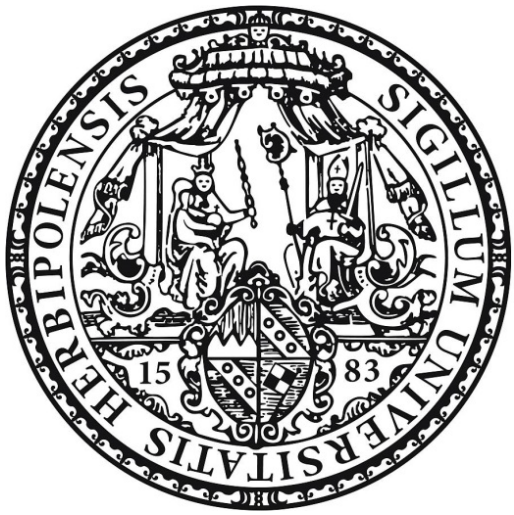
Identification of human host cell factors involved in *Staphylococcus aureus* 6850 infection



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Abstract

Staphylococcus aureus is both a human commensal and a pathogen. 20%-30% of all individuals are permanently or occasionally carriers of *S. aureus* without any symptoms. In contrast to this, *S. aureus* can cause life-threatening diseases e.g. endocarditis, osteomyelitis or sepsis. Here, the increase in antibiotic resistances makes it more and more difficult to treat these infections and hence the number of fatalities rises constantly. Since the pharmaceutical industry has no fundamentally new antibiotics in their pipeline, it is essential to better understand the interplay between *S. aureus* and the human host cell in order to find new, innovative treatment options.

In this study, a RNA interference based whole genome pool screen was performed to identify human proteins, which play a role during *S. aureus* infections. Since 1,600 invasion and 2,271 cell death linked factors were enriched at least 2 fold, the big challenge was to filter out the important ones. Here, a STRING pathway analysis proved to be the best option. Subsequently, the identified hits were validated with the help of inhibitors and a second, individualised small interfering RNA-based screen.

In the course of this work two important steps were identified, that are critical for host cell death: the first is bacterial invasion, the second phagosomal escape. The second step is obligatory for intracellular bacterial replication and subsequent host cell death. Invasion in turn is determining for all following events. Accordingly, the effect of the identified factors towards these two crucial steps was determined. Under screening conditions, escape was indirectly measured via intracellular replication. Three inhibitors (JNKII, Methyl-β-cyclodeytrin, 9-Phenantrol) could be identified for the invasion process. In addition, siRNAs targeted against 16 different genes (including CAPN2, CAPN4 and PIK3CG), could significantly reduce bacterial invasion. Seven siRNAs (FPR2, CAPN4, JUN, LYN, HRAS, AKT1, ITGAM) were able to inhibit intracellular replication significantly. Further studies showed that the IP3 receptor inhibitor 2-APB, the calpain inhibitor calpeptin and the proteasome inhibitor MG-132 are able to prevent phagosomal escape and as a consequence intracellular replication and host cell death.

In this context the role of calpains, calcium, the proteasome and the mitochondrial membrane potential was further investigated in cell culture. Here, an antagonistic behaviour of calpain 1 and 2 during bacterial invasion was observed. Intracellular calcium signalling plays a major role, since its inhibition protects host cells from death. Beside this, the loss of mitochondrial membrane potential is characteristic for *S. aureus* infection but not responsible for host cell death. The reduction of membrane potential can be significantly diminished by the inhibition of the mitochondrial Na^+/Ca^{2+} exchanger.

All together, this work shows that human host cells massively contribute to different steps in *S. aureus* infection rather than being simply killed by bacterial pore-forming toxins. Various individual host cell factors were identified, which contribute either to invasion or to phagosomal escape and

therefore to *S. aureus* induced cytotoxicity. Finally, several inhibitors of *S. aureus* infection were identified. One of them, 2-APB, was already tested in a sepsis mouse model and reduced bacterial load of kidneys.

Thus, this study shows valuable evidence for novel treatment options against *S. aureus* infections, based on the manipulation of host cell signalling cascades.

Zusammenfassung

Staphylococcus aureus kann sowohl ein Bestandteil der natürlichen Hautflora als auch ein Krankheitserreger sein. 20%-30% aller Menschen werden, permanent oder zeitweise, von *S. aureus* besiedelt, ohne Krankheitssymptome aufzuweisen. Im Gegensatz dazu kann *S. aureus* lebensbedrohliche Krankheiten wie Endokarditis, Osteomyelitis oder Sepsis verursachen. Diese Infektionen können immer schlechter behandelt werden, da immer mehr Stämme Resistenzen gegen die vorhandenen Antibiotika aufweisen. Dies führt zu einer steigenden Anzahl an Todesfällen, die auf Staphylokokkeninfektionen zurückzuführen sind. Da die Pharmaindustrie keine grundlegend neuen Antibiotika kurz vor der Marktreife hat, ist ein besseres Verständnis für das Wechselspiel zwischen Staphylokokken und ihren menschlichen Wirtszellen unbedingt notwendig, um neue, innovative Behandlungsmöglichkeiten finden zu können.

Dafür wurde in dieser Arbeit ein genomweiter RNA-interferenz basierter Screen durchgeführt. Es sollten so die Proteine identifiziert werden, die eine Rolle bei der Staphylokokkeninfektion spielen. Da 1.600 invasionsrelevante und 2.271 zelltodrelevante Faktoren mindestens 2-fach angereichert waren, musste ein Weg gefunden werden die wichtigen Faktoren herauszufiltern. Eine STRING-Pathwayanalyse stellte sich als die beste Methode hierfür heraus. In einem zweiten Schritt wurden die so identifizierten Faktoren mit Inhibitoren oder einzelnen siRNAs ein weiteres Mal herunterreguliert, um ihre tatsächlichen Auswirkungen auf den Infektionsverlauf zu untersuchen.

Im Verlauf dieser Arbeit konnte gezeigt werden, dass dem *S. aureus* induzierten Wirtszelltod mindestens zwei wichtige Schritte vorausgehen müssen. Erstens die Invasion der Wirtszelle und zweitens der Ausbruch aus dem Phagosom. Nur so können sich im dritten Schritt die Bakterien intrazellulär vermehren und die Zelle töten.

Daher wurde der Einfluss der identifizierten Faktoren auf diese beiden entscheidenden Prozesse untersucht. Der Ausbruch wurde unter Screenkonditionen indirekt über die intrazelluläre Vermehrung bestimmt. Es konnten drei Inhibitoren (JNKII, Methyl-β-cyclodeytrin, 9-Phenantrol) identifiziert werden, die die bakterielle Invasion vermindern. Darüber hinaus wurden 16 Proteine (unter anderem CAPN2, CAPN4 und PIK3CG) gefunden, deren Herunterregulation durch siRNAs, eine signifikant reduzierte Invasion zur Folge hatten. Sieben siRNAs (FPR2, CAPN4, JUN, LYN, HRAS, AKT1, ITGAM) waren in der Lage die intrazelluläre Vermehrung signifikant zu verringern. In nachfolgenden Versuchen konnte gezeigt werden, dass der IP3-Rezeptorinhibitor 2-APB, der Calpaininhibitor Calpeptin und der Proteasominhibitor MG-132 den Ausbruch aus dem Phagosom, sowie die darauffolgenden Ereignisse (intrazelluläre Vermehrung und Wirtszelltod) inhibieren können.

In diesem Zusammenhang wurden die Einflüsse von Calpainen, Calcium, dem Proteasom sowie dem mitochondrialen Membranpotentialverlust im Zellkulturmodell im Detail weiter untersucht. So konnte eine gegensätzliche Rolle von Calpain 1 und 2 bei der *S. aureus* Invasion festgestellt werden.

Die intrazelluläre calciumabhängige Signalweiterleitung spielt eine bedeutende Rolle bei der *S. aureus* Infektion, da ihre Inhibition eine normale Infektion verhindert. Das mitochondriale Membranpotential (MMP) sinkt während einer *S. aureus* infektion, ist aber nicht für den Zelltod verantwortlich. Das Sinken des MMPs kann mit einem Inhibitor, der den mitochondrialen Na^+/Ca^{2+} Austausch verhindert, signifikant reduziert werden.

Zusammenfassend zeigt diese Arbeit, dass die menschliche Wirtszelle selbst relevant zu den verschiedenen Schritten der Staphylokokkeninfektion beiträgt, und nicht einfach, wie häufig angenommen, von porenbildenden bakteriellen Toxinen zerstört wird. Entsprechend konnten einzelne Wirtszellproteine identifiziert werden, die entweder zur bakteriellen Invasion oder zum phagosomalen Ausbruch und somit zum induzierten Wirtszelltod beitragen. Überdies konnte gezeigt werden, dass Inhibitoren, die diese Wirtszellproteine hemmen, die Wirtszellen zu unterschiedlichen Zeitpunkten vor einer *S. aureus* Infektion schützen können.

Folglich liefert diese Arbeit wertvolle Hinweise für neue Behandlungsmöglichkeiten von *S. aureus* Infektionen, die auf der Manipulation von Wirtszellsignalkaskaden beruhen.

1. Introduction

1.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, coagulase positive bacterium. It belongs to the phylum Firmicutes and within this group to the class of Bacilli and order of Bacillales. Since 2010 it is added to the family of Staphylococcaceae (Euzeby, 2010). Although *S. aureus* can cause many human diseases approximately 20% of all individuals are permanent carriers of *S. aureus* without any symptoms (Lowy, 1998). However, carriers have a higher risk of developing serious *S. aureus* infections than non-carriers and they can get bacteraemia from their own colonies (von Eiff et al., 2001; Williams, 1963). In addition, carriers do not necessarily have only one strain and carried strains can change over time (Votintseva et al., 2014). Furthermore, the numbers of community-acquired methicillin-resistant *S. aureus* (CA-MRSA) infections are increasing and even children without any predestined risk are infected (Herold et al., 1998).

The most recent world health organization (WHO) report of antibiotic resistance shows clearly that it is absolutely necessary to find new antibiotics or other methods of treatment (WHO, 2014). In addition, the "Antimicrobial resistance surveillance in Europe 2012" published by the European centre for disease prevention and control (ECDC) highlights that especially in southern Europe the percentage of MRSA isolates is alarmingly high. Over 50% of the invasive strains in Portugal and Romania and between 25% and 50% in Italy, Greece and Poland are methicillin resistant strains (ECDC, 2012). As the pharmaceutical industry does not have fundamentally new antibiotics in the pipeline, it is urgent to gain more insight into the interactions between *S. aureus* and the human host cell in order to find new strategies to prevent or cure severe *S. aureus* infections.

Thus, the interaction between *S. aureus* and the human host cell has to be investigated in more detail. In the past *S. aureus* was believed to be an extracellular pathogen, which predominantly acts via its secreted toxins. Accordingly, these proteins were investigated in detail, e.g. the role of α-toxin, which is the most prominent *S. aureus* pore-forming toxin. But it is not well understood, which role these toxins have intracellularly. Therefore, not only the effect of single secreted toxins, but also the intracellular behaviour of the whole bacterium has to be investigated. The importance for this is demonstrated by the fact that among others also professional phagocytes are infected and killed by *S. aureus* (Kobayashi et al., 2010). Consequently, also the cells that are normally the first line of defence against infections, are infected by *S. aureus* and thus the bacteria can spread and cause severe damage.

1.2 *S. aureus* and its interactions with human host cells

1.2.1 *S. aureus* invasion into human host cells

Within the human body bacteria are permanently attacked by the immune system. The most common way to escape from this is to invade host cells. Here, bacteria have a second benefit: access to nutrients. *S. aureus*, even tough originally identified as an extracellular pathogen, invades host cells. In the past few years a vast number of studies verified this. Surprisingly, *S. aureus* can invade almost all types of cells e.g. endothelial cells, fibroblasts, human and bovine epithelial cells, and even osteoblasts (Almeida et al., 1996; Ellington et al., 1999; Sinha and Herrmann, 2005; Usui et al., 1992; Wang et al., 2013). One of the first hints that staphylococci might be able to invade eukaryotic cells was found by Kuusela, who observed that staphylococci can bind to fibronectin (Kuusela, 1978). More recent publications show how important this observation was. *S. aureus* interacts with fibronectin via its two fibronectin binding proteins FnBPA and FnBPB. The bound fibronectin can then interact with the integrins $\alpha5\beta1$ on the host cell surface and thereby FnBPs act as invasins (Sinha et al., 1999).

The importance of FnBPs for *S. aureus* invasion is undoubted even though it is not completely clear whether they are adhesins or invasins. This question came up, when two different groups showed that strains, which express the same level of FnBPs and bind fibronectin to the same extent, showed different invasion levels (Ahmed et al., 2001; Dziewanowska et al., 1999). Thus, an additional factor is postulated by almost all authors of studies dealing with *S. aureus* invasion. One possible co-factor is HSP60, which has been shown to interact with FnBPs directly. In addition, *S. aureus* invasion is reduced, when cells were preincubated with an antibody against HSP60 (Dziewanowska et al., 2000). Another, totally different host cell receptor is the heat shock cognate protein HSC70. It is the eukaryotic receptor for the major autolysin/adhesins (Atl) from *S. aureus*. The invasion process via Atl and HSC70 is independent of fibronectin and FnBPs (Hirschhausen et al., 2010).

Independently, inhibitor studies showed that the actin cytoskeleton is involved, since cytochalasinD reduces invasion to almost zero (Ellington et al., 1999; Jett and Gilmore, 2002; Jevon et al., 1999). To a lesser extent also microtubules are involved in the invasion process (Ellington et al., 1999; Jevon et al., 1999). Since it has been shown that actin plays a dominant role during invasion, its interaction partners gained centre stage. So inhibitors of Rho, Rac, CDC42 and NWASp reduced invasion by 50-60% (Schroder et al., 2006). Besides, Src a receptor tyrosine kinase (RTK) is involved in *S. aureus* invasion (Agerer et al., 2003). In addition, the transglutaminase inhibitor monodansylcadaverine abolished invasion into human osteoblasts almost completely. Clustering and internalization of a receptor seem to be involved in *S. aureus* invasion (Levitzki et al., 1980). This assumption is confirmed by the observation that cholesterol plays an important role during *S. aureus* invasion. As an integral component of biological membranes cholesterol contributes to clustering of receptors

and other membrane proteins. In addition, *S. aureus* co-localises with GM1, a gangliosid, which accumulates within lipid rafts. The involvement of cholesterol was shown by simvastatin, which inhibits the HMG-CoA reductase, an enzyme involved in cholesterol biosynthesis. This inhibition results in reduced invasion into HUVEC and 293T cells (Hoffmann et al., 2010; Horn et al., 2008).

But not only surface receptors and the cytoskeleton have been linked to *S. aureus* invasion. There is also evidence for intracellular signalling. ERK 1 and 2 as well as JNKs (also known as SAPK) are phosphorylated 30 min post *S. aureus* infection. As a result, the transcription factor c-JUN is phosphorylated in human osteoblasts after *S. aureus* infection (Ellington et al., 2001).

In a bovine endothelial cell line AKT1 is phosphorylated shortly after *S. aureus* infection. This phosphorylation is PI3-kinase dependent and necessary for bacterial invasion. It also leads to GSK-3α, GSK-β and p65 (a NFκB subunit) phosphorylation (Oviedo-Boyso et al., 2011).

On the bacterial side, beside FnBPs, the extracellular adherence protein (Eap) seems to contribute to the invasion process (Haggar et al., 2003).

1.2.2 *S. aureus* induces host cell signalling and host cell death

1.2.2.1 α-toxin and its effect on human host cells

S. aureus codes for a huge number of toxins, but almost all of them are under the control of *agr*, *sar* or *sae*, which are transcription factors (Giraudo et al., 1999; Recsei et al., 1986; Wesson et al., 1998). Some of these toxins are pore-forming toxins. α-toxin is the most prominent one. At high concentrations it can lyse host cells by forming pores (Walev et al., 1993). Lower concentrations cause more defined effects, like a permeability of cells for monovalent cations resulting in the activation of caspases and apoptotic cell death (Bantel et al., 2001). Howerver, the inhibition of caspases activation with a general caspase inhibitor could not rescue cells from cell death, therefore it was proposed that cells die via a special type of necrosis in which caspases are activated (Essmann et al., 2003). In contrast, α-toxin has been shown to stimulate proliferation of epithelial cells via the epidermal growth factor receptor (EGFR) that is phosphorylated (Haugwitz et al., 2006). In addition, α-toxin can interact directly with host cell receptors. It can bind to caveolin-1 (CAV1) (Pany et al., 2004). This interaction seems, at least in some types of cells, important for heptamer and thus pore formation of α-toxin (Vijayvargia et al., 2004). In line with this, membrane composition has been shown to be important for α-toxin pore formation. Therefore, either phosphatidylcholine and cholesterol or sphingomyeline and cholesterol has been shown to be necessary for pore formation (Watanabe et al., 1987). But also host cell signalling can be induced by α-toxin. It leads to the loss of intracellular K^+ and thus activates p38 (Kloft et al., 2009). α-toxin has been shown to hydrolyse phosphatidylinositol (PI) and leads to arachidonic acid release in rat PC12 cells. In addition, an increase of calcium within the cytosol was measured and the authors propose thus that α-toxin binding activates a phosphoinositide phospholipase c (PLC) resulting in the formation of IP3 and the

activation of IP3 receptors (Fink et al., 1989). ADAM10 was recently identified to be a cellular receptor for α-toxin (Inoshima et al., 2011; Wilke and Bubeck Wardenburg, 2010).

1.2.2.2 Host cell receptors of bacterial toxins

Beside α-toxin also other *S. aureus* toxins interact with host cell receptors. CCR5 is the cellular receptor for LukED and an antagonist of it can block LukED induced cell death (Alonzo et al., 2013). The receptor for LukAB is ITGAM (also known as CD11b) and for PVL two receptors are known; C5aR and C5L2 both are complement receptors (DuMont et al., 2013; Spaan et al., 2013). C5L2 is expressed in HeLa cells, too (Johswich et al., 2006). PVL is associated to mitochondria, since the isolated toxin is localised at mitochondria and able to induce the release of cytochrome c. Therefore, it is suggested that PVL can form pores in the outer membrane of mitochondria (Genestier et al., 2005). The last toxin mentioned here, is the relatively uncharted phosphatidylinositol-specific phospholipase (PI-PLC). The PI-PLC can hydrolyse PI and therefore may act directly on the host cytoplasma membrane (Low and Finean, 1976). Even though the mechanism is not clear, an involvement in virulence has been shown (Daugherty and Low, 1993; Marques et al., 1989; White et al., 2014).

The list of *S. aureus* toxins and their individual effects on host cells is long. However, studies using several toxins together or even using the whole bacterium are rare. Even signalling effects of individual toxins in host cells are poorly understood. This is nicely shown by a study performed in the lab of G. Peters. They demonstrated that isolated PVL can induce cell death within 20 min, whereas the induction of cell death during an infection with the whole bacterium needs 2-3hrs. In addition, the dynamics of cell death induction of two strains, one expressing (USA300) and one not (6850) expressing PVL, were similar (Loffler et al., 2010), suggesting that the role of individual toxins during an infection differs from the effects induced by an isolated toxin.

1.2.2.3 *S. aureus* induces via TLR2 calcium signalling within human host cells

S. aureus can increase the intracellular calcium concentration in human airway epithelial cells in an *agr*-dependent manner. The calcium signal induces p38 and ERK signalling resulting in NFκB activation and IL-8 expression. The initial molecule, with which *S. aureus* seems to interact here, is asialoganglioside-GM1 (asialo-GM1) (Ratner et al., 2001). The capacity of *S. aureus* to bind to asialo-GM1 (and gangliosides) has already been shown in a study with corneal epithelial cells (Schwab et al., 1996). IL-8 expression has been demonstrated in airway epithelial cells, too. Here, asialo-GM1 seems to co-operate with TLR2. Both receptors are mobilised after *S. aureus* exposure and end-up in lipid rafts, which are necessary for signalling indicated also by cholesterol inhibitors, which disturb them and so prevent IL-8 expression (Soong et al., 2004). The involvement of TLR2 is especially interesting since even isolated peptidoglycan (PGN) from *S. aureus* can activate TLR2 (Iwaki et al., 2002). This is in line with some findings obtained in a mouse model; here *agr* and *sarA* mutants were able to

induce IL-8 expression, but were not able to establish an invasive infection (Heyer et al., 2002). Ex vivo analysis of mouse macrophages showed, that a knockdown of TLR2 decreases JNK phosphorylation normally induced by *S. aureus*. In contrast, the induced ERK and p38 phosphorylation are not affected by TLR2 knockdown (Fang et al., 2014). Meaning that TLR2 induces JNK phosphorylation whereas ERK and p38 phosphorylation is mediated by some additional factors. This finding was confirmed in human corneal epithelial cells, in which inactivated *S. aureus* induced JNK, p38 and ERK signalling, but just JNK inhibition could inhibit IκB degradation and thus NFκB activation (Adhikary et al., 2008). A conclusion of these studies could be that signalling is induced at the bacterial attachment side, resulting in a calcium flux and the activation of p38, ERK and JNK. Whereby JNK seems to be responsible for NFκB activation and IL-8 expression.

In principal, all pore-forming toxins of *S. aureus* can induce, directly or indirectly, calcium fluxes. For example, δ-toxin activates mast cells with the help of PI3-kinases and a calcium influx (Nakamura et al., 2013).

1.2.3 *S. aureus* host cell death induction

The effect of *S. aureus* on host cells was studied in different cell lines. Beside human also bovine and mouse cells were studied. *S. aureus* invades bovine MAC-T cells and subsequently escapes from the endosome. Infected cells round up and detach from the matrix. The following DNA laddering indicates that cells undergo apoptosis (Bayles et al., 1998). All these changes are *sar* and *agr* dependent (Wesson et al., 1998). A follow-up study revealed the involvement of Caspase 8 and 3. In addition, transcription of IL1-β and TNFα are induced (Wesson et al., 2000). Cell rounding and DNA laddering was also observed in mouse osteoblasts after *S. aureus* infection (Tucker et al., 2000).

Human peripheral blood monocytes show the following alterations: caspase 8 activity, MMPL, ROS generation and caspase 3 activation (Weglarczyk et al., 2004). Infection of human endothelial HUVEC cells leads to caspase activation, ASM activation, cytochrome c release and JNK activation (Esen et al., 2001). In addition, DNA laddering has been shown (Menzies and Kourteva, 1998). In CFT-1 cells (epithelial cell line generated of cystic fibrosis tissue) *S. aureus* resides within vacuoles that do not fuse with lysosomes. The bacteria replicate in these vacuoles and induce host cell death (Kahl et al., 2000b). In contrast, in human peritoneal mesothelial cells *S. aureus* infection induces necrosis-like cell death. These cells do not become annexin positive and do not show caspase activation. In contrast, DNA laddering occurred (Haslinger-Loffler et al., 2006). Caspase activation was also not detectable in HeLa cells infected with USA300. In the same publication the succession – first bacterial replication then phagosomal escape – was proposed (Schnaith et al., 2007). Albeit several studies showed that cell death induction shares some similarities in different types of cells, induced cell death does not seem to be a classical apoptosis or necrosis. And although several studies show that *S. aureus* efficiently kills almost all types of cells, a pro-survival effect on human host cells was

described. The transcription of myeloid cell leukemia-1 (Mcl-1) was increased and the protein was stabilised in macrophages during an *S. aureus* infection. As a consequence, the macrophages showed a prolonged survival (Koziel et al., 2013). All this indicates that the interplay between *S. aureus* and its host cell is a complex, not well understood, process.

1.3 Host cell factors involved in *S. aureus* infection

In the following chapter the role of central host cell factors involved in *S. aureus* infection are described. Some of them are already linked to *S. aureus* infection, for the others their usual cellular function will be described. The latter will be important for the understanding of this work.

1.3.1 Integrins

Integrins are transmembrane proteins, which connect cells to their environment. 24 integrins are known, which have various functions. I will focus here on integrin α5β1 (van der Flier and Sonnenberg, 2001). It is, like all integrins, a heterodimer composed of the two proteins ITGA5 and ITGB1. Its primary ligand is fibronectin (FN) (Hynes, 1987). In HUVEC cells it has been shown that binding of α5β1 to FN results in an intracellular signalling cascade (including FAK, Src, PI3K, AKT1, ERK, JNK, and p38 MAPK), which finally results in the activation of the transcription factor JUN. JUN induces among others the expression of MMP-9 (Jin et al., 2011). Interestingly, the lipoteichoic acid (LA) of *S. aureus* has also been shown to upregulate MMP-9 in epithelial cells (Park et al., 2012). In general, integrins transduce signals from the environment to intracellular signalling. Therefore, they are connected to the actin cytoskeleton via talin (Pfaff et al., 1998). The binding to talin also enhances integrin activity and is even more pronounced when talin binds to PtdIns4,5-P_2 (PIP2) (Martel et al., 2001; Tadokoro et al., 2003). Binding of FN can result in integrin clustering, which results in the autophosphorylation of the protein tyrosine kinase 2 (PTK2), which belongs to the FAK subfamily (Kornberg et al., 1992). Src (or another RTK) is recruited and phosphorylates PTK2 further and therefore GRB2 is recruited. This platform then activates Ras, which can result in ERK activation (Schlaepfer et al., 1994; Schlaepfer and Hunter, 1997). But this signalling cascade is not stringent, different co-factors can modify it. In response to FN the integrin α5β1 can also induce phosphorylation of the epidermal growth factor receptor (EGFR) (Kuwada and Li, 2000). In this case, the downstream signalling of this receptor is induced, too. This results in Shr phosphorylation and ERK1 activation (Bill et al., 2004; Moro et al., 1998). A publication of Illario et al. shows how many signalling pathways can be activated during integrin activation (Illario et al., 2003). TAD-2 cells (immortalised human fetal thyroid cells) were stimulated with FN. This FN binding to integrins induces phosphorylation of PTK2 and paxilin and thus the formation of the FAK/Grb-2/Sos complex. In addition, HRAS, ERK1/2 and PI3-kinases are activated. These complex cascades induce growth (HRAS) and survival (PI3-kinase) of the cells (Illario et al., 2003). Remarkably, the integrin signalling induced during *S. aureus* invasion is still not well understood.

1.3.2 PI3 kinase

Three different classes of Phosphatidylinositol-4,5-bisphosphate 3-kinases (PI3-kinases) are known. The most prominent ones are the class I PI3-kinases, which are subdivided in class IA and IB. The class IA PI3-kinases are activated via RTKs and consist of a p85 regulatory and a p110 catalytic subunit (α,β,δ). Beside RTKs, a direct interaction between p-FAK and the PI3-kinase subunit p85 has been shown to activate class IA PI3-kinases (Chen and Guan, 1994). FAK itself can be phosphorylated through a fibronectin-integrin interaction (Guan et al., 1991).

The class IB PI3-kinases consist of the regulatory subunit p101 and the catalytic subunit p110γ (Foster et al., 2003; Stoyanov et al., 1995). They are activated via G-protein coupled receptors (GPCR). In addition, activated Ras can bind and activate PI3Kγ (Pacold et al., 2000; Rubio et al., 1997). This induces cytoskeleton remodelling and thus membrane ruffling (Rodriguez-Viciana et al., 1997). One target of the activated PI3Kγ is JNK, which is phosphorylated and thus activated. (Lopez-Ilasaca et al., 1998).

