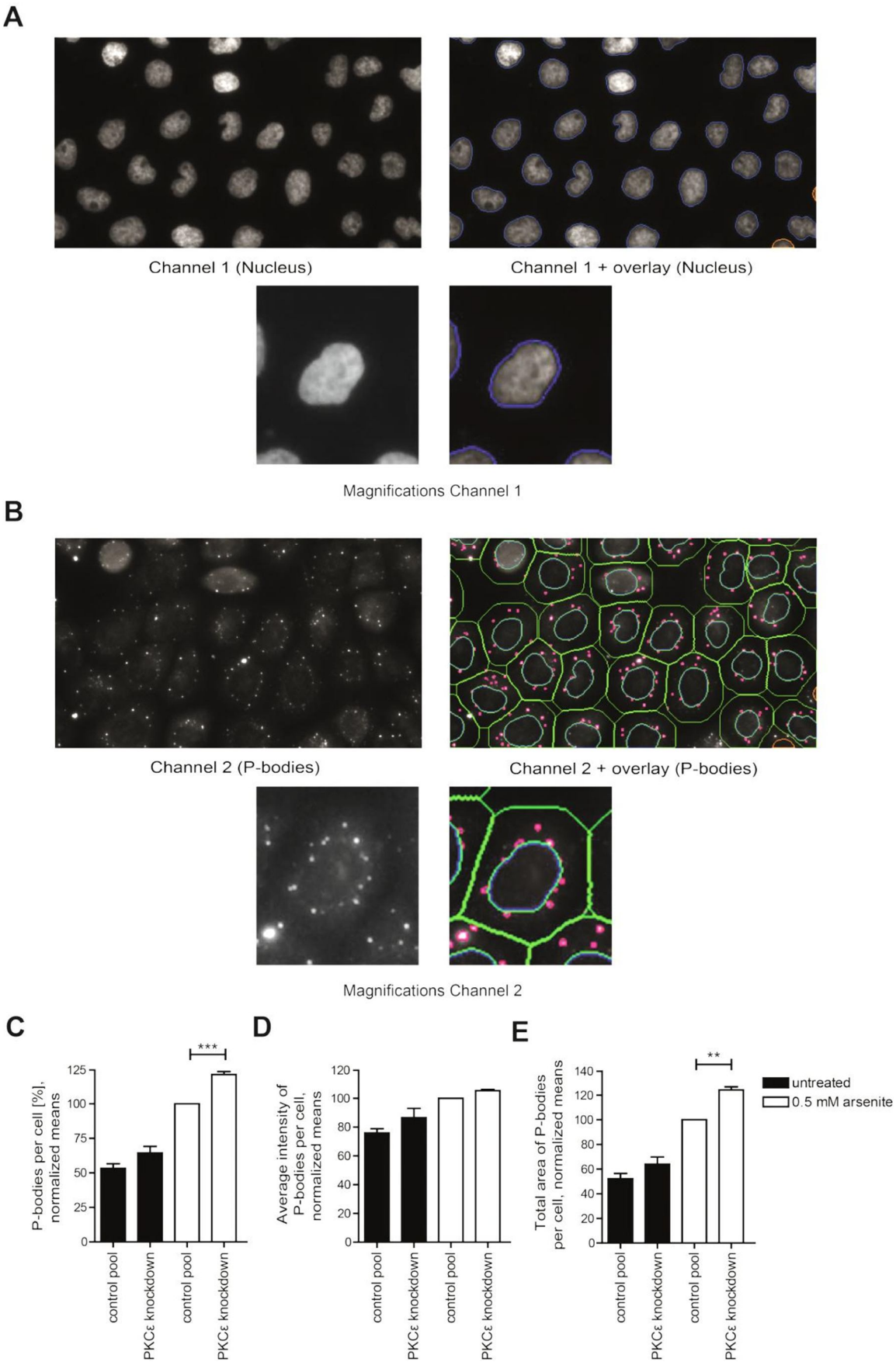

Figure 28: PKCε knockdown increases P-body formation. A) Section of view field showing nuclei identification (blue circles, right) with the Cellomics software. Orange circles are nuclei eliminated by the software due to non-matching secondary selection parameters such as the variance of pixel intensity. **B)**

Section of view field showing P-body identification. Left: HeLa cells without software-based identified regions of interest (ROIs). Right: P-body identification in HeLa cells displayed by red circles, cell nucleus by blue circles and cell shape in green. Orange circles indicate cells excluded by secondary parameters such as the variance of pixel intensity. **C)** PKCε knockdown resulted in an increase in the percentage of P-bodies per cell, which is 22 % compared to the control pool (122 ± 2 % vs. 100 ± 0 %, $p < 0.001$, ± SEM). A slight but not significant increase of 11 % was also observed in unstressed cells (64 ± 5 % vs. 53 ± 3 %, $p > 0.05$, ± SEM, black bars). **D, E)** Average intensity of P-bodies per cell was not affected by PKCε knockdown while the total area of P-bodies per cell is increased by about 24 % compared to the control pool (124 ± 3 % vs. 100 ± 0 %, $p < 0.01$, ± SEM). Three experiments were performed in triplicates with ~2000-5000 cells per well.

7.5.2 PKCε activity is not required for P-body formation

Reduction of PKCε expression as well as PKC inhibitors increased stress granule formation. Down-regulation of PKCε also increased P-body formation. Thus, next I examined, if also inhibitors of PKC had an effect on P-body formation.