The activated PI3 kinases phosphorylate the inositol ring of phosphatidylinositol 3-phosphate (PtdIns 3-P). This can result in di- or tri- phosphorylation of the ring, resulting in PtdIns 3,4-P_2 (PIP2) or PtdIns 3,4,5-P_3 (PIP3) (Carpenter and Cantley, 1996). The two products of the PI3K are therefore PIP2 and PIP3. PIP2 on its part is a substrate for PLCs. PLCs convert PIP2 to IP3 and DAG. Another function of PIP2 is to recruit and activate AKT1 and its additional activator the phosphoinositide-dependent protein kinase-1 (PDK1) (Alessi et al., 1997; Klippel et al., 1997; Watton and Downward, 1999). Thereby, PIP2 can activate a pro-survival pathway. Importantly, even though a lot of reviews suggest that PIP3 activates AKT1 I could not find any publication showing this. In contrast, Klippl et al. showed that PIP3 is not activating AKT1 (Klippel et al., 1997). Nevertheless, in vitro studies showed that PIP3 can strongly bind to AKT1 and might therefore recruit AKT1 to the plasma membrane where it is activated by PDK1, which on its part can be activated by PIP3 (James et al., 1996; Stokoe et al., 1997). In addition, it has been shown in vitro, that PIP2 can activate AKT1, whereas PIP3 inhibits its activation (Frech et al., 1997).

1.3.3 HRAS signalling

HRAS belongs to the Ras oncogene family. When located at the plasma membrane it needs cholesterol for its activation, since depletion with methyl-β-cyclodextrin (MβC) inhibits its activation (Roy et al., 1999). HRAS is involved in several signalling cascades. One of its targets is the PLCε (Song et al., 2001). It is important to mention here that the lipoteichoic acid (LTA) from *S. aureus* has been shown to activate PLCε via RTKs. This activation results in the activation of ERK in rat cortical neuronal cells (Wu et al., 2006). This is especially interesting since HRAS can be activated via GRB2 by RTKs (Lowenstein et al., 1992). HRAS can activate the two factors ERK and JNK via Raf and MEKK (Howe et al., 1992; Minden et al., 1994). In a next step ERK can lead to phosphorylation and thereby

to the activation of the transcription factor JUN (Derijard et al., 1994; Pulverer et al., 1991). Therefore HRAS, like integrins, can activate various signalling cascades.

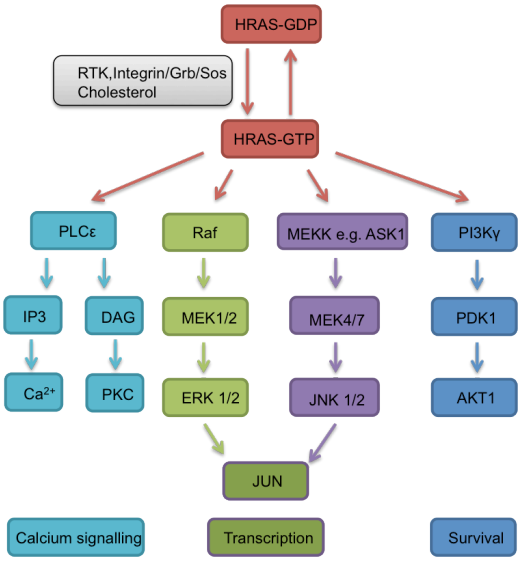

Figure 1 Scheme of HRAS signalling. HRAS is active when it is bound to GTP. RTKs, integrin signalling and cholesterol can be involved in the GDP/ GTP exchange. Activated HRAS can induce calcium signalling via the activation of PLCε. PLCε generates IP3 and DAG. IP3 induces calcium signalling and DAG antivates PKCs. In addition, JUN can be activated via the Raf/MEK/ERK pathway and JNK. HRAS can also activate PDK1 and AKT1 via PI3Kγ.

1.3.4 Protein kinase A

PRKAR1A is a regulatory subunit of the type I cAMP-dependent protein kinase A (PKA). PKAs are activated by cyclic AMP (cAMP), which is produced by adenylate cyclases (AC). ACs are often calcium regulated. Some ACs are inhibited while others are activated by calcium. That justifies to assume that calcium and PKA signalling is linked (Guillou et al., 1999). In addition, PKAs also play a role in integrin signalling and cytoskeleton rearrangements. Within the latter, they inhibit RhoA activation trough the phosphorylation of $G_{\alpha 13}$, which is a G-protein involved in RTK as well as in GPCR signalling (Manganello et al., 2003; Shan et al., 2006). RhoA can also be directly phosphorylated by PKA. This phosphorylated RhoA cannot bind to the plasma membrane anymore and therefore cannot act appropriate (Lang et al., 1996). In addition, phosphorylations of ryanodine receptors (RYR) by PKAs have been shown, but contradictory results are published. Hence, the role of this phosphorylation is uncertain (Niggli et al., 2013). Interestingly, the PKA is an antagonist of Ras /ERK signalling (Pursiheimo et al., 2002). An invovement of PKAs during *S. aureus* infection was already proposed (Miller et al., 2011). A proteome analysis of THP-1 macrophages showed that PKAs are activated 15 min post *S. aureus* infection but no further functions of PKA during *S. aureus* infection have been investigated so far (Miller et al., 2011).

1.3.5 Calpains

Calpains are cysteine proteases, which have different substrates and biological functions. For example, calpains can contribute to apoptosis induction via the cleavage of caspases (Gafni et al., 2009). Increasing calcium concentrations activate calpains, but the necessary concentrations are very high. Accordingly, it can be assumed that in vivo there are additional factors involved, which increase calcium affinity. The two most prominent calpains are calpain 1 and 2 (CAPN1 and CAPN2). These two large subunits form with the small subunit CAPNS1 (also known as CAPN4) heterodimers. The dimer is the functionally active form (Suzuki et al., 1987). Their importance for mammalian development has been demonstrated recently, since the knockout of the small subunit CAPN4 in mice leads to lethality in mid-gestation (Arthur et al., 2000).

In the context of infections, calpains can play a role during the invasion process e.g. *Cryptosporidium parvum* invasion into Caco-2 cells (Perez-Cordon et al., 2011). Also Shigella invasion is calpain dependent. Here, calpains are activated and are responsible for the important actin cytoskeleton changes during invasion of HeLa and Mef cells. In addition, calpains induce either necrosis (e.g. in HeLa) or apoptosis (e.g. HCT116) in Shigella infected cells, depending on the p53 status of the cell (Bergounioux et al., 2012).

One of the best-investigated roles of calpains is their function during tumour invasion. CAPN2 is activated via the MEK/ERK pathway and contributes to cell migration of lung cancer cells (Meng et al., 2009). In breast cancer cells CAPN2 cooperates with PTP1B and Src to regulate actin assembly and disassembly and thus facilitates cancer invasion (Cortesio et al., 2008).

A second well-investigated function of calpains is focal adhesion (FA) reassembly. CAPN2 can cleave talin and thus leads to the disassembly of FAs (Franco et al., 2004). In addition, it can cleave the focal adhesion kinase (FAK) another important protein of FA complexes (Chan et al., 2010). Overexpression of calpastatin (a cellular calpain inhibitor) or the addition of ALLN (a synthetic small-peptide calpain inhibitor) stabilises vinculin and zyxin containing FAs (Bhatt et al., 2002). Even more interesting, CAPN2 can directly cleave β-integrin subunits (Pfaff et al., 1999).

It is well known that calpains can be activated by calcium and that the activation is normally associated with calpastatin degradation, but also phosphorylation can activate calpains (Croall and DeMartino, 1991; Glading et al., 2004). ERK1 can directly phosphorylate CAPN2 and activate it in this way, even in the absence of calcium (Glading et al., 2004). One example, where this activation is important, is the stimulation of fibroblasts by the epidermal growth factor (EGF). EGF leads to the activation of MEK1 and ERK1 resulting in CAPN2 activation. This results in an increased fibroblast cell motility and disassembly of FAs. Importantly, the EGF induced calpain activity is independent of PLCγ activity and calpastatin degradation, but dependent on calpain phosphorylation via ERK (Glading et al., 2004; Glading et al., 2000; Glading et al., 2001). In contrast, the phosphorylation of CAPN2 by PKA

inhibits the activation of CAPN2 (Shiraha et al., 2002). Recently, it has been shown that the binding of CAPN2 to PIP2 is important for its activity. Artificial farnesylation of CAPN2 binds it directly to the plasma membrane and makes it resistant to PKA phosphorylation. In addition, activation of membrane-bound CAPN2 takes place even in the absence of ERK, but PIP2 is still needed for activation (Leloup et al., 2010). From this follows that calpain activation is a highly regulated process and not only calcium is responsible for its activation – in some cases it is not even necessary.

1.3.6 Calcium signalling

Calcium is an important second messenger within human cells. Beside its fundamental role during muscle contraction it is the important signal for the activation of different cells e.g. oocytes (Ridgway et al., 1977). The cytosolic calcium concentration can be increased via an influx of calcium from the environment or by an efflux from the endoplasmic reticulum (ER), the main calcium storage inside the cell. The two calcium channels in the ER, which facilitate the efflux, are Ryanodine receptors (RYR) and inositol triphosphate receptors (IP3-receptors) (Streb et al., 1983). On the other side, Sacro/endoplasmic reticulum Ca^{2+} ATPases (SERCA) ensure the calcium influx into the ER (Hogan et al., 2010). IP3-receptors can be inhibited via phosphorylation by AKT. This can rescue cells from apoptosis (Szado et al., 2008). Normally, IP3-receptors are activated by inositol triphosphate (IP3), which is a second messenger generated at the plasma membrane by phospholipase Cs (PLC) (Mignery and Sudhof, 1990). PLCs in turn, are activated via GPCRs or receptor tyrosine kinases (RTK). Some PLCs can be activated directly via PIP2 or PIP3, too (Toker et al., 1994). HRAS has been shown to activate PLCε (Song et al., 2001). RTKs, like the epidermal growth factor receptor (EGFR), can activate the PLC-γ (Meisenhelder et al., 1989). Activated PLCs hydrolyse phosphatidylinositol 4,5-bisphosphate (PIP2) and thereby generate the soluble IP3 and diacylglycerol (DAG). DAG stays at the plasma membrane and induces other signalling cascades, e. g. it can activate protein kinase c (PKC). IP3 in turn can translocate to the ER and bind to the IP3-receptors, which then release calcium from the ER into the cytosol. An increase in cytosolic calcium can have many different effects depending on the type of cell and the external circumstances. One result is the increase of calcium concentration within the mitochondria (Rizzuto et al., 1993). Interestingly, mitochondria have a low affinity for taking up calcium, but close contacts between the ER and mitochondria were observed and here local calcium concentrations are so high, that mitochondria take up calcium readily (Montero et al., 2000; Rizzuto et al., 1998). One result is that the calcium concentration within mitochondria oscillates and calcium sensitive dehydrogenases are activated leading to the activation of the mitochondrial metabolism (Hajnoczky et al., 1995). Subsequently, the ATP synthesis and O_2 consumption is increased (Jouaville et al., 1999). The calcium efflux of the ER via IP3-receptors and subsequent influx via the mitochondrial calcium uniporter (MCU) can also lead to mitochondrial membrane potential loss (MMPL) and reactive oxygen species (ROS) generation (Wiel et al., 2014). A

direct link between the IP3-receptor 3 (IP3R3) and apoptosis has been demonstrated in lymphocytes (Khan et al., 1996).

One interesting finding, which might play a role during *S. aureus* infections, is that sublytic MAC (membrane attack complex of the complemen) attacks lead to an increase in cytosolic calcium, which is at least partly induced by calcium release from internal storage. As a consequence, mitochondrial membrane potential (MMP) is lost and the NLRP3 inflammasome is activated in epithelial cells (Triantafilou et al., 2013). Independently, the NLRP3 inflammasome can be activated by PVL and α-toxin, too (Craven et al., 2009; Holzinger et al., 2012). These two pore-forming toxins of *S. aureus* might lead to the same signalling as MAC attacks do.

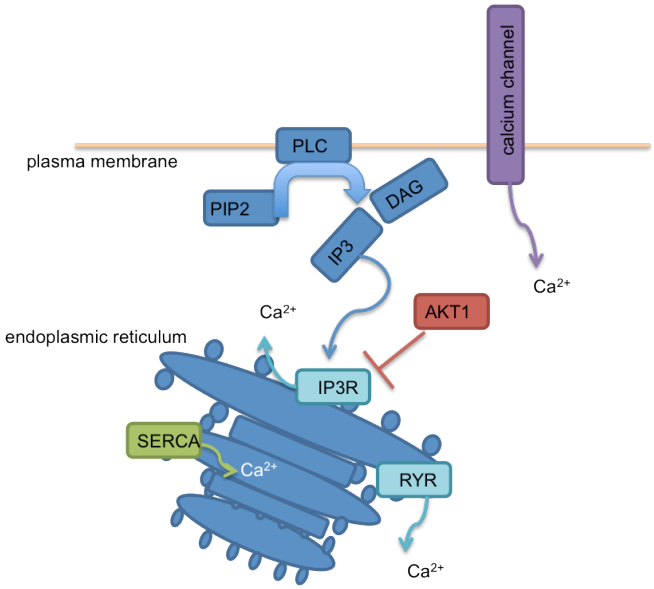

Figure 2 Scheme of intracellular calcium signalling. Activated PLCs generate DAG and IP3 from PIP2. IP3 then activates the IP3Rs in the ER and hence increases cytosolic calcium concentrations. RYRs can thereby be activated and increase the calcium concentration further. Diverse calcium channels on the plasma membrane can also increase cytosolic calcium concentrations. AKT1 inhibit IP3Rs by phosphorylation. SERCA channels in turn can increase ER calcium concentrations.

1.4 General host cell functions

1.4.1 Apoptosis and Necrosis: two ways to die

Apoptosis is a regulated way of cell death, not resulting in an immune response. In contrast, necrosis can be unregulated and leads to the leakage of cells resulting in an immune response. Beside these two classical types of cell death others like autophagic cell death are known. The different types often have some features in common. For example, cell blebbing is associated with apoptosis and autophagic cell death (Inbal et al., 2002; Kerr et al., 1972a).

Apoptosis can be triggered by extrinsic (receptor-mediated) or intrinsic (mitochondria-mediated) signalling pathways. Necrosis is normally the result of toxins or mechanical stress (Orrenius et al., 2003). The mitochondria-mediated signalling pathway results in a mitochondrial outer membrane permeabilisation (MOMP). MOMP can be accomplished by the two pro-apoptotic Bcl-2 family

members Bax and Bak or by mitochondrial permeability transition pores (MPTP), which are composed of an adenine nucleotide transocase (ANT) in the inner membrane and a voltage-dependent anion channel (VDAC) in the outer membrane of the mitochondria (Crompton et al., 1999). The association of Cyp-D with ANT results in the opening of the pore (Connern and Halestrap, 1994; Halestrap and Davidson, 1990). Besides, calcium has been shown to open this pore reversibly (Brustovetsky and Klingenberg, 1996). The opening of the ANT/VDAC pore leads to an influx of small solutes and water into the mitochondria and therefore to a swelling and rupture of the mitochondrial membrane (Garrido et al., 2006). The Bax/Bak pores as well as the ANT/VDAC channel opening lead then to an efflux of cytochrome c. Cytochrome c binds to Apaf-1 in the cytoplasm and this complex activates caspase 9 and subsequently caspase 3 (Acehan et al., 2002). Anti-apoptotic Bcl-2 family members like Bcl-2 can inhibit the Bax/Bak pore formation.

In addition, Bax can also interact with ANT to open the PT pore (Marzo et al., 1998). Bax is especially interesting since it has also been shown to induce also cell death without caspase activation (Xiang et al., 1996). This is extraordinary, since caspase activation is a hallmark of apoptosis. Normally, caspases are activated, before or after MOMP, and cleave cellular proteins. This promotes cell death induction and cell degradation (e.g. disintegration of the nucleus).

Other hallmarks of apoptotic cell death are the condensation of the nucleus and the cytoplasm. In a second step, cells show flowered-like morphology and single blebs are shedded till cells disappear. The blebs, also known as apoptotic bodies, are internalised by surrounding cells (Kerr et al., 1972b). DNA laddering was later added, as another hallmark of apoptosis and for its part it seems to be calcium dependent (McConkey et al., 1989; Wyllie, 1980). In contrast, necrotic cells show a swelling of the cytoplasm and the organelles, resulting in the permeability of the plasma membrane. These cells are internalised by surrounding cells too, but the released cellular factors can induce inflammation.

1.4.2 Mitochondrial impact on host cell death

The main function of mitochondria is to supply energy. ATP is produced via a proton gradient that is generated with the help of reduction equivalents, which are the product of the citrate cycle. Beside this important purpose, mitochondria are also a major players during life-death decisions of cells (Kroemer et al., 2007). Here, the ATP level of cells plays a prominent role. Apoptosis is induced in case of high ATP levels, necrosis in case of low ATP levels. Especially interesting is that the MPTP can be involved during apoptosis and necrosis (Eguchi et al., 1997; Leist et al., 1997; Qian et al., 1999). Furthermore, mitochondria play a role in the calcium homeostasis of the cell. There is a constant influx and efflux of calcium between mitochondria and the cytosol. When the cytosolic calcium concentration rises, it can lead to mitochondrial calcium overload, which can result in cell damage (Miyamae et al., 1996). But an uptake can also result in a reversible MMPL (Carafoli, 2002). In

addition, the cytosolic calcium increase can lead to MMPL and DNA laddering, resulting in apoptotic cell death (Miyamoto et al., 2005).

Therefore mitochondria fulfil various functions within cells and are a crucial factor during cell death induction.

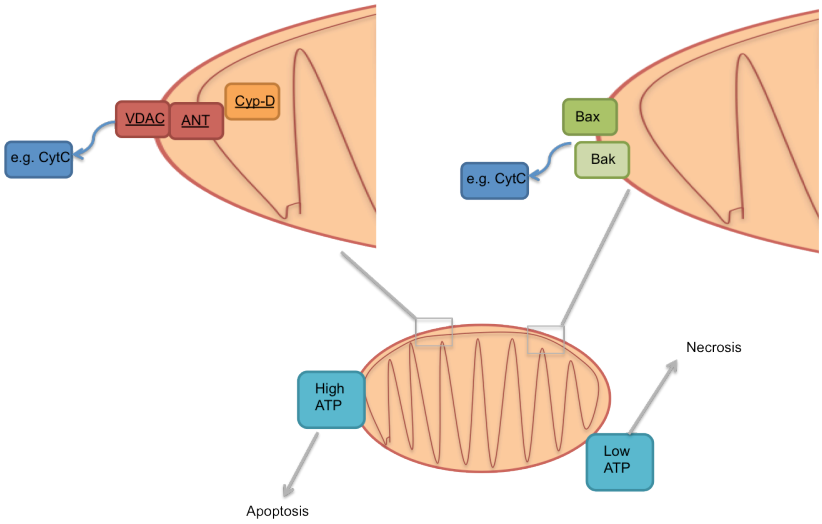

Figure 3 Mitochondria and cell death. The ATP level of a cell can determine the type of cell death a cell dies. Different models have been proposed how mitochondrial MOMP can be induced. The most prominent one is the Bax/Bak pore, which leads to a cytochrome c (CytC) efflux. In addition VDAC and ANT can lead to an influx of water into mitochondria. The conseuqnece is a swelling of mitochondria and rupture of the membrane resulting in CytC efflux.

1.5 Aims of this study

S. aureus can efficiently invade human host cells and subsequently kill them in breath-taking velocity. Bacterial toxins, which might be responsible, are heavily investigated but it is poorly understood what is going on in *S. aureus* infected cells and which host cell factors are involved.

The first aim of this study is to perform a whole genome screen to identify host cell factors involved in *S. aureus* infection. The second aim of this study is the interpretation of the screeing results.

After the successful screening process, the third aim is the validation of the screening results. Therefore the role of different factors should be determined via inhibitors and/or siRNA experiments, resulting in the identification of novel host cell factors involved in *S. aureus* infection focusing on inhibitors, which attenuate *S. aureus* infection.

For the screen as well as for the validation, new or altered methods have to be established, which are practicable under screening conditions.

2 Material and Methods

2.1 Material

2.1.1 Cell lines

Table 1 Cell lines.

Cell line	Properties	Source
HeLa	human epithelial cervical carcinoma cells	ATCC CCL-2
HEK 293t	human embryonic kidney epithelial cells	ATCC CRL-11268
HUVEC	Human umbilical vein endothelial cells	Invitrogen C01510C pooled
HeLa229-YFP-CWT	HeLa229 cells expressing YFP-CWT	M. Fraunholz

2.1.2 Bacterial strains

Table 2 Bacterial strains.

Strain	Comments	Source /Reference
S. aureus 6850	Clinical isolate septic arthritis	(Vann and Proctor, 1987)
S. aureus USA 300 Lac	CA-MRSA	(Seybold et al., 2006)
S. aureus CowanI		ATCC12598
S. aureus 6850 GFP	GFP expressing 6850	M. Fraunholz
S. aureus 6850 mRFP	mRFP expressing 6850	M. Fraunholz
Escherichia coli DH5α		M. Fraunholz

2.1.3 Plasmids

Table 3 Plasmids used in this study.

Plasmid name	Reference
psPAX	Addgene (12260)
pMD2.G	Addgene (12259)
pGIPZ	Thermo Scientific
shRNAmir library	Open Biosystems

2.1.4 Oligonucleotide

Table 4 Oligonucleotide used in this study.

Name	Sequence (5’ -> 3’)
shRNA screen	
Sense GIPZ	AATGATACGGCGACCACCGAGGACCGCGCACCTGGTGCATGAC
Reverse AGT	CAAGCAGAAGACGGCATACGAAGTCTAAAGTAGCCCCTTGAATTCCGAGGCAGTAG
Reverse TAC	CAAGCAGAAGACGGCATACGATACCTAAAGTAGCCCCTTGAATTCCGAGGCAGTAG
Reverse ATG	CAAGCAGAAGACGGCATACGAATGCTAAAGTAGCCCCTTGAATTCCGAGGCAGTAG
Reverse CGT	CAAGCAGAAGACGGCATACGACGTCTAAAGTAGCCCCTTGAATTCCGAGGCAGTAG
Reverse TGA	CAAGCAGAAGACGGCATACGATGACTAAAGTAGCCCCTTGAATTCCGAGGCAGTAG
Reverse GCA	CAAGCAGAAGACGGCATACGAGCACTAAAGTAGCCCCTTGAATTCCGAGGCAGTAG
Reverse GCA	CAAGCAGAAGACGGCATACGAGCACTAAAGTAGCCCCTTGAATTCCGAGGCAGTAG
Reverse GAC	CAAGCAGAAGACGGCATACGAGACCTAAAGTAGCCCCTTGAATTCCGAGGCAGTAG
qRT-PCR	
HMGCS1 fwd	CATTAGACCGCTGCTATTCTGTC
HMGCS1 rev	TTCAGCAACATCCGAGCTAGA
CAPN1 fwd	GAAGCGTCCCACGGAACTG

CAPN1 rev	GTGCAGGAGGGTGTCGTTG
CAPN2 fwd	CCCAACCTGTTCAAGATCATCC
CAPN2 rev	AGGCTTCCGTTACTTTCAACC
ITGA5 fwd	GCCTGTGGAGTACAAGTCCTT
ITGA5 rev	AATTCGGGTGAAGTTATCTGTGG
ITGB1 fwd	CAAGAGAGCTGAAGACTATCCCA
ITGB1 rev	TGAAGTCCGAAGTAATCCTCCT
ANPEP fwd	TTCAACATCACGCTTATCCACC
ANPEP rev	AGTCGAACTCACTGACAATGAAG
GAPDH fwd	GAAATCCCATCACCATCTTCCAGG
GAPDH rev	GACCCCCAGCCTTCCATG
PRKAR1A fwd	TTTCGGTCTCCTTTATCGCAGG
PRKAR1A rev	AACATAGACATCCGTCTCTCCTT
PCSK9 fwd	CCTGGAGCGGATTACCCCT
PCSK9 rev	CTGTATGCTGGTGTCTAGGAGA
MAPK8 fwd	TCTGGTATGATCCTTCTGAAGCA
MAPK8 rev	TCCTCCAAGTCCATAACTTCCTT
FPR2 fwd	TCTTGCTCTAGTCCTTACCTTGC
FPR2 rev	AATGACAAACCGGATAATCCCTC
AKT1 fwd	TCCTCCTCAAGAATGATGGCA
AKT1 rev	GTGCGTTCGATGACAGTGGT
HRAS fwd	GACGTGCCTGTTGGACATC
HRAS rev	CTTCACCCGTTTGATCTGCTC
JUN fwd	ACAGCTTCATGCCTTTGTAA
JUN rev	CTCAGAGTGCTCCAAATCTC

Table 5 siRNAs used in this study. All siRNAs were purchased from Qiagen. 4 independent siRNAs were used for each target.

gene symbol	gene name
ACCN3	Acid-Sensing (Proton-Gated) Ion Channel 3
ADCY9	adenylate cyclase 9
AKT1	v-akt murine thymoma viral oncogene homolog 1
ANPEP	alanyl (membrane) aminopeptidase
AP2S1	adaptor-related protein complex 2, sigma 1 subunit
ARGHEF9	Cdc42 guanine nucleotide exchange factor (GEF) 9
ASK1	mitogen-activated protein kinase kinase 5
AVIL	advillin
BCR	breakpoint cluster region
BIM	BCL2-like 11 (apoptosis facilitator)
CALM1	calmodulin 1 (phosphorylase kinase, delta)
CAPN1	calpain 1, (mu/I) large subunit
CAPN2	calpain 2, (m/II) large subunit
CAPN4	calpain, small subunit 1
Casp8AP2	caspase 8 associated protein 2
DLG1	discs, large homolog 1 (Drosophila)
FBXW8	F-box and WD repeat domain containing 8
FPR1	formyl peptide receptor 1
FPR2	formyl peptide receptor 2
GPER	G protein-coupled estrogen receptor 1
GRIK1	glutamate receptor, ionotropic, kainate 1
GRIK5	glutamate receptor, ionotropic, kainate 5
GRM5	glutamate receptor, metabotropic 5
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)
HPCAL4	hippocalcin like 4
HRAS	Harvey rat sarcoma viral oncogene homolog

HSPD1	heat shock 60kDa protein 1 (chaperonin)
ICAM	intercellular adhesion molecule 1
IP3R1	inositol 1,4,5-trisphosphate receptor, type 1
IP3R2	inositol 1,4,5-trisphosphate receptor, type 2
IP3R3	inositol 1,4,5-trisphosphate receptor, type 3
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)
ITGAM	integrin, alpha M (complement component 3 receptor 3 subunit)
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
ITPRIP	inositol 1,4,5-trisphosphate receptor interacting protein
JAK1	Janus kinase 1
JNK II	mitogen-activated protein kinase 9
JUN	Jun proto-oncogene
LDLR	low density lipoprotein receptor
LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
MAPK8	mitogen-activated protein kinase 8
MOBP	myelin-associated oligodendrocyte basic protein
TP53	tumour protein *p53*
PCSK9	proprotein convertase subtilisin/kexin type 9
PIK3CG	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma
PKN1	protein kinase N1
PLA2G4	phospholipase A2, group IVE
PPARA	peroxisome proliferator-activated receptor alpha
PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I, alpha
PRRG4	proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)
PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1
PTEN	phosphatase and tensin homolog
PTGER3	prostaglandin E receptor 3 (subtype EP3)
Rab5A	Rab5A, member RAS oncogene family
RXRA	retinoid X receptor, alpha
RYR1	ryanodine receptor 1
RYR2	ryanodine receptor 2
RYR3	ryanodine receptor 3
SCNN1D	sodium channel, non-voltage-gated 1, delta subunit
SGSM3	small G protein signalling modulator 3
SMAD4	SMAD family member 4
SYNPO2	synaptopodin 2
THEMIS	thymocyte selection associated
TRIM33	tripartite motif containing 33
TRPM3	transient receptor potential cation channel, subfamily M, member 3
TRPM4	transient receptor potential cation channel, subfamily M, member 4
TRPM8	transient receptor potential cation channel, subfamily M, member 8
VCL	vinculin
VIP	vasoactive intestinal peptide
GFP	negative siRNA control
All Star	negative siRNA control
Cell death control	transfection control

2.1.5 Kits

Table 6 Kits used in this study.