HeLa cells were pre-treated with different concentrations of BIM, Gö6976 (both 15 min pre-incubation), or Ro32-0432 (30 min pre-incubation) before 0.1 mM arsenite treatment for 1 h (Figure 29). P-bodies were stained with DCP1a. In contrast to PKCε knockdown, P-body formation was neither affected by general PKC inhibition using BIM nor by inhibition of the PKCε by Ro-32-0432 (Figure 29 A, C). Only the specific classical PKC inhibitor Gö6976 caused a significant increase in P-body formation of 14 % for 20 (1.0 ± 0 vs. 1.14 ± 0, $p < 0.001$) and of 18 % for 40 µM (1.0 ± 0 vs. 1.18 ± 0, $p < 0.001$), suggesting an influence of the classical PKC isoforms α and β (Figure 29 B). However, if classical PKCs indeed affect P-body formation, an effect of the general inhibitor BIM should have been observed as well.

I concluded that at least acute PKCε kinase activity is not required in this process. However, the impact of long-term reduction of PKCε using siRNA molecules on P-body formation may differ from acute inhibition using pharmacological inhibitors.

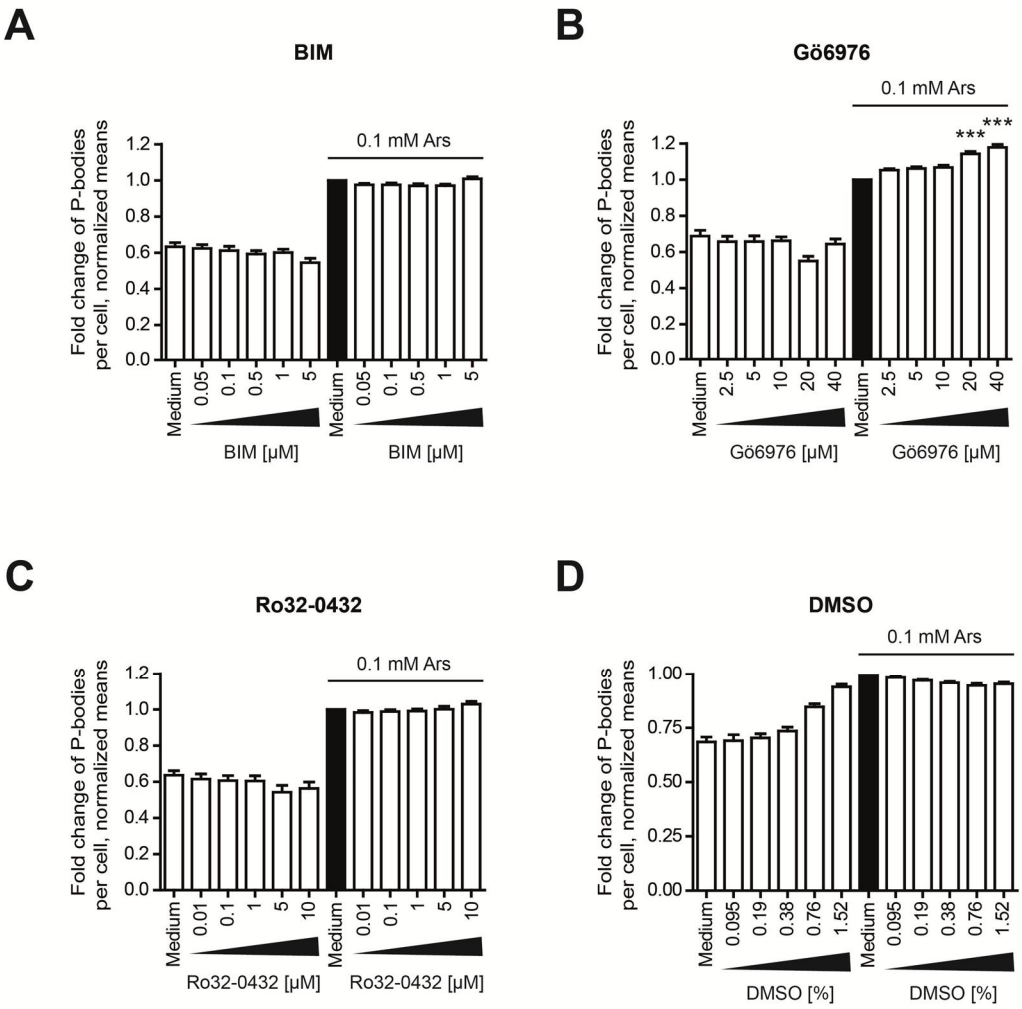

Figure 29: Inhibition of classical PKCs increase P-body formation. A) No significant effect of BIM pre-treated before 0.1 mM arsenite for 60 min on the fold change of P-bodies per cell could be detected. **B)** Significant Gö6976 increasing effects on the fold change of P-bodies per cell of 14 % for 20 µM (1.0 ± 0 vs. 1.14 ± 0, $p < 0.001$, ± SEM) and of 18 % for 40 µM (1.0 ± 0 vs. 1.18 ± 0, $p < 0.001$, ± SEM). **C)** No significant effect of Ro32-0432 pre-treatment was observed. **D)** No effect of DMSO control. 3 experiments were performed in triplicates with ~2000-5000 cells per well.