Description	Company
AxyPrep Multisource genomic DNA miniprep kit Cat. No AP-MN-MS-GDNA-50	Axygen Biosciences

Gelextractionkit QIAquick	Qiagen
Nucleobond AX	Macherey-Nagel
AxyPrep Plasmid Miniprep Kit	Axygen Biosciences
QIAquick® PCR Purification Kit Cat. No. 28106	Qiagen
QIAquick® Gel Extraction Kit Cat. No. 28706	Qiagen
Experion™ DNA 1K Kit Cat. No. 700-7163	BIO RAD
SR Cluster Generation TruSeq Kit v5 Cat. No. GD-203-5001	Illumina
GAIIx Sequencing TruSeqSBS Kit v5 Cat. No. FC-104-5001	Illumina
BD™ MitoScreen Cat. No. 551302	BD Bioscience
RNeasy Mini Kit Cat. No. 74106	Qiagen
QuantiTect Rev. Transcription Kit Cat. No. 205313	Qiagen

2.1.6 Antibodies

Table 7 Antibodies.

Antibody target	Origin	Dilution	Company
Actin (8432)	Mouse	1:5000	Sigma Aldrich
ITGB1 (4706)	Rabbit	1:1000	Cell Signaling
AKT1 (4685)	Rabbit	1:1000	Cell Signaling
pAKT1 Ser473 (4060)	Rabbit	1:1000	Cell Signaling
pAKT Thr308 (2965)	Rabbit	1:1000	Cell Signaling
Anti-mouse IgG HRP linked sc2005	Goat	1:3000	Santa Cruz
Anti-rabbit IgG HRP linked sc2004	Goat	1:3000	Santa Cruz

2.1.7 Growth media

Table 8 Cell culture and bacterial growth media.

Medium/chemical	Company
RPMI 1640	GIBCO
DMEM	Sigma Aldrich
DPBS	GIBCO or Sigma Aldrich
Fetal calf serum (FCS)	PAA
TrypLE™ Express	GIBCO
Medium 200	GIBCO
Sodium pyruvate	GIBCO
Penicillin/Streptomycin	Sigma Aldrich
Puromycine	Sigma Aldrich
Tryptic soy broth (TSB)	Sigma Aldrich

2.1.8 Buffers, solutions and media

Table 9 Buffers, solutions and media.

Media	
Annexin binding buffer	10mM Hepes/NaOH pH7.4 140mM NaCl 5mM $CaCl_2$
Cell culture medium	RPMI medium 1640 + 10%FBS + 1mM sodium pyruvate
Selection medium	RPMI medium 1640 + 10%FBS + 1mM sodium pyruvate + 100units/ml penicilin + 100µg/ml streptomycin + puromycin
Stocking medium	RPMI medium 1640 + FBS + 10%(v/v) DMSO
Polyethylenimine (PEI)	1mg/ml in H_20
LB medium (1l)	10 g tryptone, 5 g yeast extract, 10 g NaCl
LB agar (1l)	10 g tryptone, 5 g yeast extract, 10 g NaCl, 15g agar

Western blot	

10 x SDS buffer (1l)	30.275 g Tris, 144 g glycine, 10 g SDS
10x Semi dry transfer buffer (1l)	24 g Tris, 113 g glycine, 2 g SDS
1x Semi dry transfer buffer	10x semi dry buffer diluted to 1x + 20% (v/v) methanol
10x TBS (1l)	60.5 g Tris, 87.6 g NaCl, adjust to pH 7.5 with HCl
Blocking solution	1xTBS + 5% (w/v) dry milk powder or BSA
2x Laemmli buffer for lysis	100 mM Tris/HCl (pH 6.8), 20% (v/v) glycerol, 4% (w/v) SDS, 1.5% (v/v) 2-mercaptoethanol, 0.004% bromophenol blue
8% SDS lower gel solution	For 5ml:2.3 ml H_2O, 1.3 ml 30% (v/v) acrylamid-bisacrylamid mix (5:1), 0.05 ml 10% (w/v) SDS, 1.3 ml 1.5 M Tris/HCl (pH 8.8), 0.05 ml 10% APS, 0.003 µl TEMED
SDS upper gel solution	For 5 ml: 3.4 ml H2O, 0.83 ml 30% (v/v) acrylamid-bisacrylamid mix (5:1), 0.63 ml 1.0 M Tris/HCl (pH 6.8), 0.1 ml 10% (w/v) SDS, 0.05 ml 10% APS, 5 µl TEMED
ECL solution 1	2.5 mM Luminol, 0.4 mM p-coumaric acid
ECL solution 2	100 mM Tris HCl pH 8.5, 0.02 % H2O2

2.1.9 Chemicals

Table 10 Inhibitors, enzymes, marker and fine chemicals.

Chemical	Supplier
APC AnnexinV (550475)	BD Bioscience
7-AAD (559925)	BD Bioscience
2-APB	Sigma Aldrich
Calpeptin	Sigma Aldrich
CGP37157	Sigma Aldrich
JNKII inhibitor (SP600125)	Calbiochem
Methyl-β-cyclodextrin	Sigma Aldrich
Calpain Inhibitor I (ALLN)	Roth
Cathepsin Inhibitor	Santa Cruz (sc-3130)
PD150606	Sigma Aldrich
Z-VAD-FMK	BD Bioscience
Phusion DNA Polymerase	Fermentas
SYBR® Green PCR Master Mix Cat. No. 4309155	Applied Biosystems
MG-132 Cat. No. 474788	Merck
GeneRuler™ 1kb DNA Ladder	Thermo Scientific
PageRuler™ Prestained Protein Ladder	Thermo Scientific

All other chemicals were obtained from Sigma Aldrich, Roth, Serva or Merck chemicals if not stated otherwise.

2.2 Methods

2.2.1 Bacterial culture

Staphylococcus aureus strains were stored at -80°C in 25% glycerol and TSB. Every three weeks a new TSB agar plate was inoculated with *S. aureus*. This plate was incubated over night at 37°C and with 5%CO_2. For infection staphylococci were grown in 2ml TSB medium over night. A main culture was inoculated and grown till OD_{540} of 0.6 at day of infection.

2.2.2 Production of competent *E. coli* and transformation of plasmids

For the production of competent *E.coli* DH5α one bacterial colony was picked and resolved in 2ml LB medium. Bacteria were grown at 37°C till an OD_{600} of 0.5 was reached. The bacterial culture was

incubated for 20 min on ice before it was centrifuged for 10 min at 3.000g at 4°C. The bacterial pellet was resolved in 10ml of ice-cold TSS solution and froze at -80°C in aliquots (Chung et al., 1989).
For transformation one aliquot of competent bacteria was thawed. Plasmid DNA was added and the solution was incubated for 30 min on ice. After a heat shock at 42°C for 1.5 min the solution was incubated for 2 min again on ice. 800µl of LB medium were added and the bacteria were grown for 1h at 37°C while shaking. Bacteria were pelleted and plated on LB-plates with desired antibiotics. Positive clones were picked and cultivated further. With the Nucleobond AX kit plasmids were isolated (according to manufacturers insturctions) and stored at -20°C.

2.2.3 Cell culture

2.2.3.1 Cultivation

All cell lines used in this study were cultivated in a humidified atmosphere in a cell incubator. They were cultivated at 37°C and with 5%CO_2 in 75cm^2 cell culture flasks. Cells were passaged every two to three days. For this, adherent cells were washed once with PBS and incubated for 5-10min with trypsin. To stop the enzymatic reaction fresh cell culture medium supplemented with 10% FCS was added. Cells were diluted and cultured in fresh medium.

2.2.3.2 Long term storage

Cells were detached as described above resuspended in fresh medium and centrifuged at 800g for 5 min. Afterwards cells were resuspended in 1ml medium containing 10% DMSO and gradually cooled down to -80°C in an isopropyl alcohol box. Then they were stored in a liquid nitrogen tank.

2.2.4 Infection assay

One day before infection 8x10^4-1x10^5 cells were seeded in 12 well plates. The main culture was inoculated with an over night culture of *S. aureus* at an optical density (measured at 540nm) of 0.3. When bacterial suspension reached an optical density of 0.6 bacteria were harvested. Thereafter, the bacterial culture was centrifuged at 10,000g for 1 minute. The pellet was washed once with PBS and once with serum free medium. Independently, cells were washed once with PBS. For infection 0.5ml medium containing FCS was added to the cells. Cells were infected with a desired multiplicity of infection (MOI) of bacteria resolved in serum free medium. One hour post infection 20 $\frac{\mu g}{ml}$ lysostaphin was added in order to lyse extracellular staphylococci (Schindler and Schuhardt, 1964).

2.2.5 shRNA screen

2.2.5.1 Generation of the HeLa-shRNA library

293T cells were cultivated in 15cm dishes up to a density of 80%. Five dishes were transfected with 10µg of pGIPZ, 7µg of psPAX and 3.5µg of pMD2.G plasmid. PEI (1 $\frac{\mu l}{\mu g}$ of total DNA) was used for transfection. The pGIPZ vectors were a mixture of plasmids, which coded for 10,000 different shRNAs. Virus containing supernatant was collected 48h and 72h post transfection. The supernatant

was filtered sterilely and 1 $\frac{\mu g}{\mu l}$ polybrene were added. HeLa cells, which were cultivated in five 15cm dishes, were infected with the produced virus at a MOI of 0.1. 24hrs p.i. the medium was changed and cells were cultivated with puromycin containing medium for selection of virus-infected cells. These HeLa-shRNA cells were cultivated under puromycin pressure until they were used for infection.

2.2.5.2 shRNA screen implementation

One day before infection 3x10^6 HeLa-shRNA cells were seeded in seven 15cm dishes. Of these seven dishes six were infected with *S. aureus* 6850 mRFP for 1h. Subsequently, lysostaphin was added in order to kill extracellular bacteria. 4.5hrs post infection dead cells were washed away during two PBS washing steps and all attached cells were trypsinised and unified before sorting. With the help of forward scatter (FSC) and side scatter (SSC) the population of intact cells was determined. HeLa-shRNA cells expressed GFP (Emission/Excitation: 480nm/530nm) and infected cells in addition showed an mRFP signal (Em/Ex: 561nm/610nm). The genomic DNA of the sorted cells and the uninfected cells was isolated with the Multisource Genomic DNA Miniprep Kit (Axygen Biosciences). Via a PCR step the shRNA cassettes were amplified and separation from the genomic DNA was obtained via an agarose gel electrophorese. The concentrations of the shRNA cassettes were measured with a nanodrop and samples were diluted to a final concentration of 2 $\frac{ng}{\mu l}$, which corresponds to a molarity of 10 $\frac{nmol}{l}$. An Experion analysis confirmed the purity and concentration of the DNA. Samples were prepared for sequencing like propose by Illumina.

Table 11 PCR conditions for shRNA cassette recovery.

Initial template denaturation	98°C	1min
28 cycles		
	98°C	10sec
	72°C	25sec
Final extension		
	72°C	5min
	4°C	hold

2.2.6 Fluorescence-activated cell sorting (FACS)-based invasion assay

Cells were infected as described in 2.2.4. 10 min after lysostaphin treatment, cells were washed with PBS and trypsinised. Medium was added and cells were transferred into FACS tubes. Intact cells were determined via FSC and SSC. An uninfected sample was used to determine autofluorescence of the cells and signals above this value were defined as infected. The percentage of the infected cells was determined as well as the relative amount of intracellular bacteria. The latter was determined via the comparison of the mean of the fluorescence of infected cells. The relative invasion rate was calculated as the product of percentage of infected cells and mean of the infected cells. In case of

different mean values for uninfected cells (e.g. after inhibitor treatment) different autofluorescence levels were considered.

2.2.7 FACS-based assay for determination of intracellular replication

Cells were infected as described in 2.2.4. Two wells for each experimental setting were infected. 1h p.i. lysostaphin was added to each well. 10 min after lysostaphin treatment, the first well of each experimental setting was trypsinised. Medium was added and cells were transferred into FACS tubes. Intact cells were determined via FSC and SSC. An uninfected sample was used to determine the autofluorescence of the cells and signals above this value were defined as infected. The mean of the infected cells was determined. 3hrs p.i. the other wells were tripsinised and measured with FACS. The mean of the infected cells was determined. Afterwards the ratio between the mean measured 1h p.i. and the mean measured 3hrs p.i. was determined and control infection was set to 100.

2.2.8 Determination of mitochondrial membrane potential

Cells were infected as described in 2.2.4 or treated like indicated. For the determination of mitochondrial membrane potential the dye JC-1 was used. JC-1 monomers are excited at 488nm and emit at 520nm. JC-1 multimeres, which are formed in viable mitochondria show a longer emission wavelength and can be detected at 590nm. Cells were incubated with JC-1. After 15 min of incubation, they were washed, trypsinised and subsequently analysed with AriaIII.

2.2.9 Annexin/7AAD staining

Cells were infected as described above or treated like indicated. Then they were trypsinised and centrifuged for 3 min at 800g. Cells were resuspended in 50µl of AnnexinV-APC/7AAD solution (final concentration of annexin and 7AAD 18 $\frac{\mu l}{ml}$) and incubated in the dark at room temperature for 10 min. The samples were diluted with 300µl of annexin binding buffer and analysed with AriaIII.

2.2.10 siRNA transfection and screen

siRNAs were purchased from Qiagen in a 96 well plate format. The siRNAs were reconstructed with ultrapure water to a final concentration of 10µM.

1.5µl of a 10µM siRNA pool solution (four different siRNAs against the same target) were spotted in 12 well plates (final concentration of siRNA: 25nM). 100µl of a serum free medium-HiPerFect® solution (0.8µl HiPerFect® per well) were added and incubated for 15 min. $1x10^5$ HeLa cells dissolved in 700µl medium were seeded in each well and incubated for 28hrs. After that, the medium was changed and 12hrs later transfected cells were infected with *S. aureus* 6850 as described previously.

An invasion assay was performed 1h post infection. 3hrs post infection an annexin staining was performed. An siRNA, which induces cell death was used as a transfection control in all experiments.

2.2.11 SDS page and Western blot analysis

Cells were lysed directly in the well with 2x Laemmli buffer and subsequently were incubated at 95°C for 20 min for protein denaturation. Proteins were separated via gel electrophoresis on 7.5%-12% SDS gels with 120V.

Gels were blotted on PVDF membranes using a semi-dry blotting chamber. Blotting conditions were 1mA per cm^2 for 2hrs.

The membrane was blocked for 1h with 5% milk powder in 1xTBS and incubated over night at 4°C with the desired antibody (diluted in 1xTBS containing 5% milk powder). After three washing steps every 10 min the membrane was incubated with a secondary antibody (1:3000 in 1xTBS containing 5% milk powder) for 1h at room temperature.

2.2.12 Quantitative real time polymerase chain reaction (qRT-PCR)

RNA was isolated with the RNeasy Kit from Qiagen according to manufacturers instructions. The amount of isolated RNA was determined via a NanoDrop. The reverse transcription was performed with the QuantiTect Rev. Transcription Kit from Qiagen according to manufacturers instructions. The qRT-PCR was then conducted with SYBR green according to manufacturers instructions. Subsequently, the data were analysed via the comparative C_T method (Schmittgen and Livak, 2008).

2.2.13 Colony forming unit assay (CFU) assay

Cells were infected with *S. aureus* like already described in 2.2.4. 1h and 3hrs post infection cells were washed once with PBS and incubated with ultrapure water for 15 min. Eukaryotic cells were thus lysed and different dilutions of the bacteria containing solution were plated. CFU were counted the following day.

2.2.14 Animal experiments

Animal experiments were performed at the Institute for molecular infection biology (University of Würzburg) by Babett Oesterreich in the group of PD Dr. Knut Ohlsen. Ten BALB/c mice were treated with 2-APB 24hrs before and during infection with *S. aureus* USA300 (a control group of ten mice was treated with DMSO). A sepsis mouse model was used. The kidneys were isolated 3 days p.i. .

PBS was added to the isolated kidneys and the kidneys were mixed. The CFU per kidney were determined by plating different dilutions on agar plates. CFU were counted the following day (CFU determination was performed in our lab).

2.2.15 Inhibitor treatments

All inhibitors used in this study were added to the cells 30 min before infection and remained there during the whole infection process.

2.3 Technical equipment

Hera Cell 240i incubator (Thermo), Hera Safe sterile bench (Thermo), Megafuge 1.0R centrifuge (Heraeus), cold centrifuge CT15RE (Himac), TCS SPE confocal microscope (Leica), DMIL light microscope (Leica), Step One Plus RT PCR system (Applied Biosystems), Thermal cycler 2720 (Applied Biosystems), pH Electrode SenTix (WTW), Scanjet G4010 (HP), Shaker TR125 (Infors HT), Thermo mixer comfort (Eppendorf), Spectrophotometer Ultrospec 3100 pro (Amersham Bioscience), NanoDrop 1000 spectrophotometer (Peqlab Biotechnology), C6 Flow Cytometer (Accuri), FACSaria III (BD), Chemiluminescence camera system (Intas), electric balance ABS-80-4 (Kern), electric balance EW-1500-2M (Kern), Illumina Genome Analyzer II (Illumina).

2.4 Software

Microsoft Office 2011 (Mac version), BD FACSDiva™ Software, ImageJ, Step One Plus software, EndNoteX4

Online software

STRING pathway analysis: http://string-db.org/ (Franceschini et al., 2013)

WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): http://bioinfo.vanderbilt.edu/webgestalt (Zhang et al., 2005). WebGestalt uses http://www.geneontology.org/ontology/ for Gene Ontology (GO) classification.

www.genecards.org (Stelzer et al., 2011)

KEGG: http://www.kegg.jp/kegg/tool/map_pathway1.html (Kanehisa et al., 2014)

2.5 Statistical analysis

Significance was determined via the student's t-test (two-tailed) with Excel 2011 (Mac version). Stars have the following meaning: $p>0.05$= n.s., $p\leq0.05$ = *, $p\leq0.01$ **, $p\leq0.0010$ = ***.

3 Results

3.1 shRNA screen for the identification of host cell factors involved in *S. aureus* 6850 induced cytotoxicity

S. aureus is an important human pathogen, which can cause massive tissue destruction, and various publications show that *S. aureus* kills efficiently human host cells (Haslinger-Loffler et al., 2006; Menzies and Kourteva, 1998; Tarkowski et al., 2002; Tucker et al., 2000). However, it is not clearly defined how this cell death is induced. Different studies using different or identical cell types, came to different conclusions how *S. aureus* infected cells die. This indicates that cell type specialities and perhaps other unknown factors play a huge role and the results of different studies cannot be transferred easily.

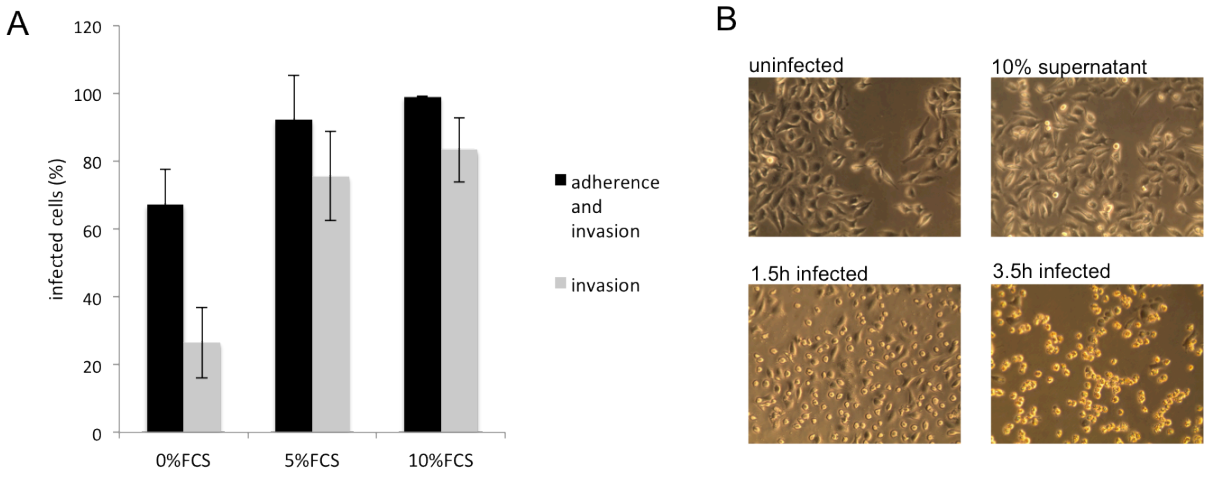

Figure 4 Invasion rate of *S. aureus* 6850 into HeLa cells and course of infection. (A) Cells were infected for 1h in the presence of different concentrations of fetal calf serum (FCS) and subsequently treated with lysostaphin in order to kill extracellular bacteria. The trypsinised cells were analysed with a C6 Flow Cytometer (Accuri). The mean of three independent experiments ± SD is shown. (B) Light microscopic monitoring of *S. aureus* infected HeLa cells. Cells were infected for 1.5hrs or 3.5hrs or treated with bacterial culture supernatant for 3.5hrs. 20x magnification.

To gain a more detailed insight into the interplay between *S. aureus* and the human host cell as well as a comprehensive picture of the involved host cell pathways, a whole genome short hairpin RNA (shRNA) screen was performed. With this method the influence of every single gene of the human host cell can be investigated. Through this approach, not only popular apoptosis or necrosis proteins, but also proteins, which are not yet linked to cell death, are investigated. It became obvious directly at the beginning that one difficulty of such a screen is to detect the effect of one single shRNA. Especially proteins, which regulate cell death, are very redundant and so another protein can undertake the task of the one, which was knocked down. Therefore it is important that screening conditions are carefully determined. Since we wanted to identify genes involved in *S. aureus* induced host cell death, it is essential that cells, which code for an irrelevant shRNA die fast and can be

removed easily, with the result that only those cells, which code for an shRNA against a cell death relevant gene, are analysed further

3.1.1 Determination of screening conditions

In a first step, optimal screening conditions were determined. Thus, the invasion rate under different conditions was evaluated. HeLa cells were infected with *S. aureus* 6850 GFP and the percentage of infected cells was determined via FACS analysis. *S. aureus* 6850 GFP infected cells also showed, beside the autofluorescence, the GFP signal of the internalised bacteria. This allows the distinction between uninfected and infected cells (**Fig. 5**). As the percentage of infected HeLa cells correlates with the fetal calf serum (FCS) concentration (**Fig. 4A**), it is obvious that invasion conditions are important and so I decided to infect cells with 10% FCS to get over 80% invasion rate and thus minimise the number of uninfected cells. A low number of uninfected cells is very important. Since we wanted to determine invasion relevant genes as well and the background (generated by the coincidentally not infected cells) increases massively, when fewer cells are infected.

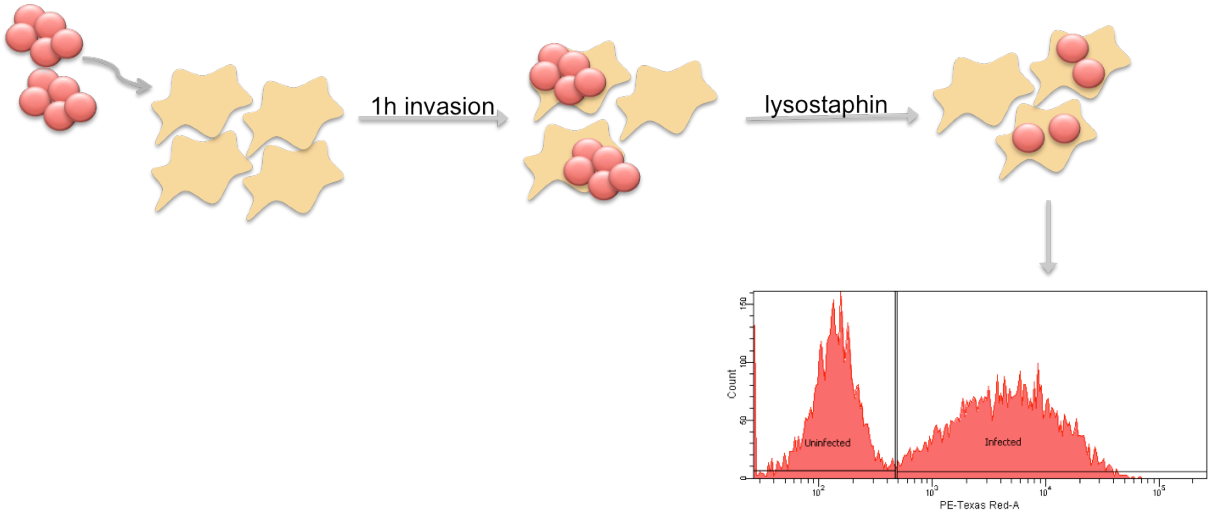

Figure 5 FACS-based invasion assay. Cells were infected with fluorochrome (here mRFP) expressing *S. aureus* strains. Lysostaphin treatment 1h p.i. lysed extracellular bacteria. Trypsinised cells were analysed with FACS (FACSaria III or C6 Accuri). The FACS blot shows uninfected cells on the left side and infected cells on the right side. The difference in fluorescence is due to the fact that infected cells show the mRFP signal of the bacteria in addition to their autofluorescence.

After invasion conditions were determined, I wanted to get a first overview of *S. aureus* cytotoxicity in this system and thus cell death was investigated in more detail. Therefore, the course of infection was microscopically monitored (**Fig. 4B**). Shortly after infection HeLa cells were rounded and showed a flowered-like morphology. In addition, rounded HeLa cells detached quickly from the surface.

Due to the fact that *S. aureus* was long described as an extracellular pathogen, the effect of supernatant on HeLa cells was determined to find out if secreted toxins are responsible for this cytotoxic effect (**Fig. 4B**). The addition of 10% bacterial supernatant did not change morphology of

HeLa cells, which suggests that not extracellular toxins but rather intracellular bacterial processes are responsible (**Fig. 4B**). To ascertain this, the course of infection with GFP expressing *S. aureus* was monitored (**Fig. 6**). All rounded cells showed intracellular bacteria, which confirms that internalised bacteria are responsible for cell death. Furthermore cells, which survived 24hrs, did not harbour bacteria and did not show morphological changes (**Fig. 6 last row**).

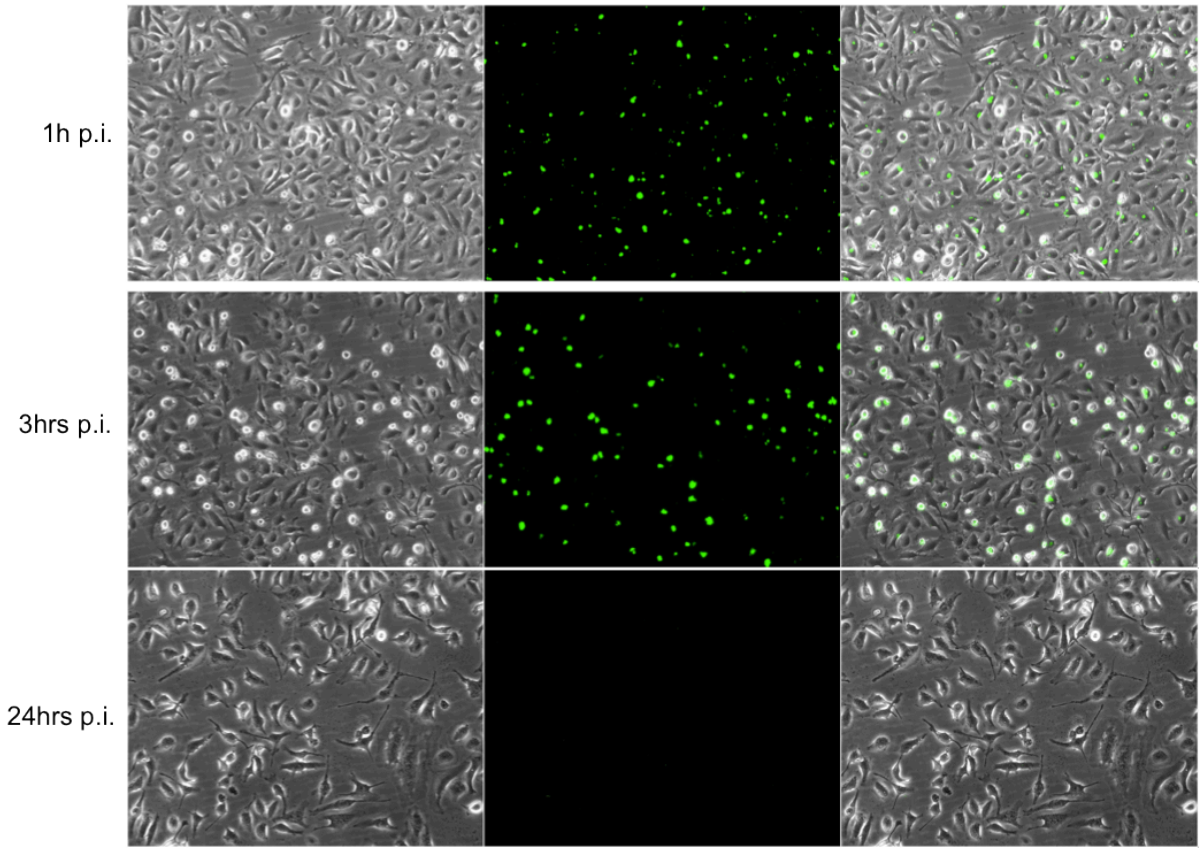

Figure 6 Microscopic observations of living cells infected with *S. aureus* 6850 GFP. Cells were infected and pictures were taken at indicated time points. The GFP signal of GFP expressing *S. aureus* 6850 is shown in green. Bacteria grew intracellularly between 1h and 3hrs p.i., this is indicated by the increase in GFP signal. The cells, which survived 24hrs did not harbour any bacteria. Magnification 20x.

To confirm further that *S. aureus* induces the death of infected cells an annexin/7AAD assay was performed. Annexin binds to phosphatidylserine, which is normally localised at the inner leaflet of the plasma membrane and is externalised as a consequence of apoptotic stimuli (van Engeland et al., 1998). 7AAD can, like propidium iodide (PI), just enter cells without an intact membrane. Infected cells are annexin positive but impermeable for 7AAD, which indicates apoptotic cell death (**Fig. 7**).

3.1.2 Generation of HeLa-shRNA library

Beside the determination of infection conditions, a preparation of the shRNA containing HeLa cells was necessary. So, HeLa cells containing the shRNAmir library (HeLa-shRNA) were created. Therefore 293T cells were transfected with the GIPZ lentiviral shRNAmir library (Open Biosystems) and

packaging vectors to create lentiviruses. With these lentiviruses HeLa cells were infected and thus five different HeLa cell pools were created. Each HeLa cell pool consists of HeLa cells, which code for over 10,000 different shRNAs. Every individual HeLa cell contains one shRNA and so one individual protein is knocked down. The single integration was ensured via a viral MOI of less than 0.3. HeLa-shRNA cells encode, beside the shRNA, a puromycin resistance and a GFP cassette for identification of shRNA containing cells.

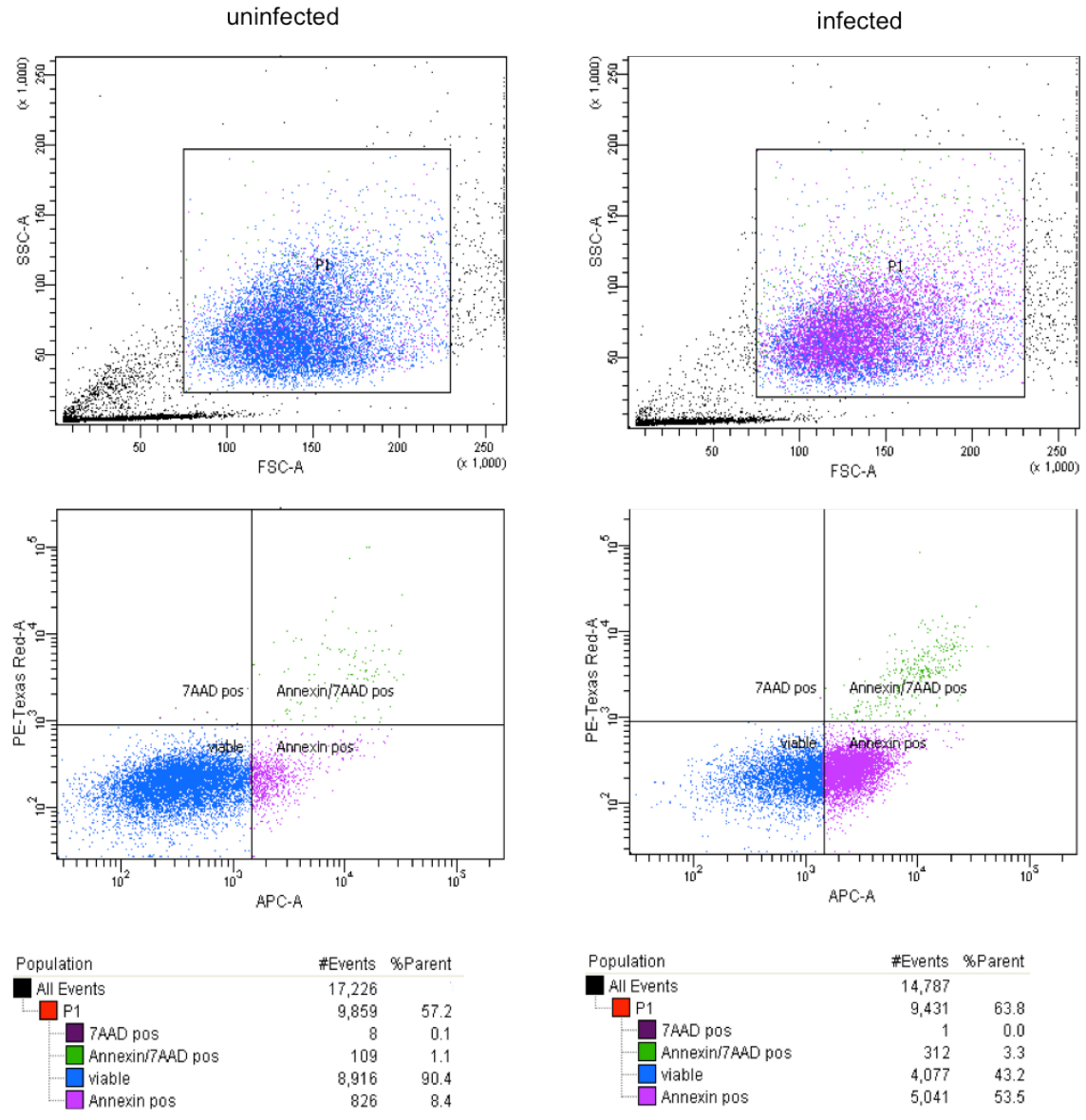

Figure 7 HeLa cells become annexin positive during *S. aureus* 6850 infection but are impermeable for 7AAD. In the first row intact cells were determined via FSC and SSC. In the second, the APC channel shows annexin staining and the PE-Texas Red channel 7AAD positive cells. In both cases none of the cells were only 7AAD positive. Annexin positive were 8% in the uninfected and 54% in the infected sample. Double positive was 1% in the uninfected and 3% in the infected sample. Viable, which means negative for both substances, were 90% of the uninfected and 43% of the infected cells.

3.1.3 Screening results

For the screen, HeLa-shRNA cells were infected with mRFP expressing *S. aureus* 6850 for 4.5hrs. Dead cells were washed away and uninfected and infected viable cells were sorted with the FACSaria III cell sorter (**Fig. 8**). For further analysis, genomic DNA was first isolated and then a PCR step amplified the shRNA, which was integrated into the genome of the sorted cells. To define the distribution of shRNAs in the HeLa cell pools, the genomic DNA of untreated HeLa-shRNA cells was also isolated and analysed and then defined as input. All PCR products were sequenced with an Illumina Genome Analyser[1].

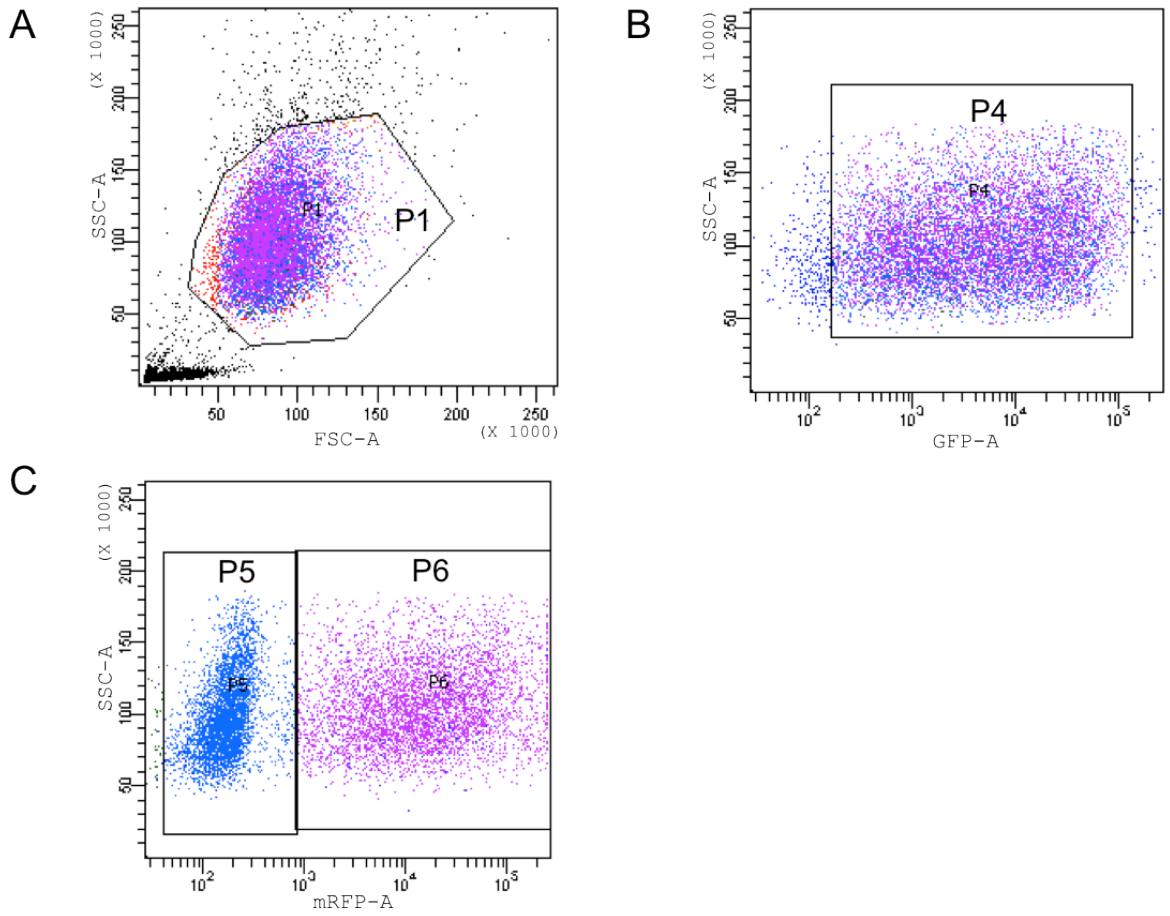

Figure 8 shRNA screen sort. (A) Intact cells were defined via FSC and SSC (P1). (B) GFP expressing cells were gated as P4. These cells should also express an shRNA. (C) Cells harbouring intracellular *S. aureus* 6850 showed increased mRFP fluorescence. Uninfected and infected HeLa cells were gated as P5 and P6. P5 and P6 were sorted in two different tubes with an AriaIII cell sorter.

[1] Performed together with Carsten Ade (Department of Biochemisty and Molecular Biology Würzburg)

3.1.4 Bioinformatics analysis of screeing results

The Illumina sequencing generated a large number of sequences, which were aligned to the GIPZ library in a first step and so the number of reads and the target for every shRNA was determined[2]. Then the ratios between infected and input as well as uninfected and input were determined and statistically analysed[3]. Even though two biological experiments were performed, no statistically significant hits could be determined. Nevertheless, a huge number of enriched shRNAs could be determined. Of the 60,000 different shRNAs used in this study, 38,050 showed more than 10 sequencing-reads. Two different data sets were generated. One shows eukaryotic genes involved in the *S. aureus* invasion process. Those are the targets of the shRNAs, which were enriched when the uninfected sample was compared to the input sample. The other set highlights cell death associated genes. These are the targets of the shRNAs, which were enriched when the infected sample was compared to the input sample. 112 cell death associated genes were enriched more than 10 fold. 70 invasion relevant genes were enriched at least 10 fold.

Due to the fact, that a lot of the top hits are genes, which are just poorly characterised and no direct connection between these hits could be observed, I decided to perform a STRING analysis. For the invasion network, I performed a STRING analysis with all at least 3 fold enriched genes in order to find relevant connections (**Fig. 9+10**) (Franceschini et al., 2013).

[2] Performed by Mark Onyango (Bioinformatics Giessen)
[3] Performed by Liang Chunguang (Bioinformatics Würzburg)

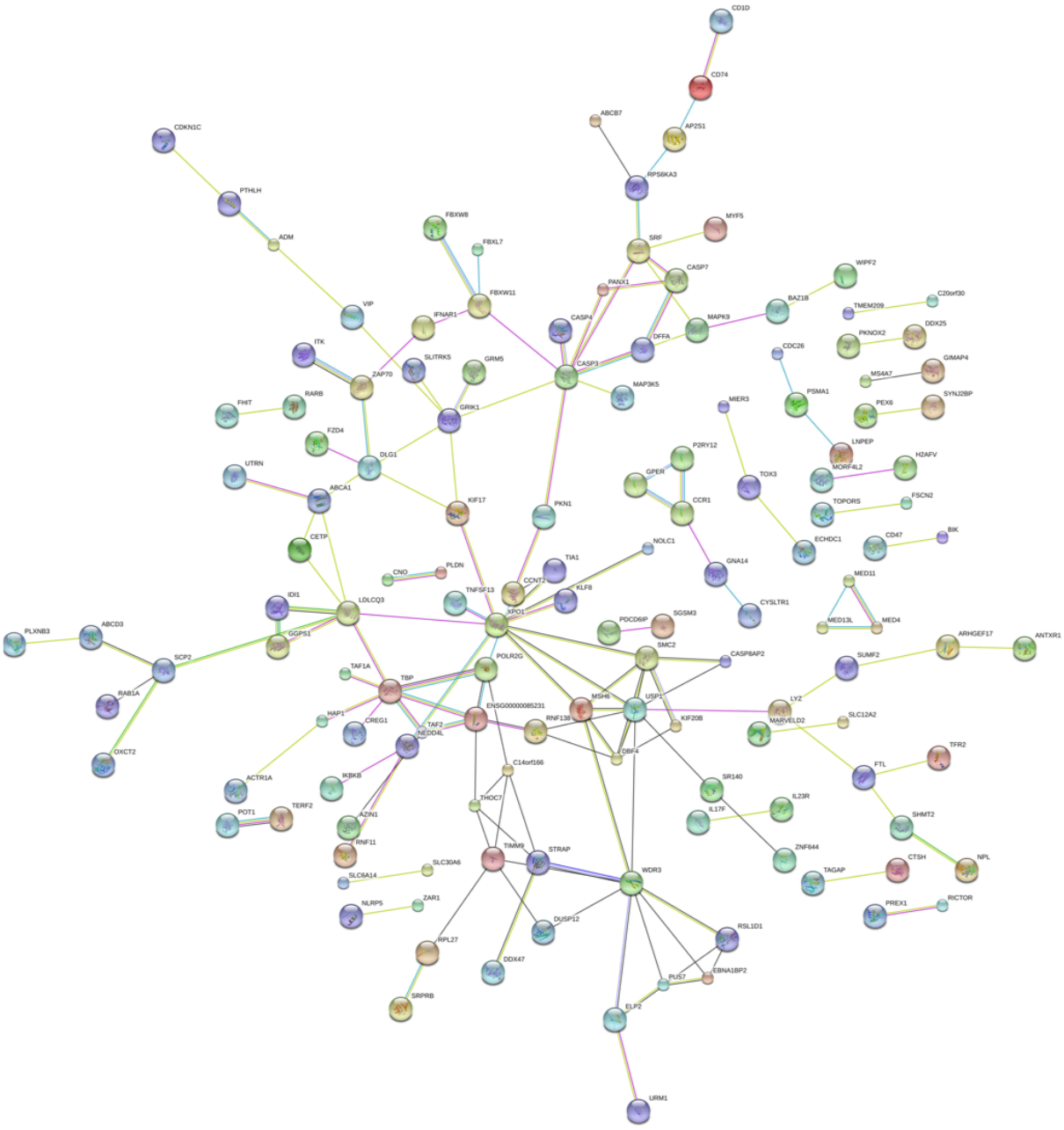

Figure 9 STRING analysis of invasion relevant genes. All at least 3 fold enriched genes were used. Disconnected genes are not shown. Colour code: green: co-mentioned in PubMed; pink: experimental/biochemical data; black: co-expression; blue: association in curated databases; purple: homology.

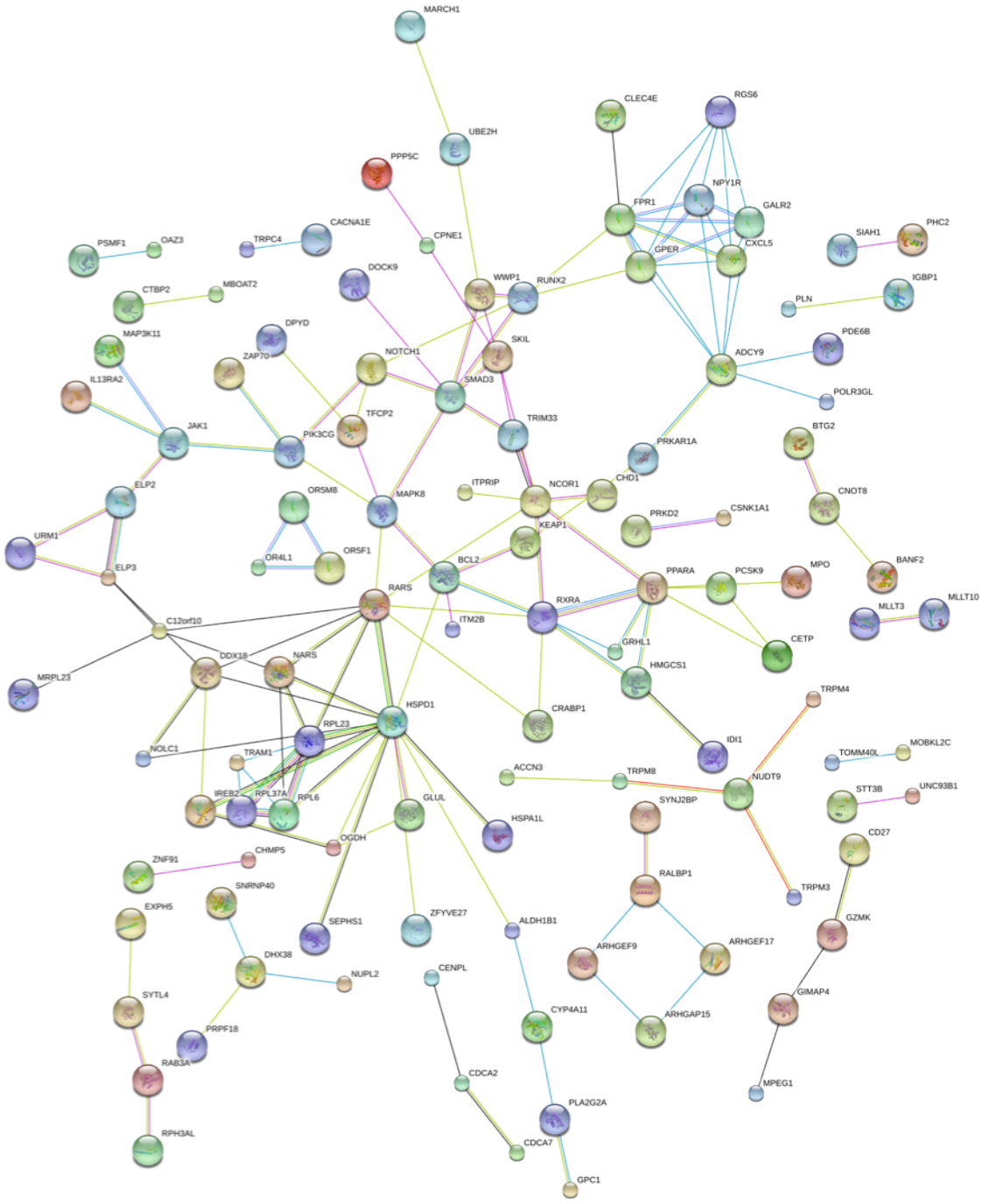

Figure 10 STRING analysis of cell death associated genes. All at least 4 fold enriched genes which are not more than 3 fold enriched in the set of invasion relevant genes were used. Disconnected genes are not shown. Colour code: green: co-mentioned in PubMed; pink: experimental/biochemical data; black: co-expression; blue: association in curated databases; purple: homology.

The genes that are shown in the STRNG network were analysed further with WebGestalt (Zhang et al., 2005). With this online tool long gene lists can be analysed. Among other things, it can automatically classify genes for different Gene Ontology (GO) classifications. Both, the invasion and the cell death relevant genes, were analysed separately.

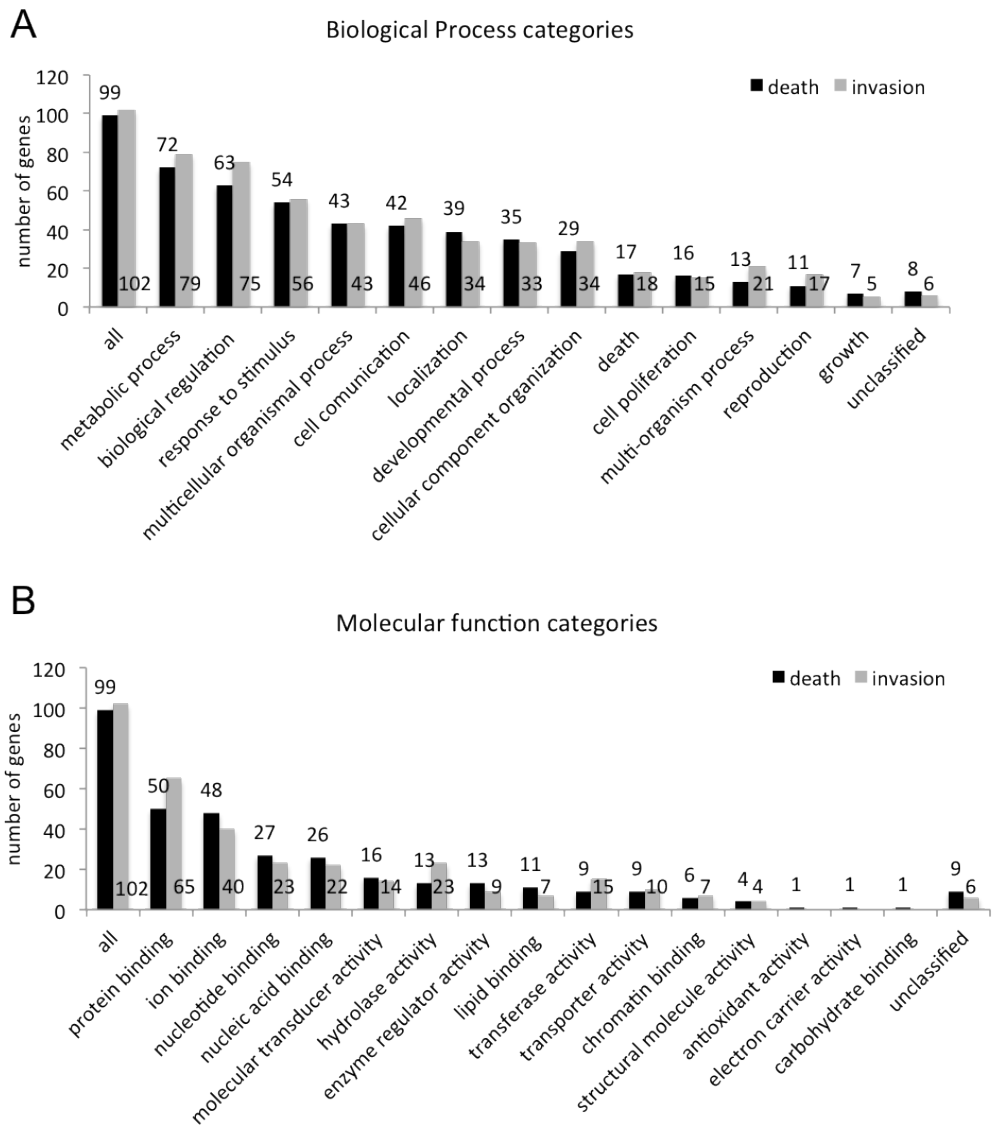

Figure 11 GO classifications of those genes, which are part of the STRING networks. (A) Biological process categories. (B) Molecular function categories.

As the invasion relevant genes are the ones, which inhibit invasion when they are knocked down. Here, especially cytoplasmic membrane proteins are expected. In addition, proteins are expected, which are involved in signal transduction, because the invasion is not only dependent on membrane receptors but also on a subsequent reorganisation of the cytoskeleton. The classification for biological processes shows that about 70% of the genes, which are involved in the invasion process, are acting as response to a stimulus (**Fig. 11A**). This supports the idea that cells interact with *S. aureus* and that invasion is a regulated process, which is inhibited when cells cannot react suitable to *S. aureus* anymore. In line with this, only 35% of the invasion relevant genes are localised at the cytoplasmic membrane (**Fig. 12**), further supporting the idea that not only plasma membrane proteins but also intracellular signalling is involved in *S. aureus* host cell invasion.