7.5.3 Summary part V

In this part of my thesis I investigated the impact of PKCε on P-body formation. I found that a knockdown of PKCε not only increases stress granule formation but also P-body formation. In this respect PKCε seems to fulfill similar functions in both RNA granules. On the other hand, PKC inhibition did not affect the formation of P-bodies while it increased the number of stress granules. In P-bodies I only found a slight increase of P-body formation upon inhibition of the α- and β-isoform of PKC by Gö6976, while acute PKCε activity is not involved in the regulation of P-body formation.

7.6 PKCε knockdown reduces protein translation

My study is based on the identification of novel substrates of PKCε, which suggest an involvement of PKCε in protein translation. So far, I described colocalization of PKCε with structures involved in protein translation control, namely stress granules and P-bodies. Further I described that PKCε expression inhibits stress granule formation and P-body formation. In the next section I asked, whether PKCε indeed affects protein translation.

I used a recently established immunofluorescence based approach referred to as FUNCAT (fluorescence non-canonical aminoacid tagging) to identify newly synthesized proteins (Dieterich, Hodas et al. 2010). Unstressed HeLa cells were 30 min starved in a methionine-free medium and then incubated with an azide-bearing artificial azidohomoalanine (AHA), which is incorporated into the proteins substituting methionine (Dieterich, Lee et al. 2007). By coupling the incorporated AHA to the fluorescence dye 5-TAMRA it is possible to detect newly synthesized proteins on a single cell basis (Figure 30 A).

In an assay control experiment co-incubation with the protein synthesis inhibitor anisomycin strongly reduced the incorporation of fluorescently labeled methionine analog by 46.85 % (0.53 ± 0.02 vs. 1.00 ± 0.00, $p < 0.001$, $n = 3$ experiments, Figure 30 B). To analyze a potential impact of PKCε in protein translation, I measured the AHA incorporation following siRNA mediated PKCε knockdown. In a knockdown control I observed about 25 % decreased PKCε signal intensity (0.78 ± 0.025 vs. 1.00 ± 0.00 or 0.62 ± 0.02 vs. 0.89 ± 0.02, $p < 0.001$, $n = 3$ experiments, Figure 30 C).

Based on my results on stress granules and P-bodies, I expected that PKCε knockdown results in decreased protein translation. Indeed, incorporation of 5-TAMRA tagged AHA was decreased by 19.36 % or 18.14 % in the absence or presence of anisomycin, respectively (0.81 ± 0.03 vs. 1.0 ± 0.00 or 0.35 ± 0.01 vs. 0.53 ± 0.02, $p < 0.001$, $n = 3$ experiments). Although this experiment was performed in unstressed cells, it indicates that PKCε has a supportive role for protein translation by inhibiting stress granule and P-body formation.