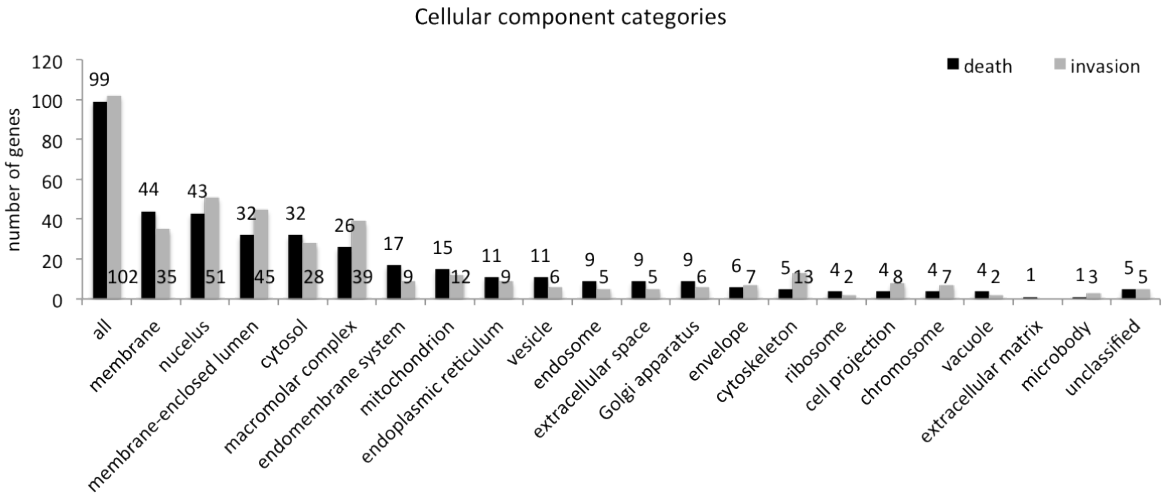

Figure 12 GO classifications of the genes, which are part of the STRING networks. Classification: cellular component categories.

It is noticeable that genes coding for ion-binding proteins play a role during invasion and host cell death, since in both cases almost 50% of all investigated genes were assigned to this category (**Fig. 11B**). The genes, which were enriched in the infected cells and therefore are important for the cell death process, are, like the proteins important for invasion, are distributed over a lot of protein classes. Here, this is expectable, as cell death, especially when it is a regulated process controlled by the cell itself, is very complex. Conspicuous is, that in the category death the same amount of proteins were sorted for both samples. One would be expecting that especially those cells, which survived the *S. aureus* infection longer, should code for more shRNAs, which promote cell death.

According to the GO classifications, ions have an outstanding importance during *S. aureus* invasion. However, other obvious hints for processes involved during invasion or cell death or localizations of events could not be observed through this approach. So in a next step, some of the central hits according to the STRING network were examined.

3.2 Validation of the invasion network

The STRING generated invasion network shows relations between enriched genes. Here, a direct protein-protein interaction as well as functional linkage is displayed. An extensive literature search revealed that cholesterol is already linked to *S. aureus* invasion (Hoffmann et al., 2010). So in a first experiment, the involvement of cholesterol during the invasion process in this system was determined. Trapping cholesterol with methyl-β-cyclodextrin (Mβc) reduced *S. aureus* 6850 invasion into HeLa cells drastically (**Fig. 13A**). This was also shown in Hek293 cells (Hoffmann et al., 2010). In addition, simvastatin, a drug, which inhibits the HMG-CoA reductase (in the invasion pathway above it is named LDLCQ3), reduces invasion remarkably (Horn et al., 2008).

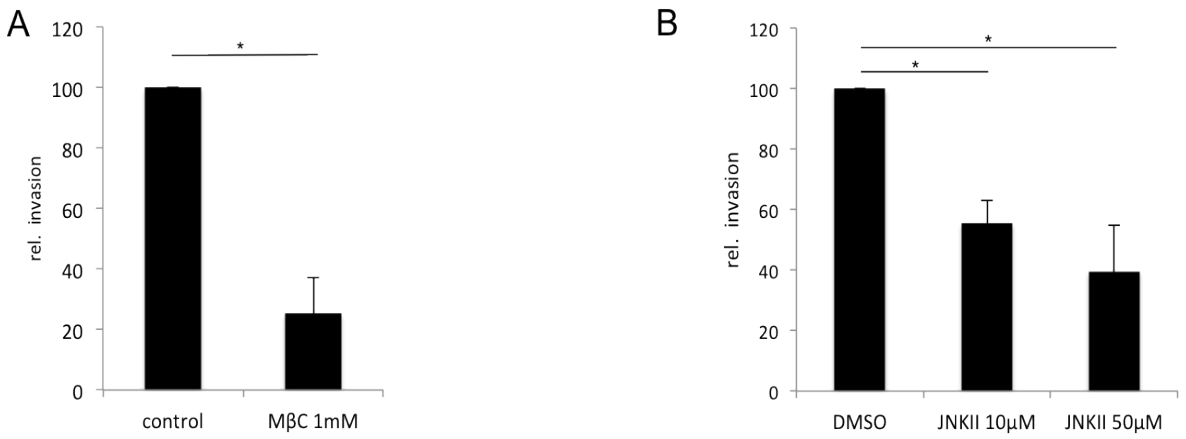

Figure 13 Involvement of cholesterol and JNK in the invasion process. HeLa cells were infected with S. aureus GFP for 1h. The addition of lysostaphin lysed extracellular bacteria and trypsinised cells were analysed with FACS (FACSaria III). The mean of two independent experiments ± SD is shown. Control infection was set to 100. (A) The invasion FACS-assay was performed ± methyl-β-cyclodextrin (Mβc). (B) The invasion FACS-assay was performed ± JNKII inhibitor.

These are first hints that the genes, which came up in the shRNA screen, are indeed important during *S. aureus* infection. Next, I looked for JNK, which has been shown to play a role during *Neisseria meningitidis* host cell invasion (Sokolova et al., 2004). *N. meningitidis*, like *S. aureus*, invades host cells with the help of fibronectin binding proteins and integrins. Therefore, it seems to be very likely that both pathogens use a similar invasion strategy and JNK could also play a role during *S. aureus* invasion. Indeed, the JNKII inhibitor reduced invasion rate in a concentration dependent manner (**Fig. 13B**). This assumes an involvement of JNK in the invasion process of *S. aureus* and a second gene of the invasion network is confirmed.

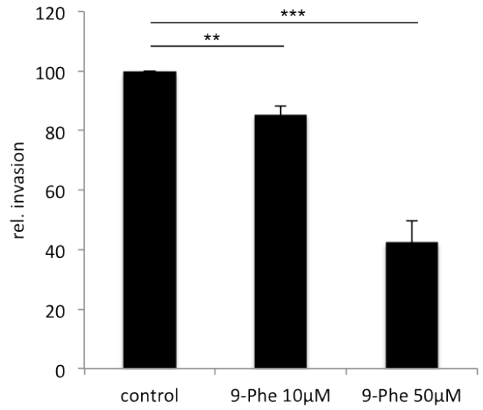

Figure 14 Involvement of TRPM4 in the *S. aureus* invasion process. HeLa cells were treated with 9-Phe and invasion of *S. aureus* 6850 GFP was monitored by FACS assay. The mean of three independent experiments ± SD is shown. Control infection was set to 100.

As a lot of transient receptor potential channels (TRP) showed up in the shRNA screen, the influence of 9- Phenantrol (9-Phe), which is a selective inhibitor for the transient receptor potential cation

channel, subfamily M, member 4 (TRPM4), was investigated (Guinamard et al., 2014). TRPM4 is a calcium activated cation channel (Mathar et al., 2014). The invasion rate of *S. aureus* was reduced in the presence of 9-Phe in a concentration dependent manner (**Fig. 14**).

To sum it up, three totally different proteins, which are part of the invasion STRING generated invasion pathway, could be verified as important players during *S. aureus* 6850 host cell invasion. The influence of Mβc, JNKII and 9-Phe on *S. aureus* itself was not tested, since it has been shown that even dead bacteria can efficiently invade host cells (Hudson et al., 1995).

3.3 Results of the siRNA screen performed for validation of the shRNA screen

Following the inhibitor studies, we decided to verify the shRNA screen by knocking down the most promising hits via siRNAs. Starting with a few, the results of finally over 70 siRNA (listed in **Table 5**) experiments are shown hereafter. The top hits of the shRNA screen were investigated first. Subsequently proteins, which are connected to hits of the screen, were added. A Dual RNA-Seq experiment performed in our lab (c.f. Dissertation M.Grosz) provided additional promising factors. For the siRNA experiments, two wells of HeLa cells were transfected with each siRNA, and invasion, cell death rate, and intracellular replication were determined within one experiment. First, 1h post infection (p.i.) the invasion rate was determined via FACS. Two hours later, cell death rate was determined via an annexin/7AAD staining. In addition, the amount of intracellular bacteria was determined with FACS. Since all cells were infected with the same bacteria and to the same time point, the number of invaded bacteria 1h p.i. can be compared with the number 3hrs p.i. in order to determine intracellular replication. The following pages will first show the percentage of infected cells (meaning how many cells are infected), then the relative amount of intracellular bacteria (meaning how many bacteria are in a single cell) and third the combination of both parameters, which I call relative invasion (rel. invasion). The rel. invasion is the mathematical product of both parameters (% infected cells x mean of infected cells), which is normalised to control infection. The rel. invasion optimises the comparability of the FACS-based invasion and the well-established CFU assay. Intracellular replication will be presented in the last section of this chapter.

3.3.1 Percentage of infected cells

One benefit of the FACS based invasion assay is that, beside the relative amount of intracellular bacteria, the percentage of infected cells can be determined. Interesting enough, this value is very robust, which is indicated by the small arrow bars and the huge number of significant genes. The amount of infected cells is very meaningful, since a knockdown of a special gene, via siRNA, which causes 50% less infected cells, must be a knockdown of a crucial gene.

The most prominent reduction of the number of infected cells was observed when calpain2 (CAPN2) was knocked down (**Fig. 15**). The double knock down of ITGB1 and ITGA5 showed slightly more infected cells and in the third position the knockdown of the alanyl (membrane) aminopeptidase

(ANPEP) was localised. Among the top 5 was, beside the knockdowns of ITGB1 and ITGA5, also vinculin (VCL). There are several other genes, which seem to be involved in the internalization process, as their knockdown reduced the percentage of infected cells significantly, but as in this cases the reduction was very low, they are not mentioned here in particular. However, two totally new invasion relevant genes, CAPN2 and ANPEP, could be identified with this method.

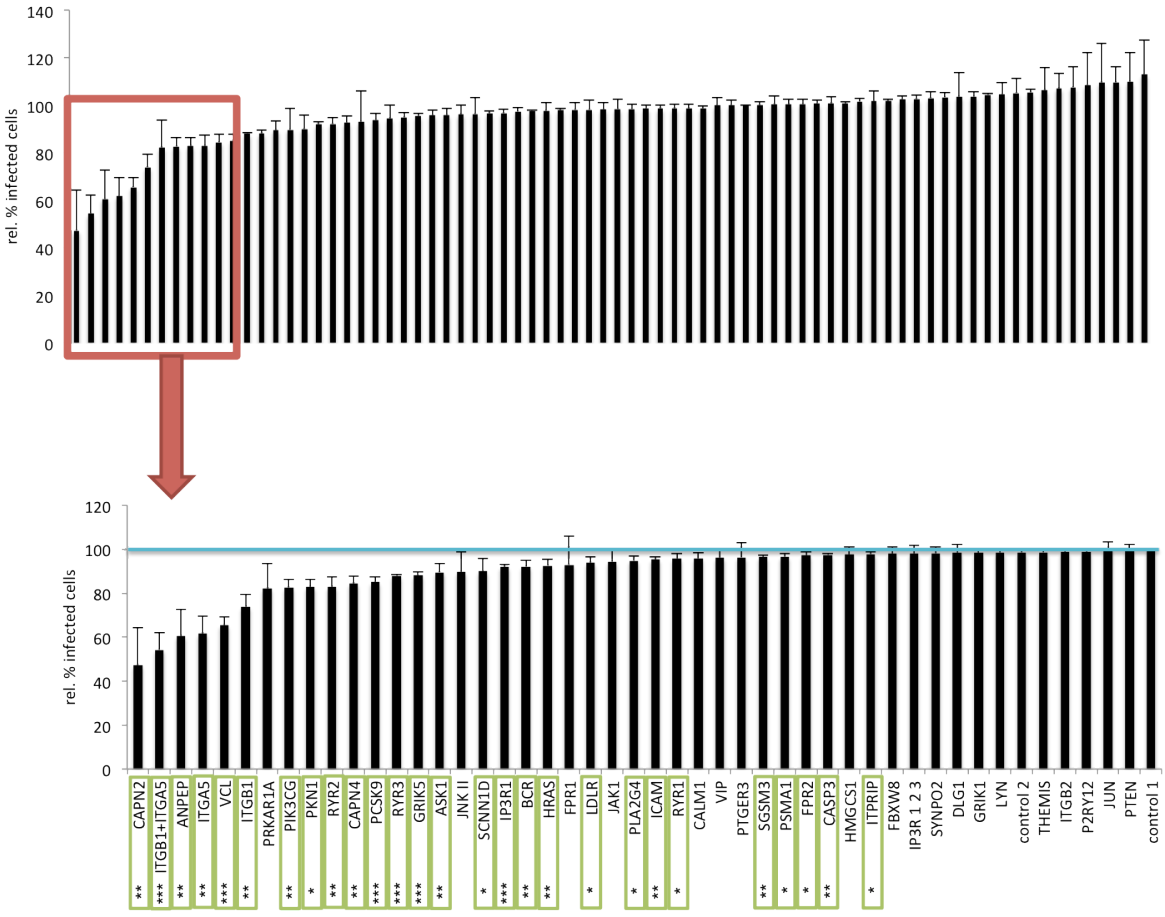

Figure 15 Genes that facilitate internalization of *S. aureus* 6850 into HeLa cells were determined via the percentage of infected cells. The overview shows the percentage of infected cells in all siRNA knockdown experiments. Below, the genes, which promote internalization, are shown. The green boxes highlight proteins, which reduce the internalization significantly, when they are silenced. The mean of three independent experiments ± SD is shown. Control infection was set to 100.

3.3.2 Relative amount of intracellular bacteria

The bacteria, which were used for the invasion assay, expressed GFP in an approximately equal amount. To ascertain this, bacteria were measured separately to verify that GFP expression is equal. Hence, the mean of the infected cells, which was measured by FACS, approximately reflects the amount of intracellular bacteria. In the following graph the relative amount of intracellular bacteria is shown (**Fig. 16**). Here, the three top hits are the double knockdown and the single knockdowns of the two integrins ITGA5 and ITGB1. CAPN2, which was the top hit of the previous graph, is found here only on position 16 and it is not significant. But the knockdown of another calpain, the calpain small subunit 1, also called calpain 4 (CAPN4), shows an effect on the amount of intracellular bacteria.

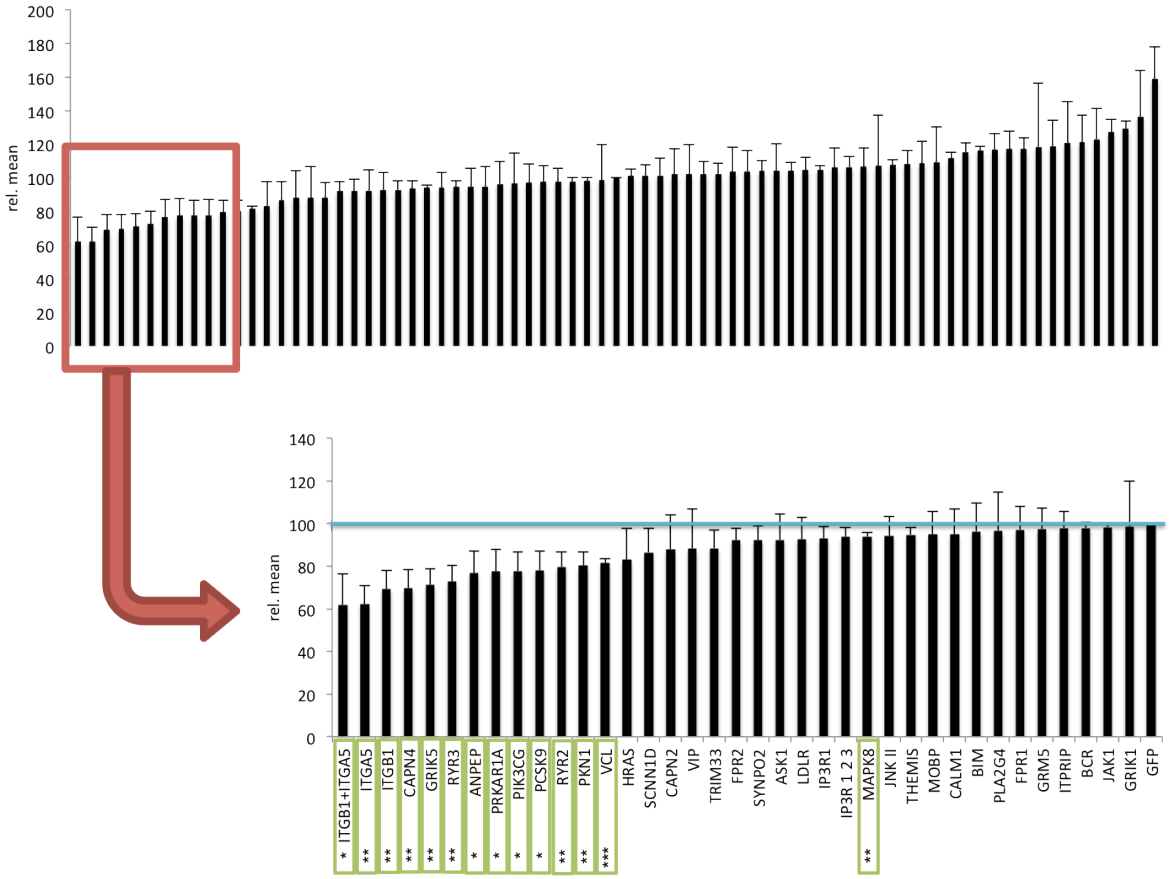

Figure 16 Genes that facilitate internalization of *S. aureus* 6850 into HeLa cells determined via the amount of intracellular bacteria. The overview shows the relative mean of the infected cells of all siRNA knockdown experiments. Below, the genes, which promote internalization, are shown. The green boxes highlight factors, which reduce the internalization significantly when they are silenced. The mean of three independent experiments ± SD is shown. Control infection was set to 100.

3.3.3 Relative invasion

What I did next, was to multiply the amount of infected cells with the amount of intracellular bacteria and normalised control infection to 100. This calculation is comparable with CFU assay calculations. In doing so, the data show that ITGA5 plays a more prominent role for invasion than ITGB1 (**Fig. 17**). In addition, the importance of CAPN2 and ANPEP becomes obvious, as the knockdown of these two proteins reduces the rel. invasion down to 40%. The knockdown of the following genes also leads to rel. invasion rates lower than 60%: VCL, CANP4, GRIK5, PRKAR1A, RYR3, PIK3CG, RYR2, PCSK9 and PKN1.

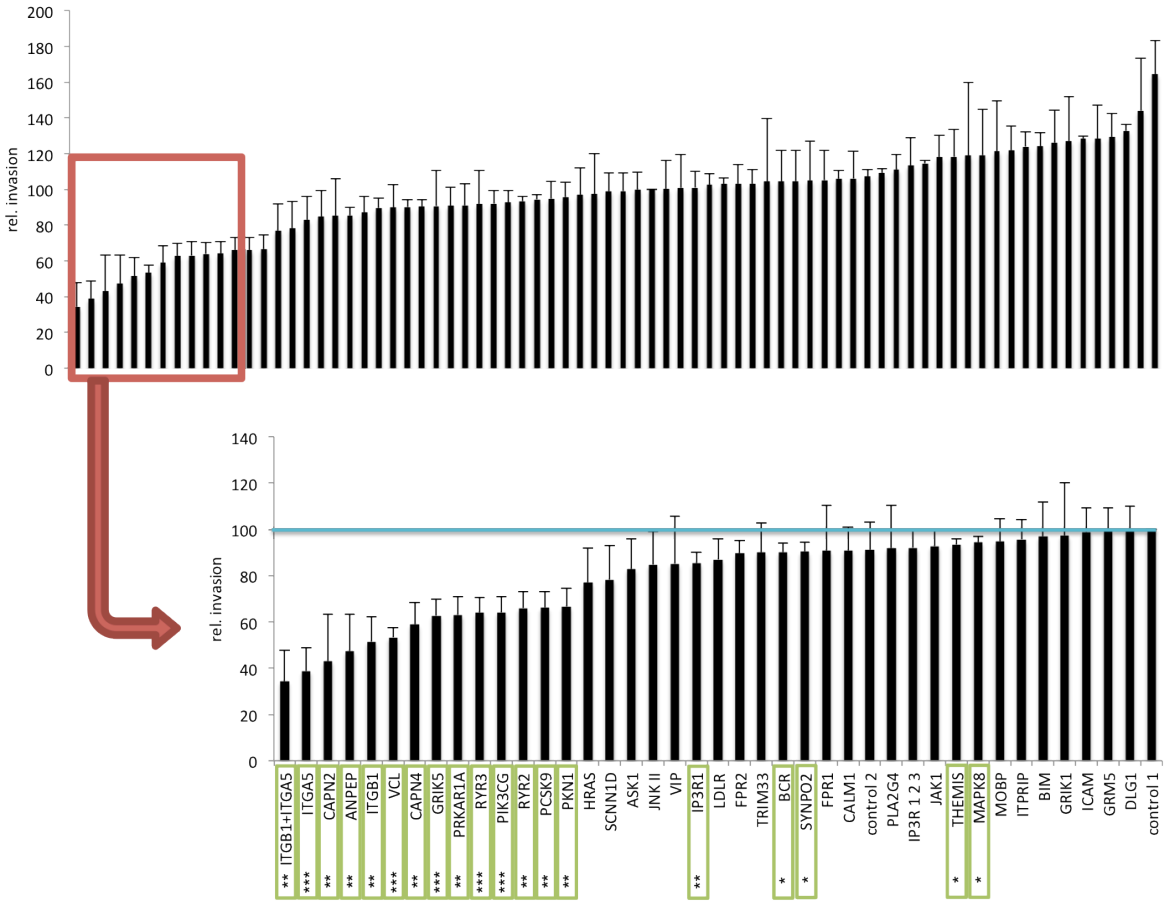

Figure 17 Genes that facilitate internalization of *S. aureus* 6850 into HeLa cells determined via the product of the amount of intracellular bacteria and the number of infected cells. The overview shows the relative invasion of the infected cells of all siRNA knockdown experiments. Below, the genes, which promote internalization, are shown. The green box highlights factors, which decrease the internalization significantly when they are silenced. The mean of three independent experiments ± SD is shown. Control infection was set to 100.

3.3.4 Intracellular replication

In addition to the invasion rate, we monitored the intracellular replication. This was possible as the replication is linked to an increase of GFP signal, when a GFP expressing *S. aureus* strain is used. The replication rate was determined via the ratio of the GFP signal 3hrs and 1h p.i. . The knockdown of FPR2 reduced the intracellular replication most, namely down to 60% (**Fig. 18**). The replication was slightly higher, when ITGB1 and ITGA5 were knocked down. This raises the question, whether a reduced invasion rate might have an influence on bacterial replication and it is not clear, whether the replication is actually reduced or if it is just reduced because of the lower amount of intracellular bacteria. Therefore, here I list only the factors, which show only a slight reduction or nor reduction in invasion rate: FPR2, JUN, LYN, HRAS, AKT1 (higher invasion) and ITGAM (much higher invasion).

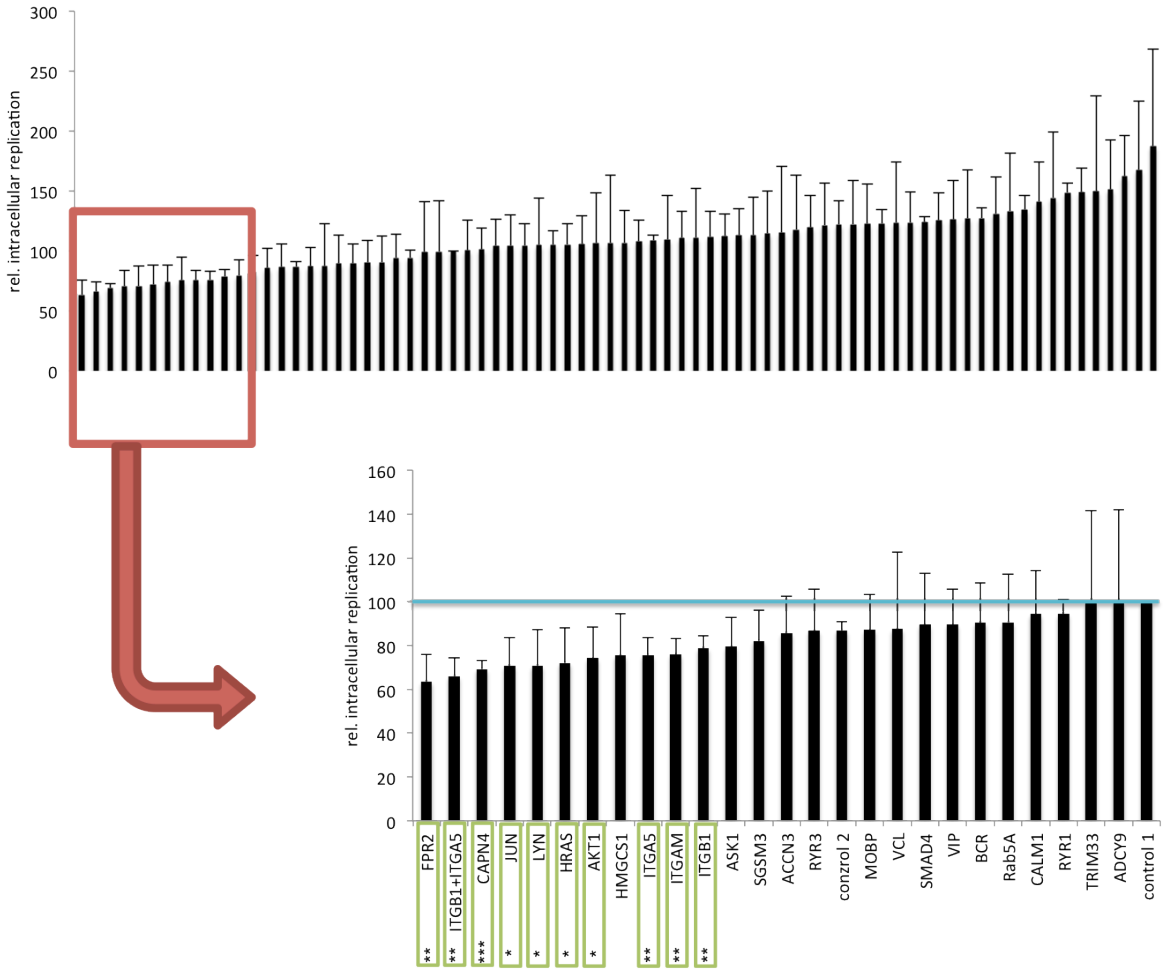

Figure 18 Genes that promote intracellular bacterial replication. The difference of the means between 1h and 3hrs p.i. were monitored and the graphs show the ratio normalised to control infection. Control infection was set to 100. The mean of three independent experiments ± SD is shown.

As the FACS based invasion assay (and the thereby resulting intracellular replication assay) was developed for and first used in this study, I performed a classical well-established CFU assay in order to validate the results generated with the FACS based assay (**Fig. 19**). Therefore I choose some siRNAs and determined the bacterial intracellular replication in the presence of these siRNAs. The results of the FACS-based assay could be confirmed with the CFU assay (**Fig. 19**). However, the relative intracellular replication measured by FACS showed smaller values, compared to the CFU assay (**Fig. 18+19**). For example, the FACS assay showed a reduction down to 60% rel. replication during the FPR2 knockdown, while the CFU assay showed reduction down to 40%. The reason for this might be that the increase in GFP signal after a cell division needs some time and thus the CFU assay shows a greater difference. Nevertheless, the FACS based assay is an excellent and swift method to screen for factors involved in intracellular replication.

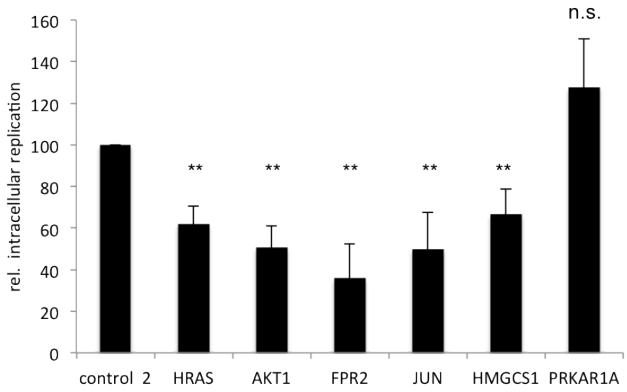

Figure 19 The reduced intracellular replication after knockdown with five different siRNAs confirmed via CFU assay. Cells were infected with *S. aureus* 6850 GFP and 1h and 3hrs p.i. CFU were determined. 1h p.i. lysostaphin was added in order to kill extracellular bacteria. The mean of three independent experiments ± SD is shown. Control infection was set to 100.

Intracellular replication of bacteria is an important step, because pathogens can better survive and injure their host sustainably, when they are able to replicate within the host. Therefore, I was also interested in genes inhibiting *S. aureus* intracellular replication.

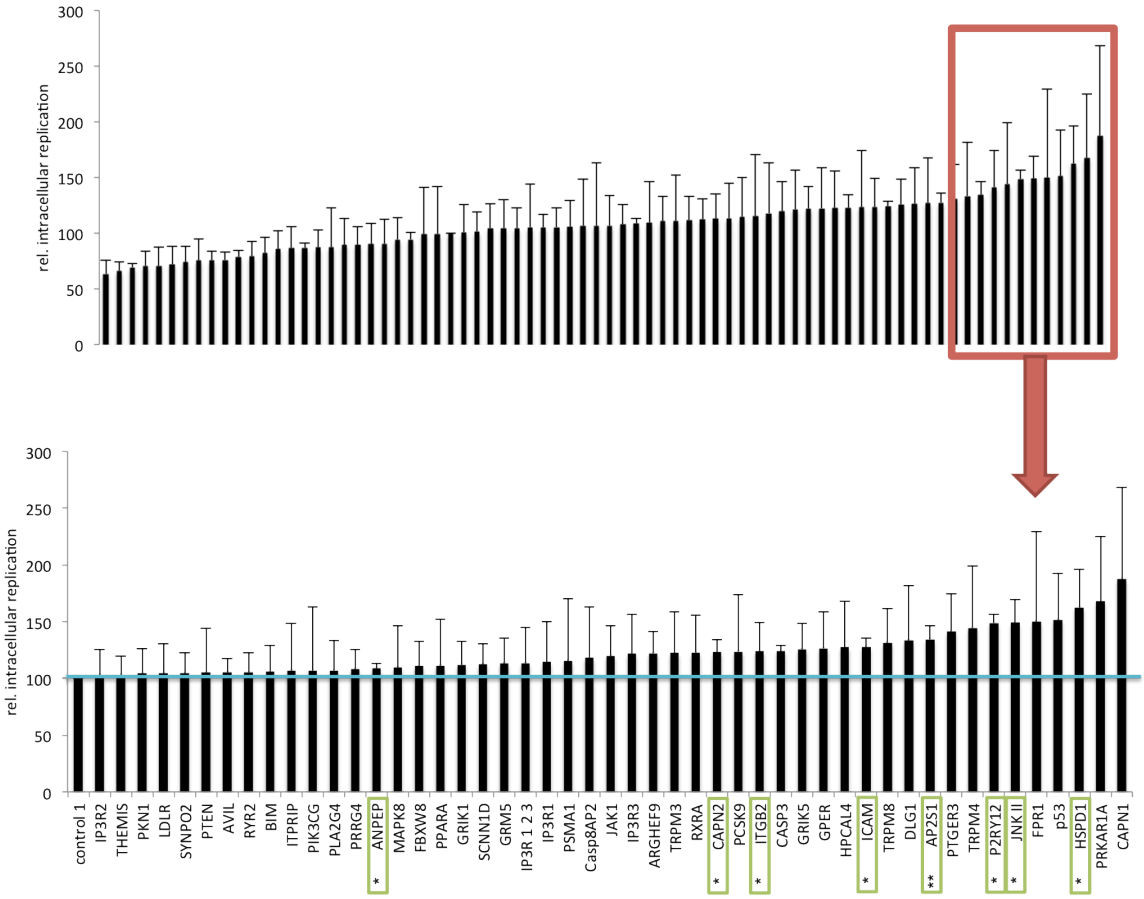

Figure 20 Genes that inhibit intracellular bacterial replication. The difference of the means between 1h and 3hrs p.i. were monitored and the graph shows the ratio normalised to control infection. The mean of three independent experiments ± SD is shown. Control infection was set to 100 and so 150 means a 1.5 fold increase of bacterial replication.

Proteins that inhibit bacterial intracellular replication under normal circumstances, promote it, when they are knocked down. HSPD1 knockdown drives replication most (and significantly) (**Fig. 20**). It is also called HSP60 and already linked to *S. aureus* invasion. It interacts with FnBPs and is therefore proposed as a co-receptor for fibronectin dependent internalization of *S. aureus* (Dziewanowska et al., 2000).

Further factors are: JNKII, P2RY12, AP2S1, ICAM, ITGB2, CAPN2 and ANPEP.

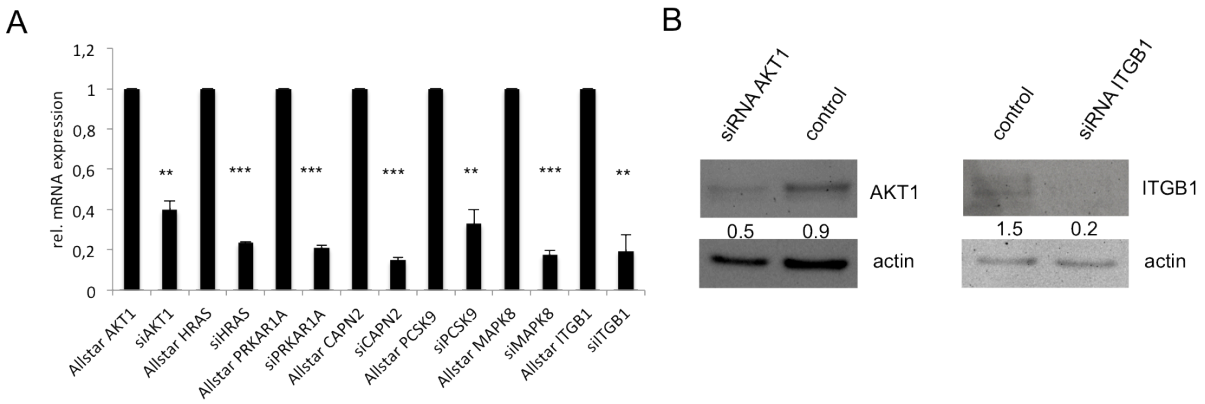

Figure 21 Validation of the siRNA knockdown. (A) HeLa cells were either transfected with AllStar negative control or with indicated siRNAs. The amount of mRNA was determined via qRT-PCR. Bar graphs show the mean of two independent experiments ±SD. Control conditions were set to 1. (B) Western blot analysis of HeLa cells either transfected with AllStar negative control, AKT1 or ITGB1. The ratio between the protein of interest and actin is shown.

In order to control the transfection efficiency during all siRNA experiments, a siRNA, which kills transfected cells (Cell death control) was used. In all cases, at least 80% of the cell death control transfected cells died. To confirm further that the siRNAs really silence their target, we used four different siRNAs, a so-called siRNA pool. The siRNAs in the pool are different, but targeted against the same gene. To demonstrate the knockdown, a few siRNA targets were picked out. All qRT-PCR experiments confirmed a knockdown of over 50% (**Fig. 21A**). On protein level I checked AKT1 and ITGB1, which were both obviously reduced in cells transfected with an siRNA against these genes (**Fig. 21B**).

3.4 2-APB inhibitor studies

Since the GO classification and the KEGG analysis (data not shown) suggested that ions and here especially calcium plays an important role, I decided to inhibit the IP3 receptors with 2-aminoethoxydiphenyl borate (2-APB). This chemical compound is known to inhibit the IP3 receptors as well as several TRPM channels (Dobrydneva and Blackmore, 2001; Maruyama et al., 1997; Togashi et al., 2008). Bacterial invasion rate was dose dependently reduced (**Fig. 22A**). Importantly, the FACS-based assay showed that the intracellular replication was completely blocked (**Fig. 22B**). This was still the case, when the amount of intracellular bacteria in 2-APB treated cells 1h p.i. was twice as high as in control infection. This indicates that the prevention of replication was not

due to the reduced invasion rate. Next, I was interested, if HeLa cells still die, when they are infected with *S. aureus* 6850 in the presence of 2-APB.

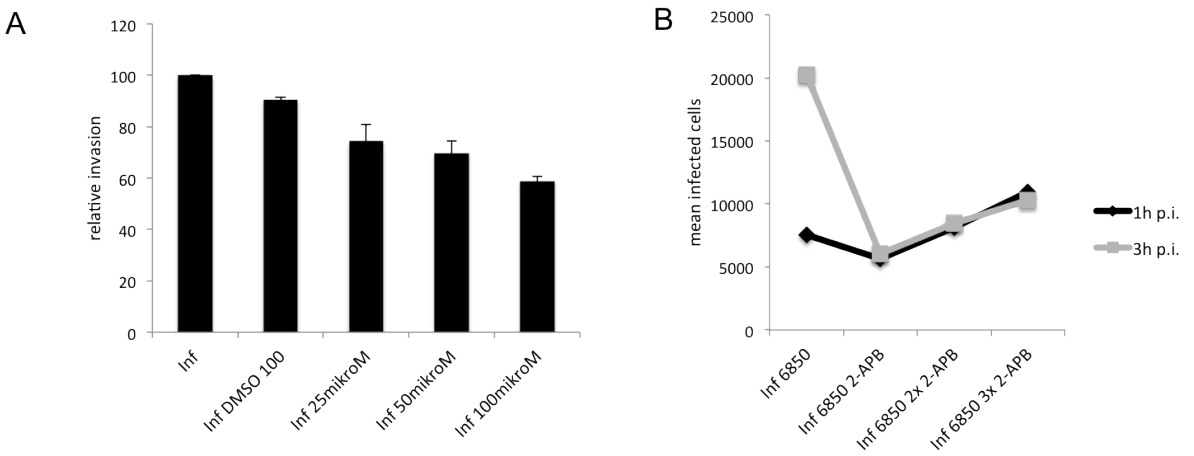

Figure 22 Invasion and intracellular replication of *S. aureus* 6850mRFP in 2-APB (30µM) treated cells. (A) Invasion rate 1h p.i. was determined via FACS-analysis. Invasion rate decreased in the presence of 2-APB in a concentration dependent manner. Control infection was set to 100. (B) Relative amount of intracellular bacteria was monitored 1h and 3hrs p.i. . Control infection showed markedly increase in mRFP signal whereas 2-APB pre-treated cells showed a constant mRFP signal. 2-APB treated cells were infected with the same amount as control infection. In addition, two and three times more bacteria were added to 2-APB treated cells in order to exclude that replication is decreased because of decreased invasion rates.

An annexin/7AAD staining showed that the cell death rate is reduced in cells treated with 2-APB prior to infection (**Fig. 23**). The percentage of annexin positive cells is reduced down to 50%. The amount of 7AAD permeable cells is constant under all tested conditions. This is an impressive result, since cell death induced by *S. aureus* happens so fast. Therefore, 2-APB must inhibit a crucial step of *S. aureus* infection. Therefore I wanted to gain more insight into the mechanism of action of 2-APB during infection.

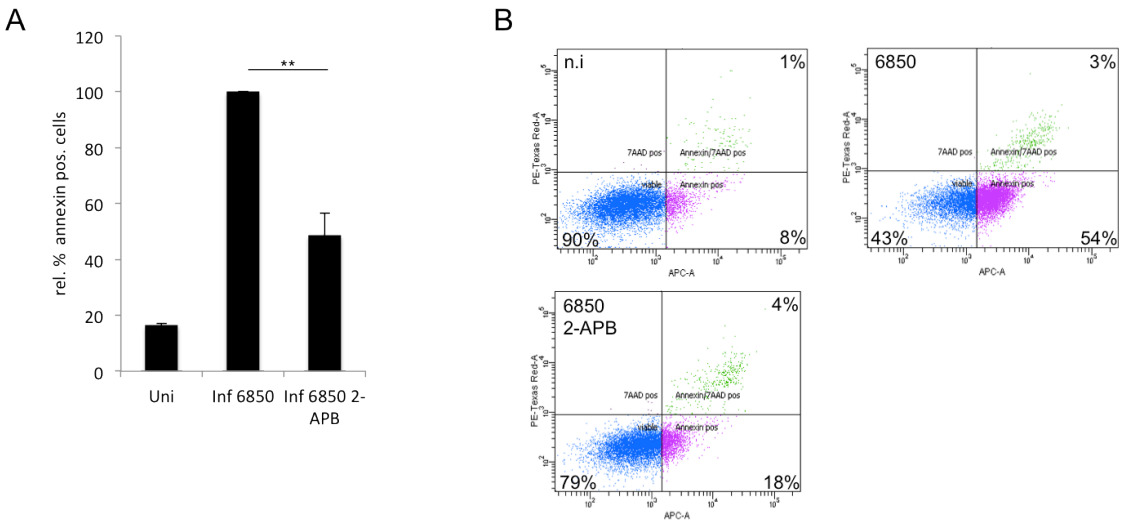

Figure 23 2-APB inhibits *S. aureus* induced host cell death. (A) Amount of annexin positive cells 3hrs post *S. aureus* infection ± 30µM 2-APB. Mean of three independent experiments ±SD is shown. Control infection was set to 100. (B) Examples for FACS blots. X-axis shows annexin, y-axis 7AAD staining.

Next, the course of infection was monitored by fluorescence microscopy. The amount of intracellular bacteria decreased in the presence of 2-APB, since the number of GFP dots decreased during infection (**Fig. 24**). In contrast, the number of bacteria increased markedly during the control infection. These observations suggested that not only intracellular replication is blocked by 2-APB, but also host cells are able to kill intracellular staphylococci better.

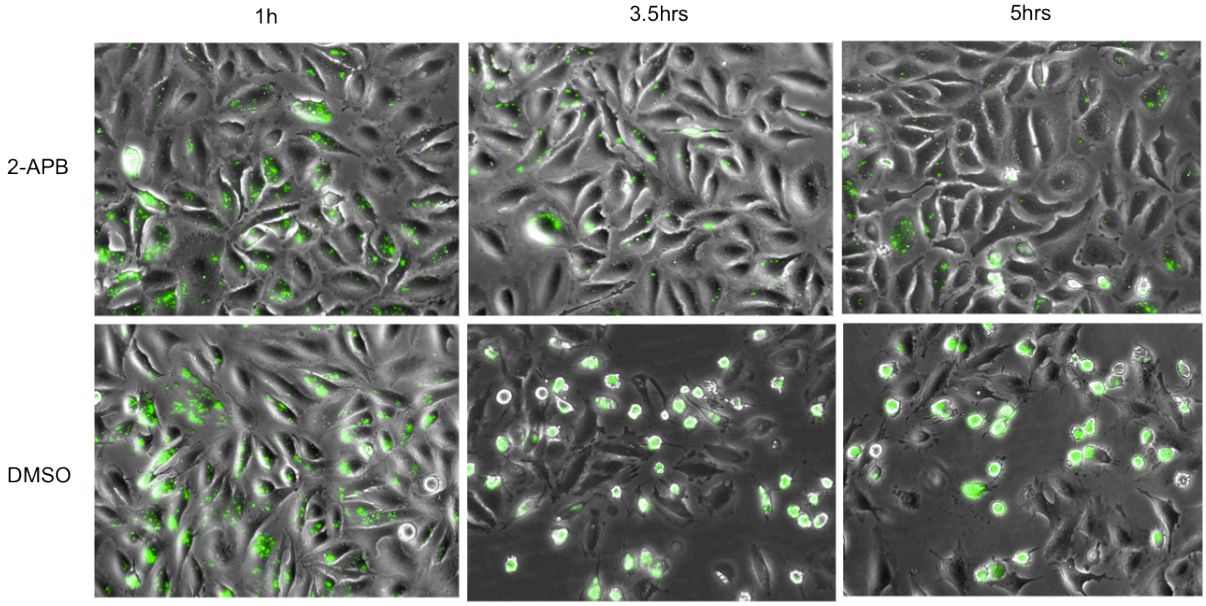

Figure 24 Inhibition of *S. aureus* induced host cell death. HeLa cells were infected with *S. aureus* 6850 GFP. In the presence of 30µM 2-APB intracellular bacterial replication and host cell death was inhibited. 40x magnification.

Next, the question arose, whether 2-APB has a direct effect on *S. aureus*. Bacterial growth was not affected in TSB culture medium, when 2-APB was added. Thus, a direct toxic effect of 2-APB on *S. aureus* could be ruled out (**Fig. 25A**).

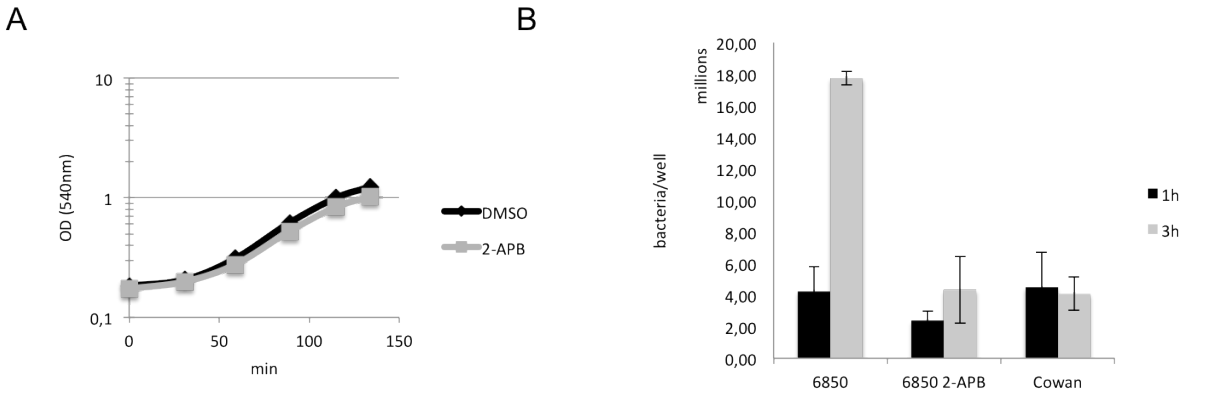

Figure 25 2-APB (30µM) cannot inhibit *S. aureus* growth in vitro, but intracellularly. (A) Growth curve of *S. aureus* 6850 in TSB ±2-APB. DMSO was used as control since 2-APB is solved in it. (B) CFU assay for determination of invasion and intracellular replication rate. CFU were determined 1h and 3hrs p.i.

Since the FACS assay used for determination of intracellular replication was first used in this study, I performed a CFU assay in addition. Here, 1h and 3hrs p.i. HeLa cells were lysed and the bacteria containing solution was plated to determine the CFU. Like the FACS based assay, the CFU assay showed that there is no bacterial replication in the presence of 2-APB (**Fig. 25B**).

As it has been shown (in cooperation with M. Grosz) in our lab before that the escape deficient *S. aureus* mutants cannot replicate within the cytosol anymore. So phagosomal escape was monitored next (Grosz et al., 2014).

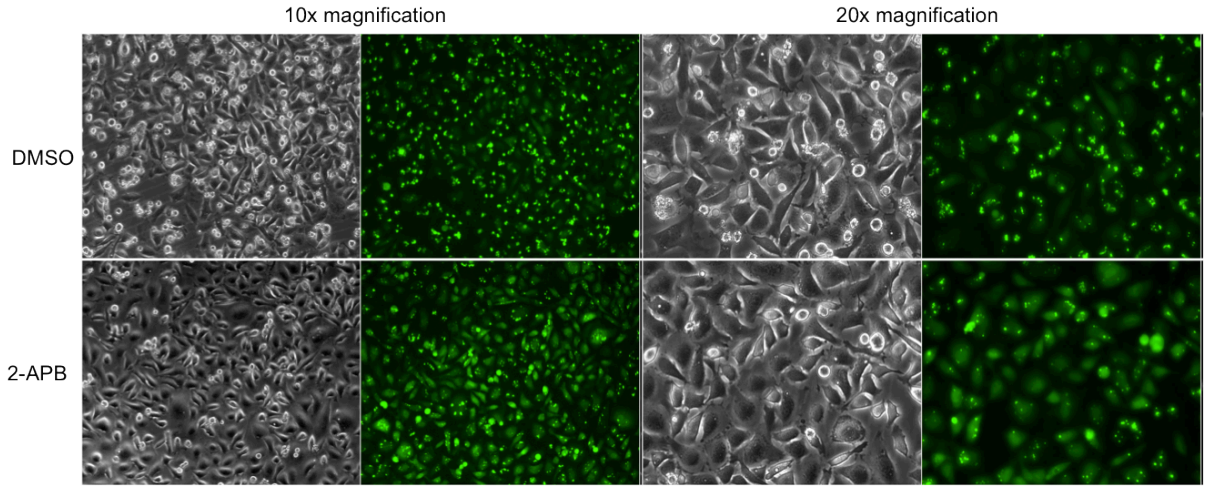

Figure 26 2-APB reduced phagosomal escape of *S. aureus* 6850 markedly. HeLa-YFP-CWT cells were infected ±2-APB with *S. aureus* 6850. The YFP-CWT protein is normally localised diffuse in the cytosol, upon phagosomal escape the CWT part binds to the bacterial peptidoglycan, which is localised on the surface of *S. aureus*. Therefore, the GFP signal increases at one point of the cell and distinct bright dots appear.

HeLa cells expressing YFP-CWT, which binds to the cell wall of *S. aureus* after escape from the phagosom, were infected ± 2-APB (**Fig. 26**). Cells treated with 2-APB showed markedly reduced escape rates. A pH assay performed by M.Grosz confirmed these results in HUVEC cells (data not shown).

As 2-APB inhibits phagosomal escape, intracellular replication and host cell death, a mouse experiment was performed [4].

[4] Performed by Babett Oesterreich (Institute for Molecular Biology of Infectious Diseases, University of Würzburg, AG Ohlsen).

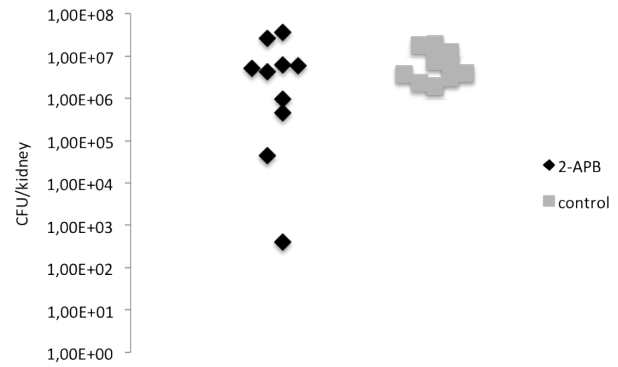

Figure 27 Mice were treated with 2-APB or DMSO (solvent of 2-APB). The kidneys were isolated and CFU were determined.

The CFU, which were isolated from the kidneys of the mice treated with 2-APB and infected with *S. aureus* in a sepsis model, were reduced in some of the mice (**Fig. 27**). Since the variations of the isolated CFU were very high within the 2-APB treated group, there is no significant difference, but some mice showed a markedly visible reduction of number of bacteria.

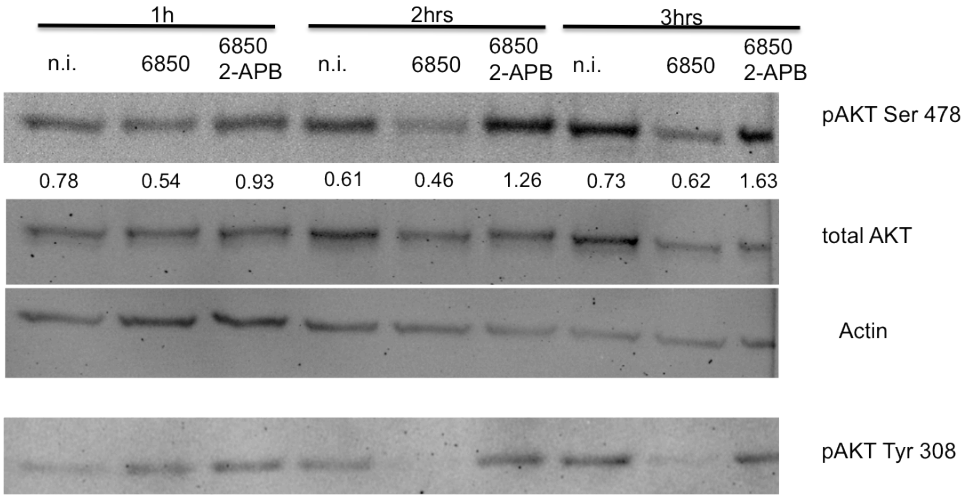

Figure 28 AKT1 is dephosphorylated during *S. aureus* infection. AKT1 phosphorylation pattern was determined via western blot analysis. Serine (Ser) and tyrosine (Tyr) phosphorylation sites both showed the same phosphorylation pattern. AKT phosphorylation decreases during S. aureus infection but can be rescued by 2-APB. Two independent experiments were made, which showed the same results.

Since 2-APB has such a strong effect on host cell survival, I was interested, how high the phosphorylation level of AKT1 is within 2-APB treated cells. Phosphorylation of AKT1 is a strong pro-survival signal. AKT1 was already identified with the siRNA screen and therefore seemed to be important for *S. aureus* infection. Interestingly, it is dephosphorylated after *S. aureus* infection but phosphorylation increased again in infected cells treated with 2-APB (**Fig. 28**). This was true for the serine (Ser) and the tyrosine (Tyr) phosphorylation site. The increase of AKT1 phosphorylation in 2-

APB treated infected cells increased between 1h and 3hrs p.i. , whereas the level in infected cells stayed constantly low.

3.5 The role of calpains during *S. aureus* induced cell death

As 2-APB, a calcium channel blocker, can inhibit *S. aureus* infection so efficiently, calcium related genes, which can act downstream and directly induce host cell death, were investigated.

The most obvious link between ion fluxes and cell death are calpains. Calpains are cysteine proteases, which can, like caspases, cleave proteins to activate them and thus can induce indirect apoptosis or necrosis (Harwood et al., 2005). Importantly, a connection between *S. aureus* infection and calpains was already drawn since they play a role during transmigration of *S. aureus* across keratinocytes (Soong et al., 2012). Therefore, an involvement of calpains under our infection conditions was further investigated.