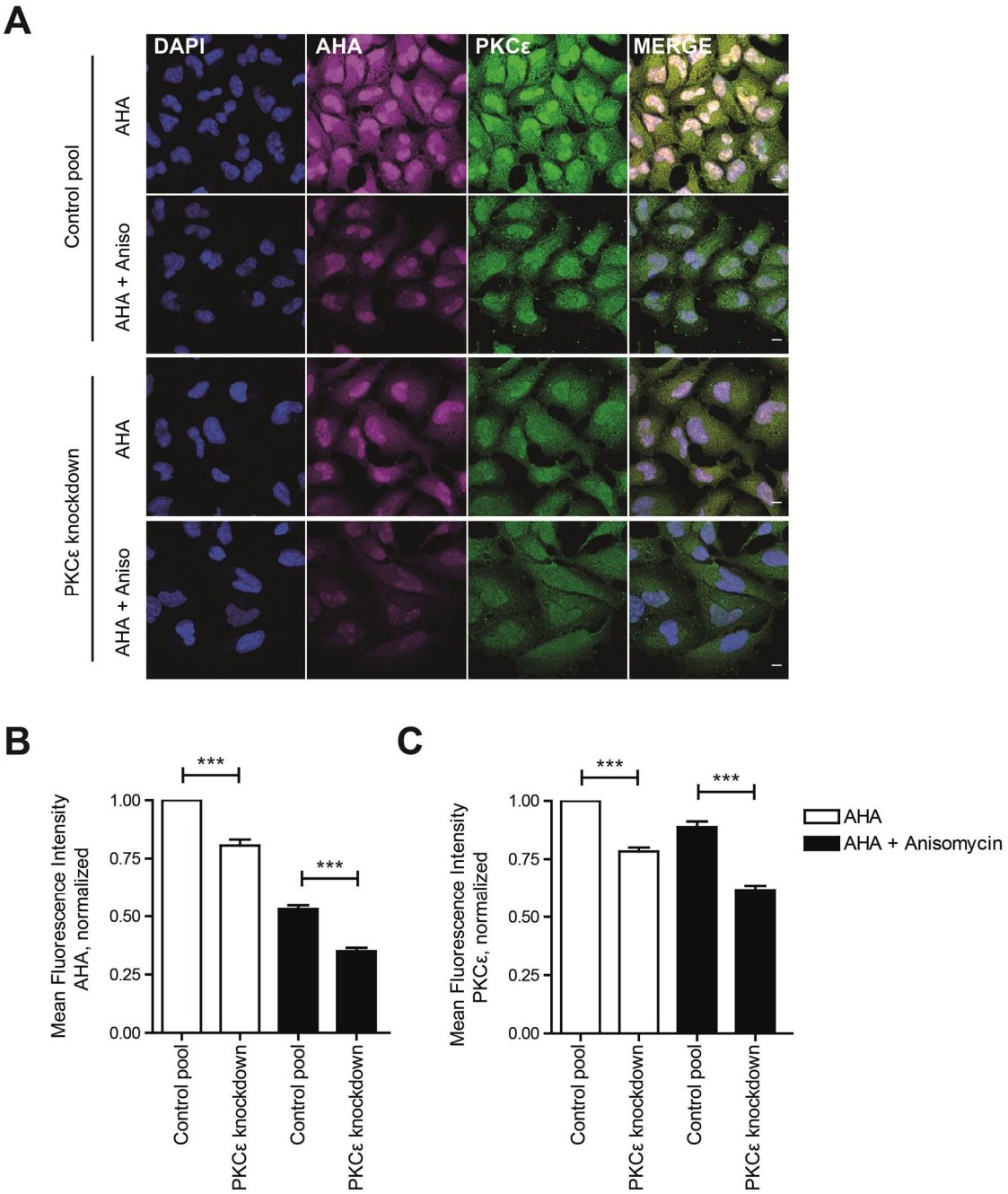

Figure 30: PKCε knockdown reduces protein translation. A) Confocal images of AHA and AHA + anisomycin (both purple) treated HeLa cells transfected with non-targeting siRNA pool or PKCε siRNA (green). Nuclei were stained with DAPI (blue). Scale bar is 10 µm **B)** Mean fluorescence intensity reflecting newly synthesized proteins. Upon PKCε knockdown protein translation was significantly reduced in case of AHA treatment (20 %, ***P < 0.001) as well as AHA + anisomycin treatment (18 %, ***P < 0.001) compared to the control pool. Data were normalized to control pool of AHA treated cells. **C)** Fluorescence based PKCε knockdown control. PKCε knockdown significantly decreased PKCε mean fluorescence intensity of both AHA (22 %, ***P < 0.001) and AHA + anisomycin (28 %, ***P < 0.001) treated HeLa cells. Data were normalized to control pool of AHA treated cells. Three experiments were performed in duplicates. ~50 cells per coverslip were analyzed.

7.6.1 Summary part VI

In this part, I tested if PKCε directly affects protein translation. Using the fluorescence based method FUNCAT to label newly synthesized proteins I could show that PKCε is directly involved in protein synthesis, an aspect so far not known for this isoform of PKCs.

7.7 Protein kinase A subunit RII alpha is recruited to stress granules and P-bodies

As I could show in section four and five, PKCε has an inhibitory role on the formation of stress granules and P-bodies. I suggest that under conditions of environmental stress and decreased PKCε availability the translation of “housekeeping” proteins is diminished and thus transcripts have to be stored in stress granules as well as P-bodies. This as a consequence leads to an increase in the number of both RNA granules.

When I screened the literature concerning other protein kinases with an inhibitory role on stress granules or P-body formation I found that protein kinase A (PKA) was shown to be involved in the regulation of P-bodies (Ramachandran, Shah et al. 2011). Activation of PKA leads to decreased P-body aggregation by phosphorylation of Pat1, thereby disrupting protein-protein interactions which are necessary for the formation of large P-bodies.

Nevertheless, they did not speak about a localization of PKA in P-bodies or even stress granules, which could also hint to another role of PKA there as I suggest it for PKCε. This would also indicate similar roles for both protein kinases in mRNA granules which could be an important part in stress response.

Therefore I tested if PKA is also recruited to P-bodies and stress granules. I found that the RII subunit, more precisely, the RIIα subunit of PKA is localized to P-bodies with and without stress and to stress granules upon arsenite treatment (Figure 31 A, B).

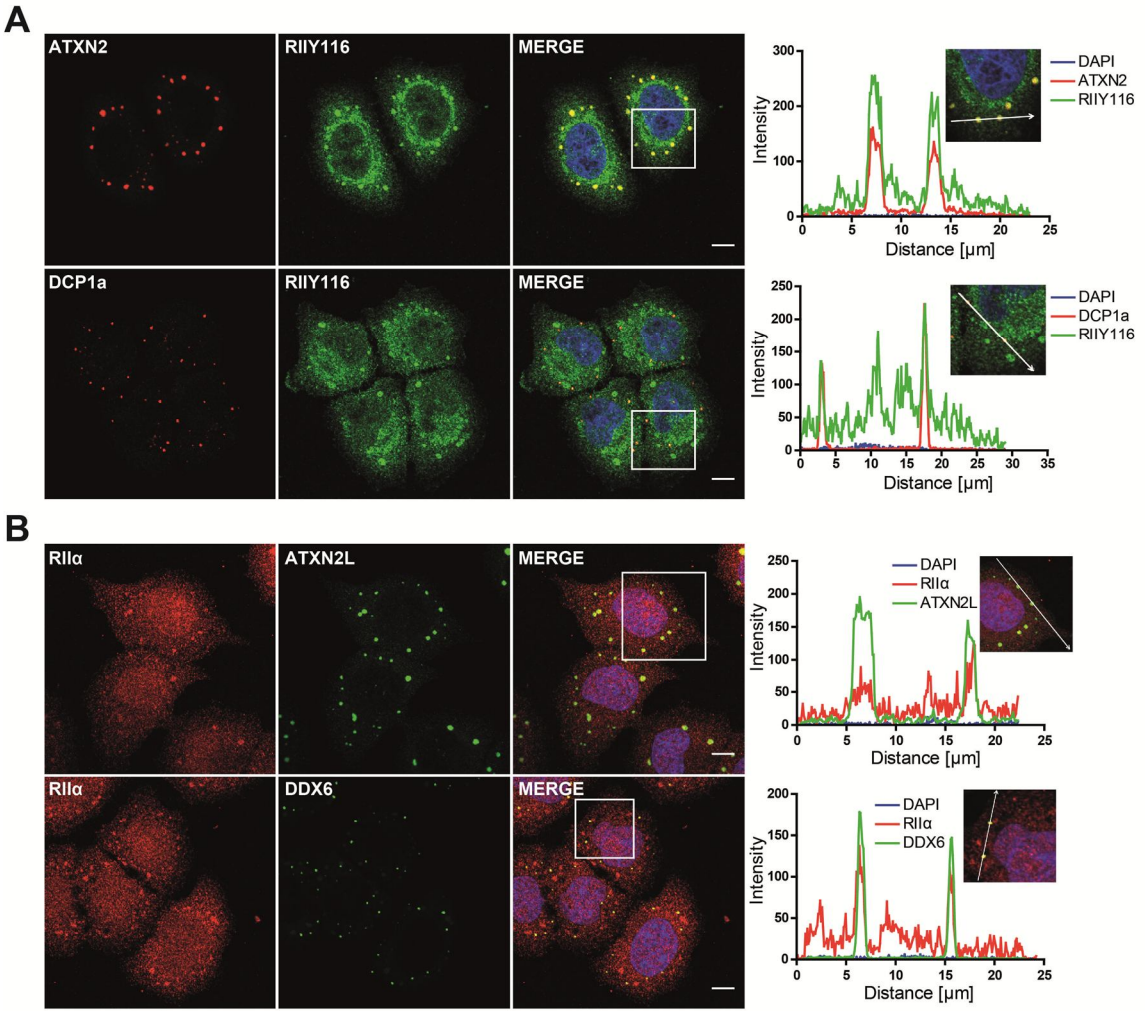

Figure 31: PKA subunit RIIalpha is recruited to stress granules and P-bodies. A) HeLa cells were stressed with 0.5 mM arsenite for 1 h and stained for RIIY116 (green) and the stress granule marker ATXN2 (red, upper panel) or the P-body marker DCP1a (red, lower panel). RIIY116 was recruited to stress granules (upper panel) and P-bodies (lower panel), which is illustrated with colocalization profiles of the line scan in the magnification (right-hand). Nuclei were stained with DAPI, scale bar is 10 µm. **B)** HeLa cells were stressed with 0.5 mM arsenite for 1 h and stained for RIIα and the stress granule marker ATXN2L (green, upper panel) or the P-body marker DDX6 (green, lower panel). RIIα was recruited to stress granules (upper panel) and P-bodies (lower panel), which is illustrated with colocalization profiles of the line scan in the magnification (right-hand). Nuclei were stained with DAPI. Scale bar is 10 µm.

Thus, both kinases are localized to stress granules and P-bodies and both have inhibitory roles on the formation of P-bodies. If PKA also inhibits stress granule formation has not been investigated so far.

7.7.1 Summary part VII

In the last part of my thesis I asked if the recruitment of kinases to translation modifying granules is a larger phenomenon. I could show that also the PKA subunit RII alpha is localized to stress granules upon arsenite stress and to P-bodies with and without arsenite

stress. As PKA like PKCε was shown to inhibit P-body formation they seem to have similar functions on at least this RNA granule type.

8 Discussion

I set out to investigate, if PKCε plays a role in protein translation in general. I tested further if PKCε is involved in particular in the formation of stress granules and P-bodies, two structures of high importance for the modulation of protein translation. I found that PKCε is recruited to stress granules in response to various cellular stresses and that this effect is cell type independent. Beyond the recruitment to stress granules, PKCε is also localized in P-bodies. I corroborated a functional role for PKCε by finding also the receptors of activated PKCs, RACK1 and RACK2, to be recruited to stress granules and P-bodies. PKCε down-regulation as well as inhibition of its activity increased the formation of stress granules. In contrast, activation of PKCε did not affect stress granule formation. Similarly, down-regulation of PKCε also enhanced P-body formation. In contrast, inhibition of PKC did not alter P-body formation. Using a fluorescence-based method to visualize newly translated proteins on a single cell basis (FUNCAT) I found that down-regulation of PKCε decreased protein translation. Thus, indeed, PKCε plays a role in protein translation and in translation-regulating stress granule and P-body formation. In the last part of my thesis I showed that also other signaling proteins such as classical PKC isoforms and the RII alpha subunit of PKA are involved in stress granule regulation in part by being recruited to stress granules and P-bodies.

First, I will now discuss the potential functions of PKCε and its anchoring proteins RACK1 and RACK2 in stress granules and P-bodies. Afterwards I will reflect on the role of PKCε in protein translation. In the end I will suggest PKA as a second player in the modulation of protein translation beyond PKCε.

8.1 Captured in stress granules – harmful or helpful?

Localization to a subcellular domain can mediate two effects: 1) it could lead to a depletion of that protein from other cellular regions and thereby modulate a process at this other cellular region. Or 2) it could result in an action on the subcellular domain it is recruited to. An example for the former has been reported recently. Arimoto et al. found that the recruitment of RACK1 to stress granules reduces its concentration in the cytoplasm. Thereby, MTK1-SAPK signaling is inhibited and apoptosis is blocked (Arimoto, Fukuda et al. 2008). This process is of high relevance as prevention of apoptosis can provide time to regaining functional homeostasis. On the other hand it can become detrimental if cell death is attempted for example during the course of cancer therapy. Radiation and chemotherapeutics are optimized to induce apoptotic cell death. However, cancer tissue often develops hypoxic microenvironments, which leads among others to

RACK1 recruitment to stress granules and thereby to a block of the therapy-induced apoptosis (Arimoto, Fukuda et al. 2008). This poses a serious therapeutic challenge.