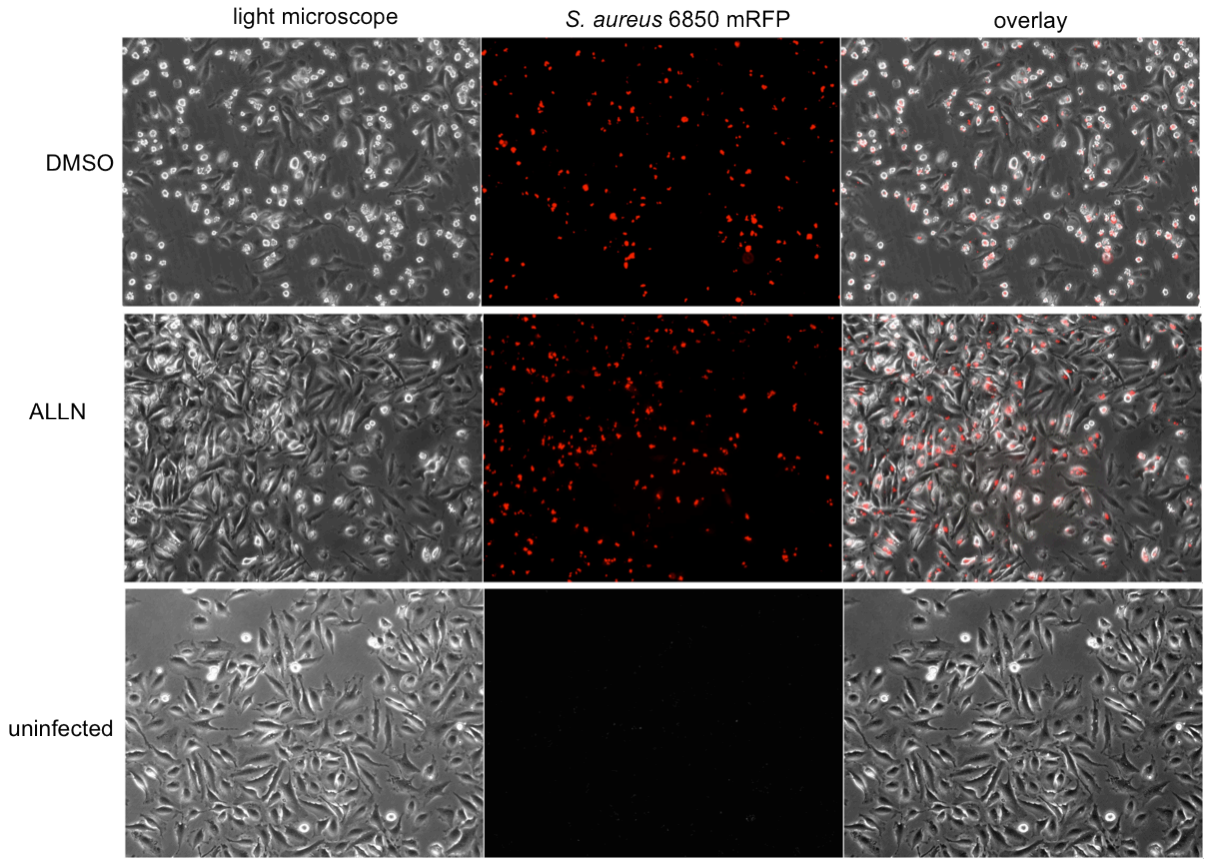

Figure 29 Inhibition of *S. aureus* 6850 induced morphological changes with 20µM ALLN. Fluorescence microscopy of living cells 3.5hrs p.i. . HeLa cells were infected with *S. aureus* 6850 mRFP. Magnification 20x.

HeLa cells were preincubated with the calpain inhibitor ALLN and the course of infection was monitored. ALLN inhibited the typical type of cell death, which is normally caused by *S. aureus* 6850 (**Fig. 29**). 3.5hrs p.i. HeLa cells are, although highly infected, still attached to the surface and their morphology is comparable to uninfected cells. But in contrast to the effect of the 2-APB inhibitor, bacteria seem to replicate within HeLa cells in the presence of ALLN. To prove this further, a FACS

based replication assay was performed, which showed that *S. aureus* could replicate in the presence of ALLN (**Fig. 33**). This result suggests that bacteria are able to escape in the presence of ALLN, which could be confirmed with the YFP-CWT expressing HeLa cell line (**Fig. 34**). So, both results suggest a role of calpains in host cell death. It seems that the ALLN inhibition acts downstream of 2-APB, since ALLN inhibits only cell death, but not phagosomal escape and intracellular replication.

To investigate this further, a time course experiment was performed. Therefore, HeLa cells were infected with *S. aureus* 6850 and at different time points ALLN was added to the infection. The addition of ALLN rescued cells till 2hrs p.i. . The addition of ALLN after 2hrs resulted in increasing cell death rates. Indicating, that calpains are active approximately 2hrs p.i. (**Fig. 30**). This further confirms that ALLN blocks cell death relevant events. As inhibitors often have side effects and especially protease inhibitors are not really specific, I looked for other proteins that could be affected by ALLN.

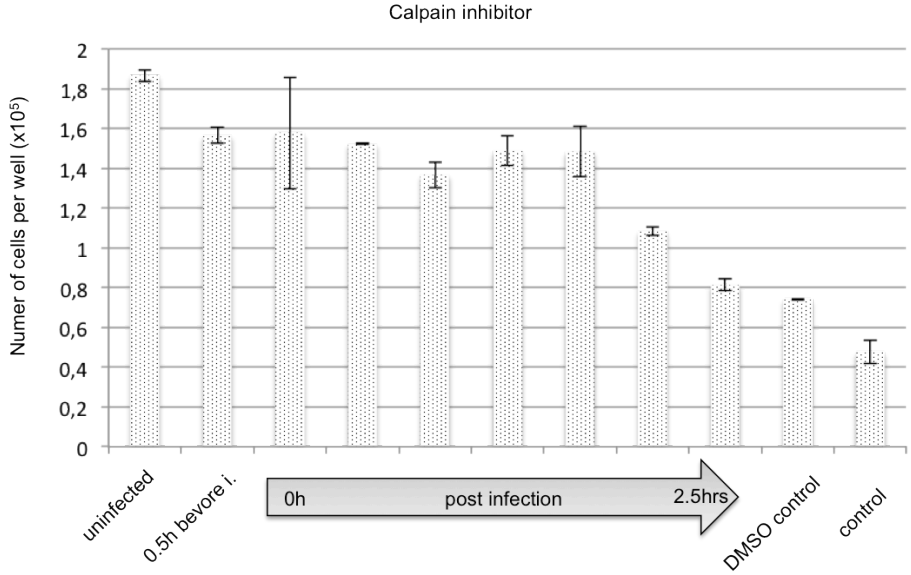

Figure 30 Determination of the time point when ALLN acts. ALLN was added at indicated time points p.i. . When ALLN was added till 1.5hrs p.i. the number of attached cells did not change. But when ALLN was added later then 2hrs p.i. the number of viable cells decreased strongly.

ALLN can also inhibit cathepsin B, L and papain. Cathepsins are proteases localised e.g. in lysosomes and, even more important, have shown to induce cell death after release from the lysosome. One example, where this happens, is an infection with *Mycobacterium tuberculosis*. *M. tuberculosis* can induce a caspase independent but cathepsin dependent type of cell death in macrophages (Lee et al., 2006).

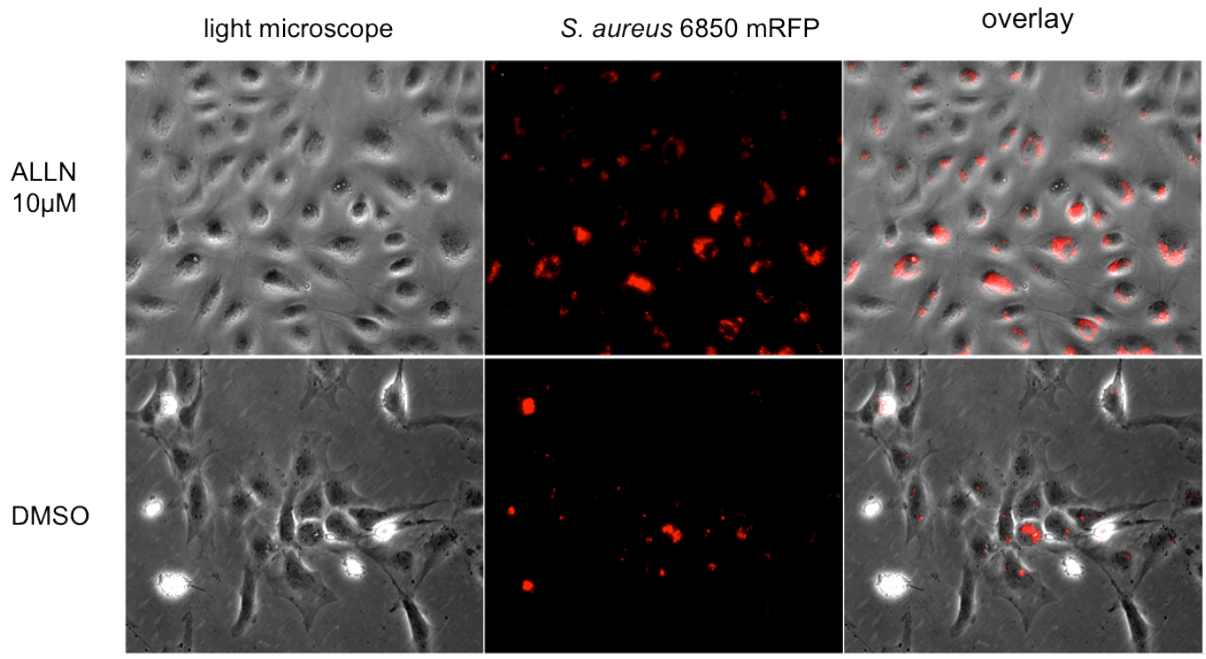

Figure 31 ALLN reduces cell death in HUVECs. *S. aureus* 6850 mRFP efficiently killed HUVEC control cells but not cells treated with ALLN. Fluorescence microscopy of living cells 5.5hrs p.i. . Magnification 40x.

As *S. aureus* escapes the phagosome, it is likely that cathepsins enter the cytosol during this process. This release of cathepsins could then induce host cell death. To exclude or confirm this possibility, the effect of a cathepsin inhibitor, which inhibits Cathepsin B, L, S and papain, was tested.

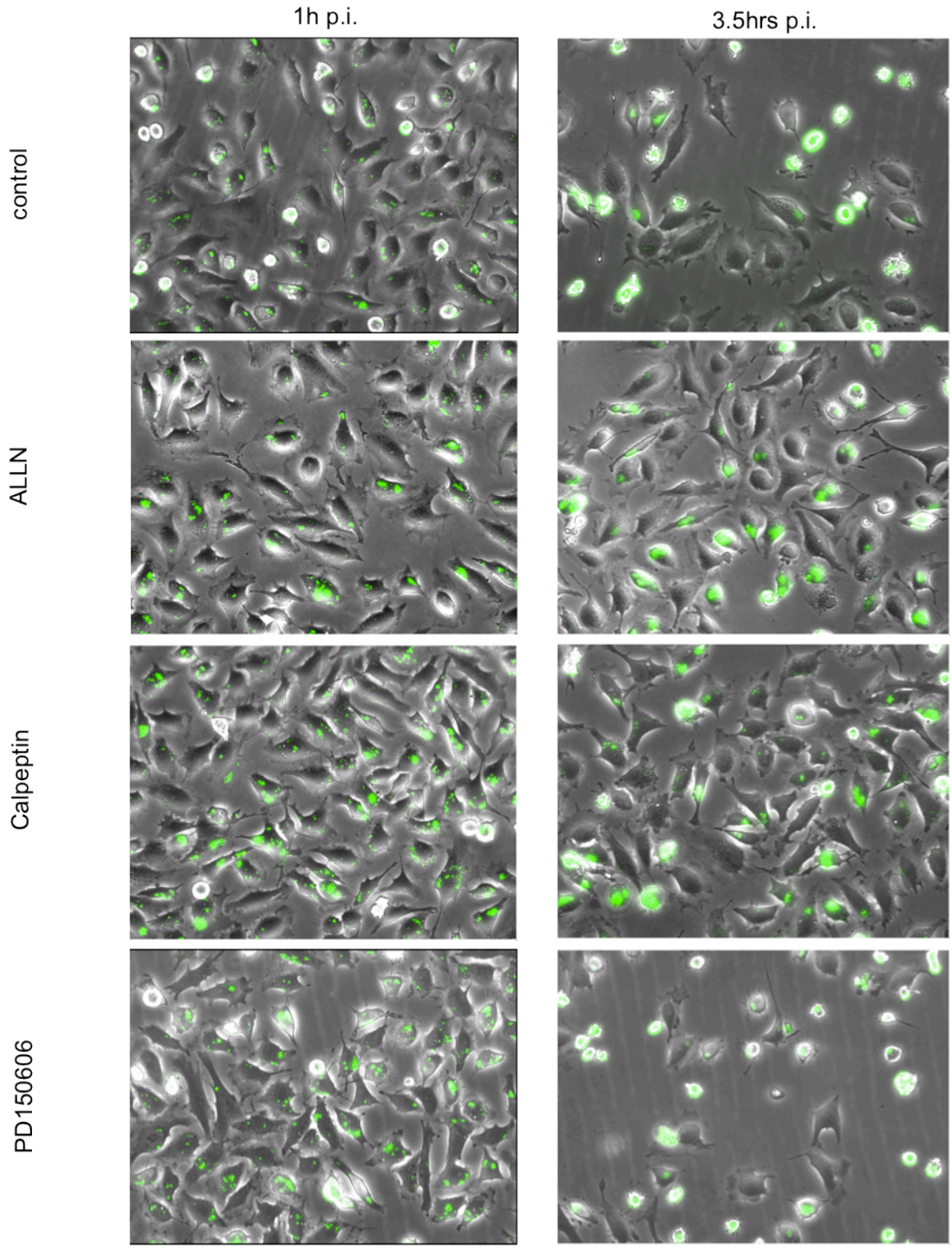

Figure 32 Calpain inhibitors have different effects on host cell survival after *S. aureus* infection. Cells were incubated with indicated inhibitors, infected with *S. aureus* 6850 GFP and pictures were taken 1h and 3.5hrs p.i. . Cells treated with PD150606 (50µM) and control cells looked alike. Calpeptin and ALLN (both 50µM) reduced rounding up und detachment of infected cells distinctly. 40x magnification.

The cathepsin inhibitor did not show any effect on the course of infection (data not shown). Indicating that at least these cathepsins (B, L and S) are not involved in *S. aureus* induced host cell death. Since the calpains are crucially involved in *S. aureus* induced cell death, their role during

infection of a primary cell line was tested. Like HeLa, also HUVEC cells could be rescued from *S. aureus* induced death (**Fig. 31**).

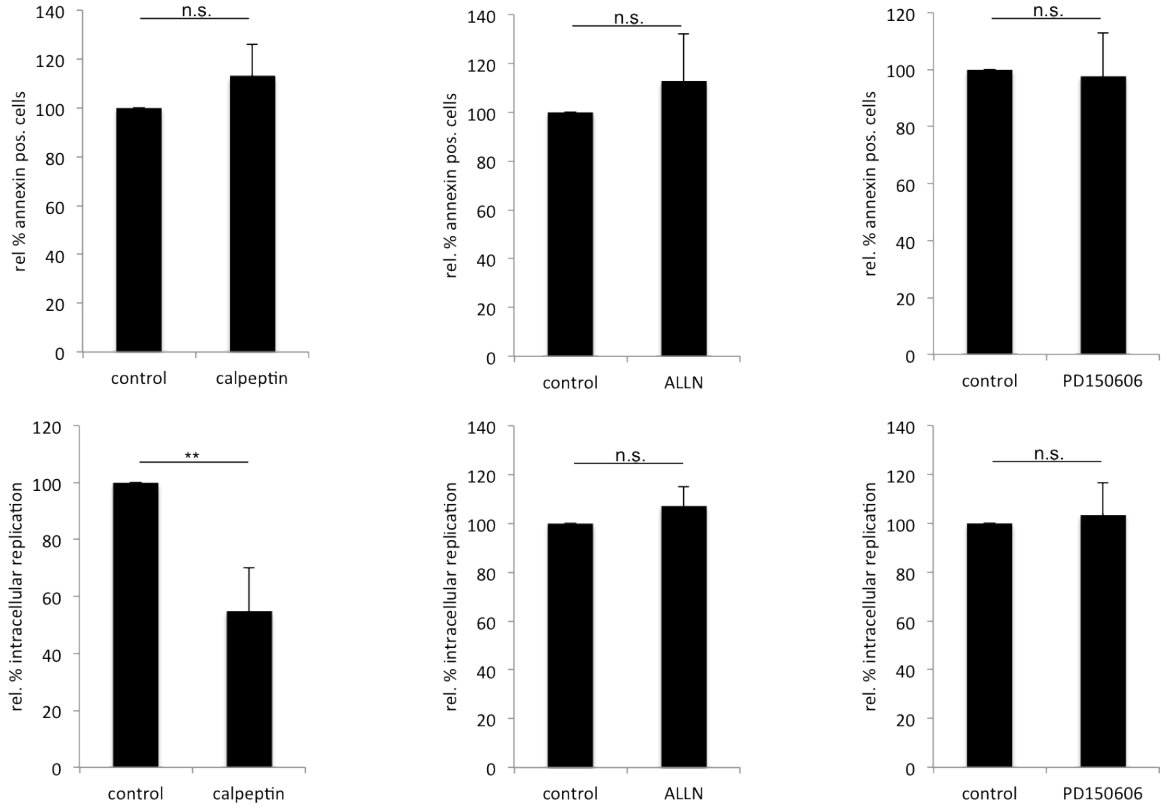

Figure 33 Calpain inhibitors do not inhibit annexin staining and only calpeptin blocks intracellular replication. In the first row, annexin staining in the presence of different calpain inhibitors is shown. The second row shows the intracellular replication of *S. aureus* 6850 GFP in the presence of different calpain inhibitors measured by FACS. The mean of three independent experiments ±SD is shown. Control infection was set to 100.

The identification of such a potent inhibitor motivated us to conduct some in vivo experiments in a mouse model, since ALLN seems to have a strong potential to cure *S. aureus* infections. An extensive literature search displayed that not ALLN was commonly used in mouse experiments but calpeptin, another calpain inhibitor. Hence, cell culture experiments were repeated with calpeptin. Calpeptin, like ALLN inhibited cell rounding and detachment of infected cells (**Fig. 32**), but remarkably, also reduced the intracellular replication of bacteria (**Fig. 33**). In line with our previous results, showing that intracellular replication depends on phagosomal escape, *S. aureus* 6850 was not able to escape from the phagosome in the presence of calpeptin.

So the two different calpain inhibitors showed different effects on *S. aureus* infection. This is very remarkable, since both are peptide inhibitors that have nearly the same action spectrum (**Table 12**). To find out which of the effects of the inhibitors do have an effect on calpains, a third inhibitor was tested. PD150606 is not a peptide but rather a small compound inhibitor. Surprisingly, the addition of

PD150606 resulted in a course of infection similar to control infection (**Fig. 32**). Hence, bacterial intracellular replication as well as phagosomal escape was comparable to control infection (**Fig. 33+34**). To exclude that 10µM, a concentration at which ALLN and calpeptin works, is only to slight, I tried PD150606 with various concentrations up to 50µM, but no effect was visible.

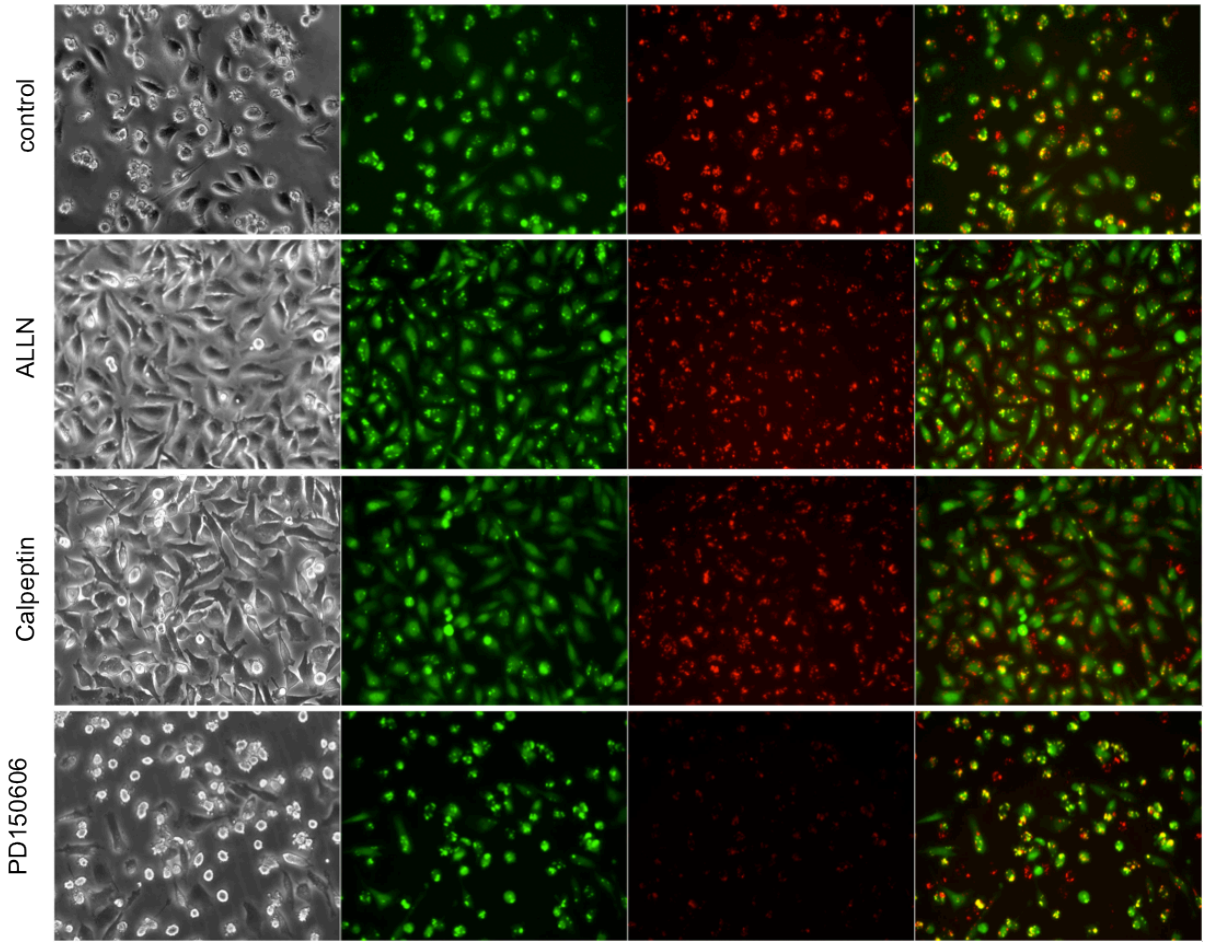

Figure 34 Calpeptin is the only tested calpain inhibitor that decreases phagosomal escape rate. HeLa229-YFP-CWT cells were infected with *S. aureus* 6850mRFP. The green signal shows the escape marker and only escaped bacteria give a bright dot signal. The red signal is mRFP, which is expressed by all bacteria. Only calpeptin treated cells showed a diffuse GFP signal indicating that bacteria were not escaped. 20x magnification.

Subsequently, I was interested, which of the different calpain inhibitors are able to prevent the annexin positivity of the cells. Unexpectedly, none of the inhibitors led to a reduction of the number of annexin positive cells (**Fig. 33**). Since some of the calpain inhibitors showed a promising effect on *S. aureus* infection, I knocked down the two major calpains, CAPN1 and CAPN2, via siRNAs. Both siRNAs had an effect on bacterial invasion.

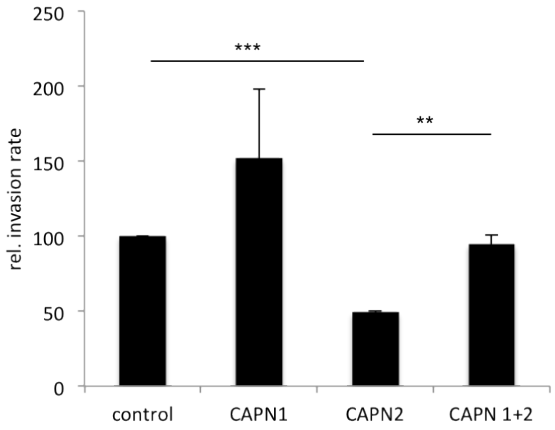

Figure 35 siRNA knockdown of calpains. HeLa cells were transfected with siRNAs against CAPN1 and/or CAPN2 and invasion of *S. aureus* 6850 GFP was monitored by FACS (FACSaria III). The mean of three independent experiments is shown ±SD. Control infection was set to 100.

The knockdown of CAPN1 increased invasion, whereas the knockdown of CAPN2 decreased invasion significantly. The knockdown of both calpains led to an invasion rate comparable to the control infection (**Fig. 35**).

As a conclusion, calpeptin protects the host cells most. This seems plausible, since it is the most potent inhibitor of the calpains (**Table 12**). The second potent inhibitor is ALLN, which also prevents host cell detachment but cannot inhibit escape and replication of bacteria. PD150606 has K_i values comparable with ALLN but does not show any effect on *S. aureus* infection. Therefore, it is uncertain whether the effect on *S. aureus* infection is really calpain dependent or if other proteases are involved.

Table 12 Targets of calpain and proteasome inhibitors.

Compound	Targets
ALLN	CAPN1 (K_i=190nM), CAPN2 (K_i=220nM), Cathepsin L/B (K_i=500pM/ K_i=150nM), proteasome (K_i= 6μM)
Calpeptin	CAPN1 (K_i= 7nM), Cathepsin K (IC_{50}=0.11nM), CAPN1 (IC_{50}=52nM), CAPN2 (IC_{50}=32nM) (Tsujinaka et al., 1988)
PD150606	CAPN1 (K_i=210nM), CAPN2 (K_i=370nM) (Wang et al., 1996)
MG-132	Proteasome (IC_{50}=100nM) (K_i=4nM) (Bregegere et al., 2003), calpain (measures via casein degradation) (IC_{50}=1.2μM) (Tsubuki et al., 1996)

Involvement of the proteasome

The calpain inhibitor studies suggested that proteases play an important role during *S. aureus* infection of epithelial cells. But varying results with these inhibitors raised the question, if actually calpains are the important proteases or rather other proteins, which are additionally inhibited by these compounds. One of these additionally inhibited proteins could be the proteasome. The shRNA screen data showed that several subunits of the proteasome and ubiquitin ligases were identified as

top hits. So, next the impact of the proteasome inhibitor MG-132 on the course of infection was monitored.