8.1.1 Why is PKCε recruited to stress granules?

Beyond the role of stress granules as a signaling sink for RACK1 (Arimoto, Fukuda et al. 2008), my data now suggest a functional role for RACKs also at the stress granules itself. I found not only RACK1 but also RACK2 in stress granules. Both are “receptors for activated C kinases” and serve therefore as anchoring platform for activated PKCs. Thereby the activity of PKCs is focused on a subcellular region and the potential substrates there. Both RACKs and in particular RACK2 bind activated PKCε, which I found to translocate to stress granules. Thus, the localization of RACKs in stress granules in combination with our recent finding, that PKCε phosphorylates proteins involved in protein translation, suggest that RACKs in stress granules bind active PKCs. They keep them in close vicinity to their substrates, thereby allowing phosphorylation of the PKC substrates in the stress granules.

The translocation of RACKS and PKCε to stress granules appears as a general mechanism. I observed translocation not only in F-11 cells but also in HEK293T and HeLa cells. In DRG neurons, it was not possible to observe a consistent localization of PKCε to stress granules. This was due to two potential challenges: 1) there is strong proliferation of glia cells in the cultures. Although it would have been possible to distinguish between immunofluorescence signals from stress granules and glia cells using z-stacks, influence on stress granule formation by signaling events in glia cells could not have been excluded. 2) We suggest that DRG neurons contain high amounts of RNA due to a multitude of ongoing translational processes. Thus, stress granules are potentially located so closely spaced or fused together that the detection of a typical single stress granule is not possible.

Translocation to stress granules occurs not only in many cell types but also in response to various cellular stressors. I found PKCε to translocate to stress granules upon oxidative and temperature stress. As both stressors require strong and immediate reaction of the cell, this suggests an active role of RACKs and PKCε for the regulation of stress granule formation and the modulation of protein translation. RACKs may regulate the extent of stress granule formation by binding of PKCε which is recruited to stress granules. This process requires the activity of PKCε, as not only the down-regulation but already a block of PKCε is sufficient to alter this process.

Interestingly, I found an increased number of stress granules upon inhibition of the classical PKC isoforms α and β as well. This extends the recent observation, that also

classical PKCs regulate stress granule formation. Kobayashi et al. published that down-regulation of PKCα resulted in delayed stress granule assembly upon heat shock (Kobayashi, Winslow et al. 2012). Upon arsenite stress they did not find any significant effect, which suggests that the function of PKCα in stress granules depends on the kind of stress. I now found also an effect after arsenite stress. I believe this discrepancy to be based on my use of a more sensitive method for detection of stress granules. The method used by Kobayashi et al. for stress granule analysis was not based on the high sensitive single cell granule identification, which was used for the analysis in my thesis. In contrast to the analysis used here, Kobayashi et al. analyzed in 200 cells if stress granules were present or not in an all or nothing manner. In contrast, I now evaluated the number of stress granules within each cell for more than 6000 cells per single experiment using high content screening microscopy. This allows the analysis of the distribution of stress granules in much larger number of cells and enables due to its sensitivity the analysis of subtle modifiers of stress granule formation.

Similar to RACKs, the presence of PKCε in stress granules can have several effects. PKCε could have a supportive role for protein translation. Therefore it could be localized to stress granules upon translational arrest due to environmental stress. On the other hand, the localization of PKCε in stress granules may free energy from other PKCε induced signaling events. This would be important since under stress the whole signal transduction capacity is demanded for an adequate stress response. Alternatively, PKCε could be “captured” in stress granules to 1) directly prevent its signaling and thereby cell damage or 2) to allow processes, which are normally blocked by PKCε activity. Although my data support the hypothesis, that PKCε is located to stress granules to inhibit protein translation in general or to interrupt the synthesis of certain proteins during stress, other reasons for the localization of PKCε to stress granules such as the inhibition of apoptosis are still feasible and have to be investigated more in detail.

8.2 Effects of PKCε localization in P-bodies

P-bodies, another type of RNA granules, have functions similar to stress granules concerning mRNA storage. Nevertheless, also other processes such as protein degradation occur in P-bodies. My results reveal that PKCε not only is localized to stress granules but also to P-bodies. Whether the cell is exposed to stress or not does not influence the localization of PKCε to P-bodies. However, the number of P-bodies and thereby also the number of PKCε granules colocalizing with P-bodies is increased upon stress such as arsenite treatment. This shows that PKCε is also recruited to newly formed P-bodies.

The presence of RACK2 in P-bodies suggests that active PKCε is anchored to its binding partner RACK2 to be close to its downstream substrates. Thus, a role of PKCε for P-body formation and/or participation in other processes occurring in P-bodies is suggestive. In the literature, a function of RACK2 in P-bodies was so far not discussed.

Down-regulation of PKCε increased the amount of P-bodies. Similar to stress granules, this indicates an inhibitory role of PKCε for their formation. According to the main function of P-bodies, PKCε localization to these structures strongly suggests a connection between PKCε and protein translation. Nevertheless, in contrast to stress granules, an acute kinase activity is not required. The inhibitors used showed no influence on the number of P-bodies. This indicates a role for PKCε in P-bodies, which is not exclusively related to stressful situations. What other tasks could PKCε have in P-bodies? The main function of P-bodies is to harbor translationally silenced mRNA, which can then be released again for further translation (Anderson and Kedersha 2009). A second function of P-bodies is to recruit mRNAs targeted for deadenylation and degradation (Kulkarni, Ozgur et al. 2010). While I could not investigate this aspect in more detail, interestingly, we found ubiquitin specific peptidase (USP) 10 as a substrate candidate of PKCε (Schreier, Isensee et al., under review at MCB). USP10 belongs to the ubiquitin-specific protease family of cysteine proteases. It catalysis the cleavage of ubiquitin-conjugated proteins like p53/TP53, SNX3 and CFTR and regulates p53/TP53 stability (Yuan, Luo et al. 2010). Also these proteins are connected with stressful situations. Thus, a potential function of PKCε in P-bodies could be to mediate USP10 induced degradation of stress connected proteins.

8.3 PKCε modulates protein translation

The regulation of protein translation is an important response to cellular stressors such as oxidative stress, hypoxia or viral infections. It quickly stalls major parts of translation, thereby minimizing the risk from aberrant gene expression during stress conditions and preventing the cell from apoptosis. Further, proteins necessary for acute response and the recovery from stress damage by signaling transduction need to be provided in an appropriate amount, which is ensured by a stress-adapted regulation of gene-expression (Duncan and Hershey 1989, Anderson and Kedersha 2002, Kim, Back et al. 2005, de Nadal, Ammerer et al. 2011). Therefore, the proteins localized to stress granules and P-bodies and the processes regulating their formation are of high interest for cell biologists.

There are a number of indications for an involvement of PKCε in protein translation. Without investigating specific PKC isoforms, PKC activity in general was found to be necessary to stabilize interleukin-1 mRNA and to regulate GAP-43 mRNA (Gorospe, Kumar et al. 1993, Perrone-Bizzozero, Cansino et al. 1993). Moreover PKCε was found to

interact with an RNA-binding protein localized in RNA granules. The PKCε dependent induction of hyperalgesic priming in isolectin B4-positive primary afferent nociceptors was shown to be inhibited by the cytoplasmic polyadenylation element binding protein (CPEB) (Bogen, Alessandri-Haber et al. 2012). CPEB, which coimmunoprecipitated with PKCε (Bogen, Alessandri-Haber et al. 2012), is an RNA-binding protein also located in stress granules and P-bodies and responsible for the translation regulation of other mRNAs (Wilczynska, Aigueperse et al. 2005). This suggests that PKCε directly regulates protein translation by activating downstream substrates such as CPEB. Nevertheless, a direct role of PKCε in translation was not corroborated so far. Also the identified substrates of PKCε have not been suggesting translational control. A growing number of methods to identify new protein kinase substrates have been published (Johnson and Hunter 2005, Meng, Michaud et al. 2008). Recently a new computational biology based method to predict kinase substrates based on phosphorylation motif conservation over evolution was published (Lai, Nguyen Ba et al. 2012). Without focusing on the prediction of substrates of PKCε, they verified their method by predicting substrates of Cdc28, which was in accordance with published substrates. Although a computational based prediction method can serve a starting point for further research, it does not substitute experimental laboratory work. In our recently submitted study we used a kinase phosphorylation assay, which revealed among others substrates of PKCε involved in protein translation (Schreier, Isensee et al., under review at MCB). Of these, ribosomal protein and stress granule component S6 is the best studied S6K substrate. S6K represents a very important molecule in protein biosynthesis (Magnuson, Ekim et al. 2012). Among others it phosphorylates eIF4B inducing eIF4B binding to eIF4A, which in the end promotes the formation of pre-initiation complex (Raught, Peiretti et al. 2004, Shahbazian, Roux et al. 2006). Additionally it phosphorylates and thereby inactivates PDCD4, an eIF4A inhibitor (Dorrello, Peschiaroli et al. 2006).

My results now corroborate the previous circumstantial evidence. Using fluorescent non-canonical amino acid tagging, which is a procedure not reliant on radioactivity, I could directly show that PKCε down-regulation reduces the amount of newly synthesized proteins. This suggests that at least some proteins cannot be synthesized anymore during reduced PKCε availability or are less stable. It will be interesting to check, if single proteins are not translated anymore or if translation processes are inhibited in general. This can be achieved by using bio orthogonal non-canonical amino-acid tagging (BONCAT) followed by mass spectrometry (Dieterich, Lee et al. 2007)). There, lysates of AHA incubated cells are coupled to an affinity tag and undergo affinity chromatography. Upon digestion of purified proteins, peptides are analyzed by mass spectrometry.

8.4 PKA – a second stress related protein kinase in the function as protein translation modulation player?

Beyond PKCε, I found PKCα and β to alter the formation of stress granules as well as P-bodies. Thus, it was suggested that the recruitment of protein kinases to RNA granules is a general phenomenon. Indeed, PKA activation was found to prevent the formation of large P-bodies by inhibiting small P-body aggregation events (Ramachandran, Shah et al. 2011). Similar to PKCε, PKA is described to have a function in stressful situations. For example, several biological metabolic processes are regulated by PKA activation, which is mediated by extracellular nutrients (Fuller and Rhodes 2012). If PKA signaling is I impaired, metabolic disorders such as diabetes will follow (Netticadan, Temsah et al. 2001). Although the functions of PKA and PKCε differ from each other, both of them appear in stress related situations and both of them regulate the formation of P-bodies. So far PKA was not shown to be localized to RNA granules. In my study I found that PKA, similar to PKCε, not only regulates P-body formation but is also located to this type of RNA granules. Moreover, I could also show that the same PKA subunit RIIα, which I found in P-bodies, is also localized to stress granules upon arsenite stress. This suggests a role for PKA in stress granules and P-bodies, which is similar to PKCε. Thus, PKA may function as a second player in the modulation of the balance between protein translation and RNA granule formation.

8.5 Outlook

An important role of PKCε in protein translation regulation is more and more suggested, although the molecular mechanisms underlying this process are still not clear. One way to shed light on those mechanisms would be to find and validate in detail the novel PKCε substrates identified in our group. Moreover, investigating the functions of PKCε's interaction partners such as RACK1 and RACK2 offers a second challenging opportunity to understand the complex mechanisms. Both can be accomplished by e.g. further knockdown experiments. Also, the identification of the exact phosphorylation site of PKCε as well as their mutation should allow further insight about the mechanistic role of PKCε in translational control. My work also opened the door for another approach of analysis. As I find PKCε to be translocated to RNA-granules specifically after stress stimuli, one now can analyze the transcripts modulated by cell stress in a PKCε-dependent manner. The analysis could be based for example on a technique called BONCAT, which is similar to FUNCAT but AHA labeled proteins are tagged with a biotin-flag-alkyne to be purified by affinity chromatography. Afterwards newly translated proteins can be identified using mass spectrometry. Moreover, since one of the PKCε substrate candidates is USP10, it

would be interesting to investigate a function of PKCε in the degradation of stress connected proteins.

9 Conclusion

In this thesis I demonstrated a novel function of Protein Kinase C epsilon, namely its involvement in RNA granule formation and protein translation. I showed in different cell lines that PKCε is recruited to stress granules upon arsenite and heat stress by potentially anchoring it to RACKs. PKCε is localized to P-bodies in arsenite stressed as well as unstressed cells. Diminished PKCε availability affected stress granule and P-body quantity under conditions of oxidative stress. This indicates reduced synthesis of new proteins without PKCε. Indeed, I found direct effects of PKCε knockdown on protein translation in unstressed cells. Therefore I suggest a regulating role for PKCε in protein expression.

These data serve a starting point to investigate to what extend protein translation can explain the transition of acute activation to long-lasting effects and how in detail PKCε is implicated in this mechanism. As PKCε is involved in a large number of clinically relevant conditions, further elucidation of PKCε's function in stress related signaling can help to develop novel therapeutic approaches for various acute and chronic diseases.

10 References

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11 Abbreviations

ADAR1	Adenosine deaminase acting on RNA 1
AHA	azidohomoalanine
ars	sodium arsenite
ATXN2	Ataxin-2
ATXN2L	Ataxin-2-like
BIM	Bisindolylmaleimide I
BSA	bovine serum albumin
CMyBPC	cardiac myosin binding protein C
DAG	diacylglycerol
DAPI	4‘,6-diamidino-2phenylindol-dihydrochloride
DCP1	DCP1 decapping enzyme
DCP1a	DCP1 decapping enzyme homolog A
DCP2	DCP2 decapping enzyme
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	dimethylsulfoxide
DRG	dorsal root ganglia
eIF2A	eukaryotic translation initiation factor 2A
eIF4E	eukaryotic translation initiation factor 4E
ERK	extracellular regulated kinase
Fig.	figure
FUNCAT	fluorescence non canonical aminoacid tagging
GABA	gamma-aminobutyric acid
h	hour
IQGAP1	Ras GTPase-activating-like protein 1
LAGeSo	Landesamt für Gesundheit und Soziales
Lck	lymphocyte-specific protein tyrosine kinase
MAPK	mitogen-activated protein kinase
min	minute

mTORC2	mammalian target of rapamycin complex 2
NDS	normal donkey serum
NGS	normal goat serum
P/S	penicillin/streptomycin
PBS	phosphate buffered saline
PDK1	phosphoinositide-dependent kinase 1
PDLIM5	PDZ and LIM domain protein 5
PFA	paraformaldehyde
PKA	protein kinase A
PKC	protein kinase C
PKD	protein kinase D
PMA	phorbol 12-myristat 13-acetat
RACK1	receptor for activated C kinases 1
RACK2	receptor for activated C kinases 2
RNP	ribonucleoprotein
ROS	reactive oxygen species
RT	room temperature
s	second
SAPK	stress-activated protein kinase
SDS	sodiumdodecylsulfate
SDS-PAGE	sodiumdodecylsulfate-polyacrylamid gel electrophoresis
SEM	standard error of mean
Tab.	Table
TBTA	Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
TCEP	Tris(carcoxyethyl)phosphine
TEMED	N,N,N',N'-Tetramethylethylenediamine
Thr	threonine
TIA-1	T-cell internal antigen-1
TIAR	TIA-1 related protein

TRAM	translocating chain-associated membrane (protein)
Tris	trishydroxymethylaminoethane
TRPV1	transient receptor potential cation channel subfamily V member 1
Tudor-SN	Tudor Staphylococcal Nuclease

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13 Publication list

Publications

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Protein kinase C epsilon in protein translation

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14 Curriculum Vitae

For reasons of data protection,

the curriculum vitae is not included in the online version