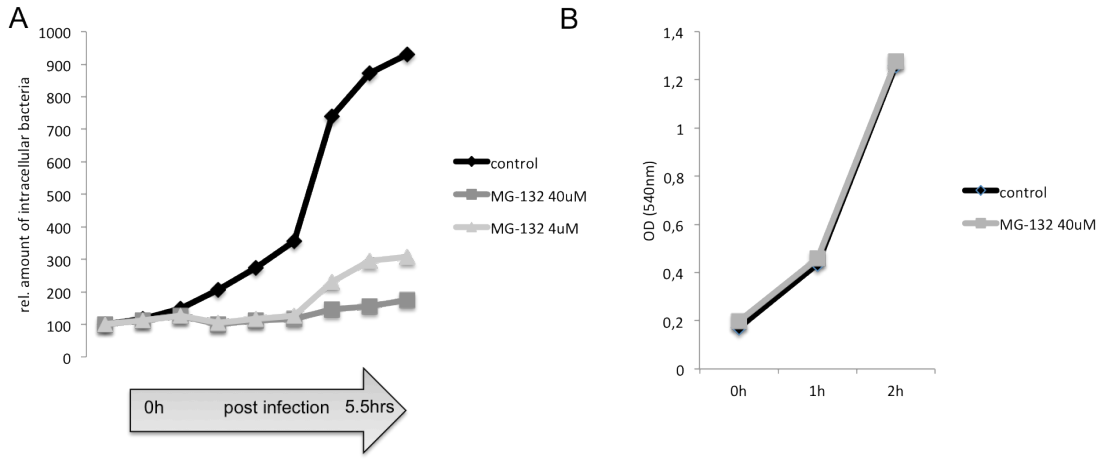

Figure 36 The proteasome inhibitor MG-132 inhibits intracellular replication. (A) Intracellular replication was monitored via FACS. HeLa cells were infected with *S. aureus* 6850 GFP and the increase in GFP signal was measured over time. (B) A growth curve experiment was performed in TSB. The growth ± MG-132 was identical.

The FACS based assay showed that *S. aureus* 6850 replication within HeLa cells is dose dependently reduced in the presence of MG-132 (**Fig. 36A**). A direct bacteriostatic or bacteriotoxic effect could be excluded as bacteria grow normally when MG-132 is added to the growth medium TSB (**Fig. 36B**). This is a first hint that the proteasome is important for *S. aureus* infection and therefore the effect of MG-132 was investigated further. Bacterial escape was monitored with the HeLa-YFP-CWT cell line. Interestingly, ethanol, which is the solvent of MG-132, seems to accelerate bacterial escape as 1h p.i. a vast number of bacteria already escaped. This is true not only for the highly cytotoxic strain 6850 (data not shown) but also for USA300 Lac (**Fig. 37**). The addition of MG-132 inhibited the fast bacterial escape, since 1h p.i. no escape signal was visible (**Fig. 37 row 1**). 2hrs p.i. few escaped bacteria were also visible in the MG-132 treated sample. This is in line with our assumption that intracellular replication is a result of phagosomal escape, since at later time points bacteria replicated even in the presence of MG-132 (**Fig. 36A**).

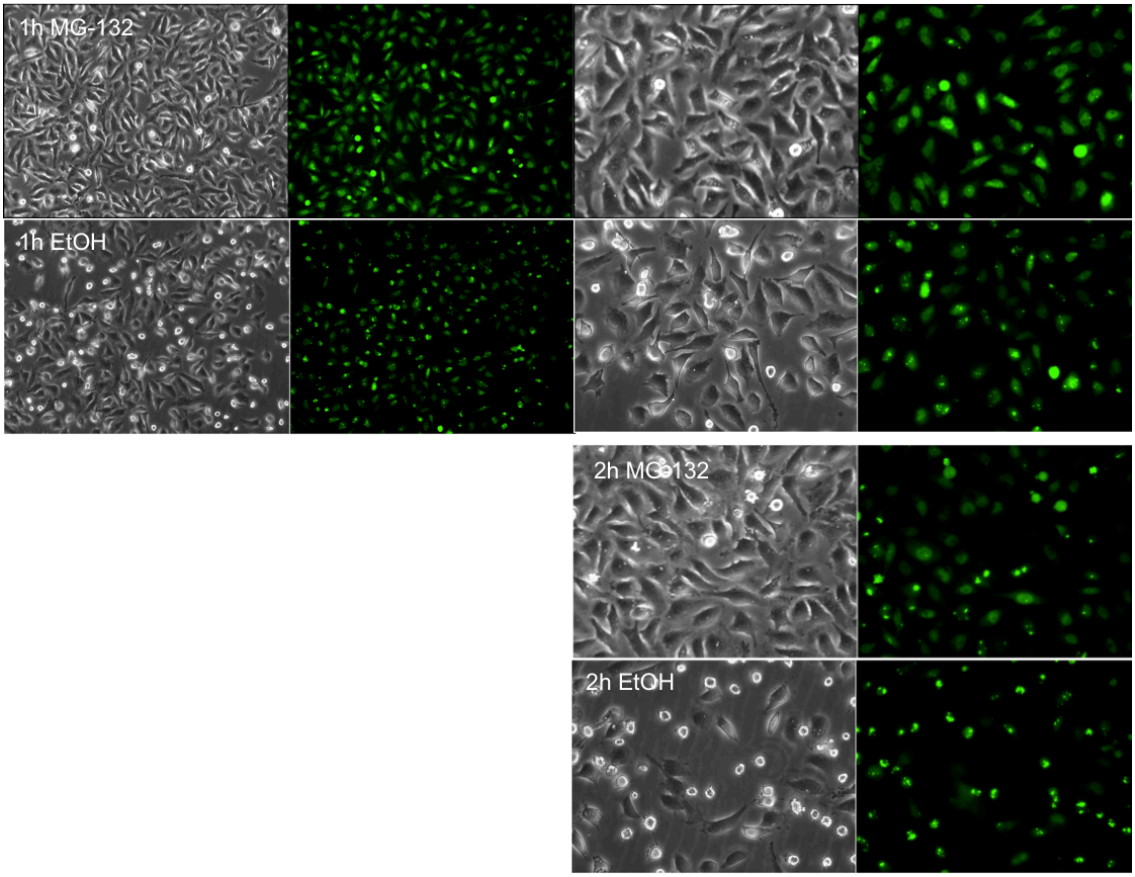

Figure 37 Escape of *S. aureus* USA300 Lac in cells treated with MG-132. HeLa229-YFP-CWT cells were infected with *S. aureus* USA300 Lac ±MG-132 and escape rate was monitored. Diffuse GFP signal within the cell indicates that no bacteria escaped, bright dots show escaped bacteria. 40x magnification.

The proteasome degrades proteins, marked with ubiquitin. Ubiquitin can be added to almost all cellular proteins via ubiquitin ligases. Ubiquitination is a post-translational modification and it changes the role of the protein to which it is bound.

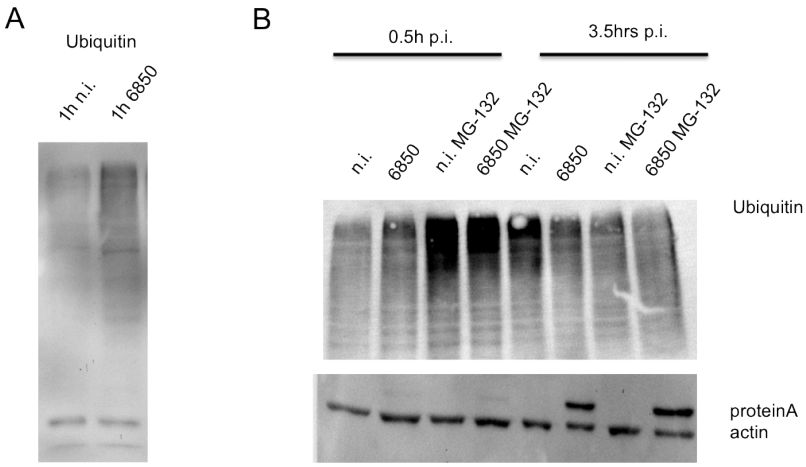

Figure 38 Western blot analysis showing the ubiquitination of proteins during *S. aureus* infection. (A) Amount of ubiquitinated proteins before and after *S. aureus* infection. (B) Ubiquitination pattern during *S. aureus* infection in presence of MG-132.

In most of the cases the ubiquitination of a protein leads to its degradation via the proteasome. To ascertain that the proteasome is important for *S. aureus* infection, I tested whether the total ubiquitination pattern within HeLa cells is changed during *S. aureus* infection.

Western blots incubated with an antibody against ubiquitin showed a big smear. This is normal, since a lot of different proteins are ubiquitinated within the cell. Interestingly, the over-all ubiquitination is increased 1h after *S. aureus* infection (**Fig. 38A**). This is true even 0.5h p.i. . As a control MG-132 was used. The inhibition of the proteasome inhibited the degradation of the ubiquitinated proteins, thus these proteins accumulated within the cell and so an even more prominent smear was visible (**Fig. 38B**).

Tu sum upthe data presented here suggest that the proteasomal degradation of proteins at an early time point of *S. aureus* infection is important for escape, subsequent replication of bacteria and finally host cell death.

3.6 Mitochondrial membrane potential loss (MMPL) can be rescued by a mNCX inhibitor

Mitochondrial membrane potential (MMP) can be destroyed as a result of calcium overload (Miyamae et al., 1996). The shRNA screen showed, beside an important role of calcium, an involvement of mitochondria. So I was interested if *S. aureus* 6850 lead to a loss of MMP. Mitochondrial viability was measured at different time points p.i. with JC-1. JC-1 is a membrane permeable fluorescent dye, which emits in the monomeric form light at 520nm, in the multimeric form the emission spectrum shifts to a more red fluorescence (590nm). The dye is trapped in viable mitochondria and so the red fluorescence (590nm) signal here is much stronger than in cells where MMP is lost. For the determination of viability, the ratio between the 590nm and the 520nm signal is used to exclude different basal levels of staining.

S. aureus 6850 infected cells rapidly lost their MMP 1.5hrs p.i. and the resulting potential was comparable to the potential of cells treated with the uncoupler Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (**Fig. 39A**). The slight increase in MMP at 4hrs p.i. was a result of the fast dying of the infected cells. At this time point, cells were already detached and so in relative terms more uninfected cells were analysed.

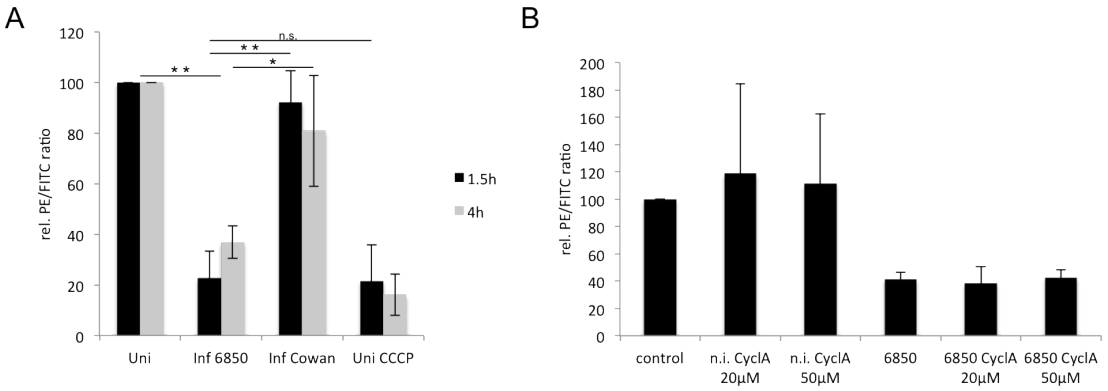

Figure 39 Loss of mitochondrial membrane potential during *S. aureus* infection. (A) *S. aureus* 6850 infected cells rapidly loose their mitochondrial potential, whereas the control strain CowanI did not induce this loss. (B) CsA could not rescue MMPL. MMP was measured with JC-1 by FACS. Three independent experiments ±SD are shown. Control was set to 100.

Since *S. aureus* infection induced a loss of MMP, I was interested if this loss can be inhibited. Therefore I tested cyclosporinA (CsA) (**Fig. 39B**), an inhibitor of cyclophilinD (CyP-D) (Connern and Halestrap, 1992; Crompton et al., 1988). CyP-D is an important member of the mitochondrial permeability transition pore (MPTP) and CsA inhibits the opening of the MPTP via its interaction with CyP-D and thus MMPL. Interestingly, CsA did not inhibit the MMPL. Thus, I tested a second well-known inhibitor, the adenine nucleotide translocator (ANT) inhibitor Bongkrek acid (B. acid) (**Fig. 40A**) (Klingenberg et al., 1970). ANT is also a component of the MPTP and therefore is also involved in MMPL during apoptosis. Even though tested in different and very high concentrations B. acid was not able to inhibit MMPL induced by *S. aureus*.

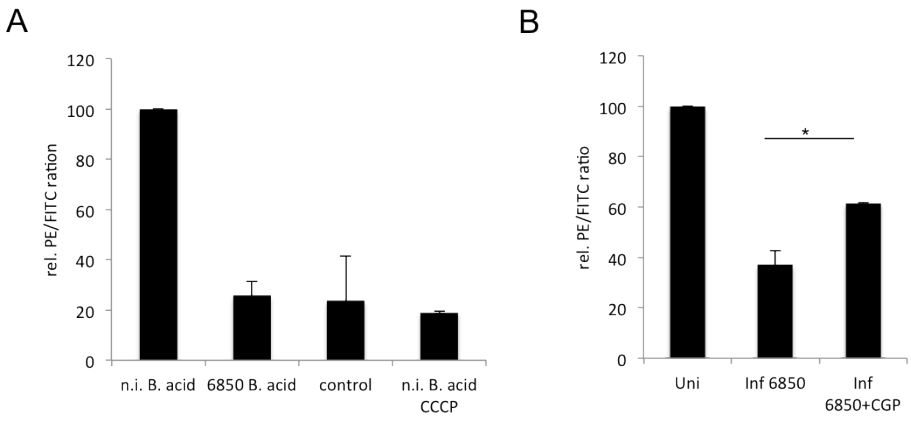

Figure 40 B. acid cannot rescue MMPL but CGP37157 can do so. (A) MMP after *S. aureus* infection in the presence of B. acid. (B) MMP after *S. aureus* infection in the presence of CGP37157. CCCP was used as positive control. MMP was measured with JC-1 by FACS. Three independent experiments ±SD are shown. Control was set to 100.

Since none of the well-known inhibitors of MMPL could rescue the MMP during *S. aureus* infection I started to test all inhibitors, which I used during my studies. But none of them was able to rescue the

MMP 1.5hrs p.i. . However, MG-132 was able to rescue it 4hrs p.i. (**Fig. 41**). Hence, cells treated with a proteasome inhibitor can somehow rescue their MMP.

One explanation for this rescue 4hrs p.i. could be that the mitochondria are not seriously damaged, but rather the MMP is reversibly disbalanced. One obvious reason for this could be the calcium overload (Carafoli, 2002) and since the 2-APB experiments and the shRNA screen already suggested an involvement of calcium, I looked for calcium transporters within the mitochondria. For one of them, the mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX), a specific inhibitor, CGP37157, is available (Nicolau et al., 2009). Actually, the MMP was significantly higher in the presence of CGP37157 than in control cells 1.5hrs p.i. (**Fig. 40B**).

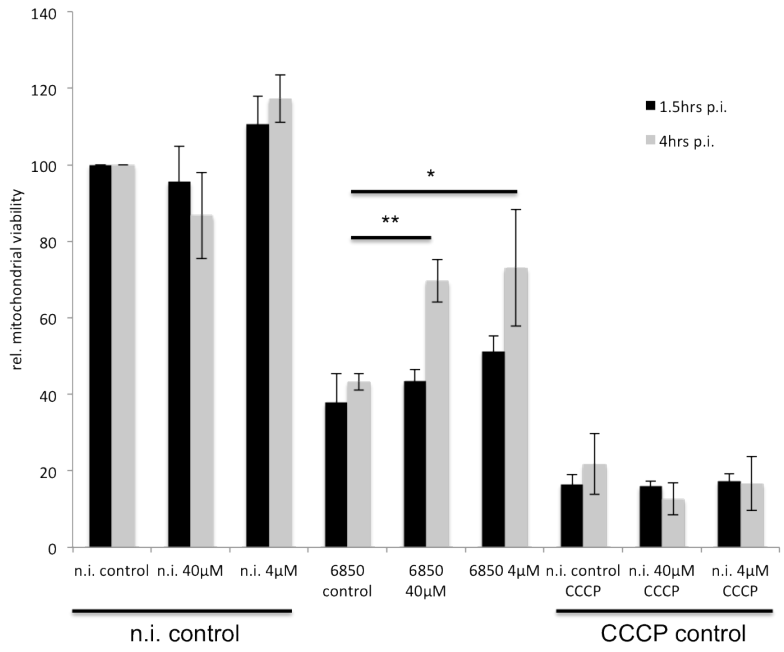

Figure 41 MMP can be rescued during infection in presence of MG-132. MMP was measured with JC-1 at indicated time points. The MMP increased between 1.5hrs and 4hrs p.i. . CCCP was used as positive control. The mean of three independent experiments is shown ±SD. Control was set to 100.

To sum up, the MMP is drastically reduced after *S. aureus* 6850 infection and the inhibition of the mNCX reduced this loss significantly. In addition, MG-132 treated cells could rescue their potential 4hrs p.i. . Therefore, MMPL seems to be a reversible event, which is not accountable for host cell death. This is confirmed by the fact that CGP37157 rescued MMP but does not rescue infected cells from death (data not shown).

4 Discussion

4.1 Identification of human host cell factors involved in *S. aureus* 6850 infection with an shRNA and a follow-up siRNA screen

S. aureus is a well-known human pathogen, which kills different types of cells very efficiently. Since community-acquired (CA) as well as hospital-acquired (HA) *S. aureus* strains develop more and more antibiotic resistances, it is important to find other innovative approaches and treating options. One first step is to gain more inside into the interaction between *S. aureus* and human host cells. Proteomic studies, which identified phosphorylation patterns, reveal that *S. aureus* infections change protein phosphorylation in human host cells drastically and therefore induce host cell signalling cascades. As an example, JNK is phosphorylated in THP cells 1h post infection (p.i.) (Miller et al., 2011). In addition, microarray experiments in HUVEC and Hep-2 (a HeLa derivative) cells showed basic expression changes within host cells during *S. aureus* infection (Grundmeier et al., 2010; Li et al., 2009; Matussek et al., 2005). Studies like this suggest that *S. aureus* does interact with human cells rather than just lysing them via its pore-forming toxins.

In this work, a loss-of-function approach was used to identify factors, which contribute to bacterial invasion and subsequent intracellular events. With this method, host cell factors, which definitively contribute to the investigated processes, can be identified. In contrast, proteome and transcriptom analyses describe on-going processes but cannot define the impact of the respective process. Knockdown experiments can actively influence a process, and in this way show that the silenced factors play a essential role during this process.

With the help of lentivirus 60,000, different shRNAs were integrated into the genome of HeLa cells. Therefore in theory, every human gene should be silenced at least once. A low virus MOI minimised the risk of integration of two different shRNAs within one cell. 38,050 of these different shRNAs could be rescued, meaning they showed at least ten sequencing-reads.

The sequencing-reads were mapped and a statistical analysis was performed. Although working with two biological replicates, the resulting data structure did not allow to identify significantly enriched genes. We were however able to organise the hits by their fold changes.

The comparison of shRNAs rescued from input control cells and the ones, which were infected with *S. aureus* 6850 mRFP but did not harbour any bacteria, showed, which genes are critical for bacterial invasion. 1,600 genes were at least two fold enriched. This is an impressive number and it is not likely that all of them are involved in *S. aureus* invasion. Therefore, I focused on the 69 at least ten fold enriched genes first (**Table 17**). Unfortunately, most of them are poorly characterised or do not seem to be likely invasion relevant genes. The same was true for genes, which are supposed to be involved in cell death induction. These genes were determined via the comparison of control cells and cells infected with *S. aureus* 6850 but survived this infection for more than 4.5hrs. Approximately 70% of

the infected cells were already dead at this time and so the integrated shRNA was directed against a factor involved in the cell death process. 2,271 shRNAs were enriched at least two fold and 112 ten fold (**Table 18**).

The challenge now was to identify the relevant genes within these big data sets. First, I tried a KEGG analysis (data not shown). This online tool assigns genes to known pathways. Here, the endocytosis pathway or pathways involved in different diseases were top hits, meaning a lot of proteins, which came up in the screen are also involved in other diseases. But except for calcium signalling (its role will be discussed below), no interesting pathway, with a considerable amount of proteins assigned to, could be identified by this method.

Second, I classified the hits into GO categories with WebGestalt (**Fig. 11+12**). But again the only reliable information was that ion-binding proteins are overrepresented.

So I tried a third principle of classification. STRING is an online tool that shows connections between the genes of interest. Some of them are more robust like direct protein-protein interactions; others are rather soft connections like co-mentioned in PubMed.

I performed a STRING analysis to order the genes involved in the invasion process and received a highly interconnected network. Even more important, sub-networks were discernible (**Fig. 9+10**). One of the invasion network clusters showed that cholesterol and its transport might play a role during bacterial invasion. Trapping cholesterol with methyl-β-cyclodextrin (MβC) then indeed reduced the number of intracellular *S. aureus* strongly (**Fig. 13A**). This is in line with a study that showed that *S. aureus* invasion into 293T cells is reduced in the presence of MβC (Hoffmann et al., 2010). An additional role of cholesterol during *S. aureus* infection seems to be likely, since it is an important component of biological membranes. Cholesterol has a determining influence on the fluidity of membranes and is an integral component of specialised membrane parts. Recently, it has been shown that the phenol soluble modulins (PSMs) from *S. aureus* can lyse vesicles best, when they contain between 10 and 30mol% cholesterol (Laabei et al., 2014). Therefore the cholesterol content might influence the activity of other pore-forming toxins, too. This is of particular interest, when *S. aureus* escapes the phagosome with the help of its pore-forming toxins.

Since the trapping of cholesterol has an effect on *S. aureus* invasion I chose two other, totally different proteins of the STRING network: the C-Jun N-Terminal Kinase 2 (JNK2 also known as MAPK9) and the transient receptor potential cation channel, subfamily M, member 4 (TRPM4). The JNKII inhibitor, which inhibits all three JNK proteins, showed a concentration dependent reduction of bacterial invasion (**Fig. 13B**). The same was observed with 9-Phenanthrol, a specific inhibitor of TRPM4 (**Fig. 14**) (Guinamard et al., 2014). The involvement of JNK during host cell invasion has been shown for *N. meningitidis* but not for *S. aureus* so far (Sokolova et al., 2004). This is especially interesting since *N. meningitidis* like *S. aureus* uses α5β1 integrins for internalization. In addition, the

JNK inhibitor, as well as genistein, a protein tyrosine kinase (PTK) inhibitor inhibits *N. meningitidis* invasion (Sokolova et al., 2004). PTKs also play a role during *S. aureus* invasion into bovine MAC-T cells (immortalised mammary alveolar cells) (Dziewanowska et al., 1999). Interesting enough, the *S. aureus* invasion process into human host cells shares important similarities with the one of *N. meningitidis*, which is a gram-negative bacterium.

TRPM4 has to my knowledge never been connected to bacterial invasion so far and is therefore the first unexpected *S. aureus* invasion factor identified by the shRNA screen.

Since all inhibitors showed a prominent effect, but always have the disadvantage of distinct side effects, some siRNA knockdown experiments were performed next.

4.1.1 Host cell factors promoting *S. aureus* 6850 internalization

The first important step of a *S. aureus* infection is the invasion of the host cell. This cannot be taken for granted since *S. aureus* has long been described as an extracellular pathogen. The fact that it possesses a lot of pore-forming toxins supports the idea of a bacterium, which acts predominantly via its secreted toxins. But there are already a number of publications, which indicate that *S. aureus* kills host cells from within. Accordingly, it has been shown that bacterial supernatant cannot kill human peritoneal mesothelium cells (HMC) (Haslinger-Loffler et al., 2006). Studies with bovine MAC-T cells showed that the addition of *S. aureus* to cells does not result in death of all MAC-T cells. The authors therefore concluded that not extracellular factors kill the cells but rather the bacteria do (Bayles et al., 1998). A further indication is that an invasion deficient mutant cannot kill CFT-1 cells (a respiratory epithelial cell line derived from a cystic fibrosis patient) as efficiently as wild-type bacteria do (Kahl et al., 2000a).

Since all theses studies were done in different cell lines, with different bacterial strains, I conducted some observation experiments in my experimental setup. Here, I observed that the supernatant of a bacterial over night culture was not able to kill HeLa cells, whereas viable bacteria do (**Fig. 4B**). In addition, time course experiments showed that cells harbouring *S. aureus* die and HeLa cells, which survive 24hrs, do not harbour any bacteria (**Fig. 6**). These data indicate that bacterial invasion is an essential first step for further infection.

With the siRNA screen performed in this study 24 proteins, whose downregulation leads to a significant decrease of the percentage of infected cells, could be identified (**Fig. 15**). In addition, the knockdown of 11 genes leads to a significant reduction of the amount of intracellular bacteria (**Fig. 16**). In both cases the two integrins ITGA5 and ITGB1 served as positive controls and showed a significant reduction of bacterial invasion, when knocked down.

The siRNA directed against calpain 2 (CAPN2) showed the most prominent effect on the percentage of infected cells. In contrast, the knockdown did not show any effect on the number of intracellular *S. aureus*. The reason for this could be that not all cells are transfected with an siRNA and the

knockdown in individual cells is different. So it could be that cells, which showed a good knockdown, are uninfected whereas cells with a poor knockdown are normally infected. The role of calpains during *S. aureus* infection will be discussed in more detail below. The other siRNAs, which lead to fewer than 80% infected cells, compared to control infection, are ANPEP, VCL and the two mentioned integrins.

4.1.1.1 Alanyl (membrane) aminopeptidase (ANPEP)

The involvement of ANPEP is especially interesting since it has been shown to be a receptor for the human coronavirus 229E (Yeager et al., 1992). In addition, ANPEP is attributed to play a role in plasma membrane protein organization (Petrovic et al., 2007). It is also involved in phagocytic processes of human dendritic cells and macrophages. During phagocytosis ANPEP is internalised and ends in the phagosome of the internalised particle e.g. zymosan (Villasenor-Cardoso et al., 2013). An expression study showed that ANPEP is rarely expressed in culture cell lines, so it is not expressed in A549 and HEK293 cells, but it is expressed in HUVEC cells (Fukasawa et al., 2006). In our HeLa cells ANPEP is expressed and slightly downregulated 3hrs post *S. aureus* infection (**app. Fig. 45B**). To sum up, the siRNA screen identified ANPEP clearly as an important human protein for *S. aureus* 6850 invasion into HeLa cells as its knockdown reduced the invasion rate down to 50%. The subsequent literature search showed that ANPEP is already linked to virus internalization and is involved in endocytosis processes, which strongly supports an involvement during *S. aureus* invasion into human host cells. To confirm this, in vitro interaction studies or pull down experiments should be performed. Especially, since beside ANPEP, ITGB1 seems to be downregulated during an ANPEP knockdown (**app. Fig. 45A**).

4.1.1.2 Vinculin (VCL)

VCL can couple integrins to the cytoskeleton and thereby form the core of focal adhesions (FA) (Ezzell et al., 1997). It can directly bind to several actin binding and FA proteins e.g. to α-actinin and talin. They are both necessary for VCL activation, too (Chen et al., 2006; Humphries et al., 2007). VCL also plays a role during FA turnover. Here, PIP2 is important, since a VCL mutant deficient in PIP2 binding massively stabilises FA. Therefore the generation of PIP2 might destabilise FAs (Chandrasekar et al., 2005; Saunders et al., 2006).

In contrast to studies performed in the lab of C. Hauck, the siRNA knockdown of VCL in HeLa cells reduced *S. aureus* invasion markedly (Borisova et al., 2013). The reason for this discrepancy might be that mouse fibroblasts were used in the experiment showing that *S. aureus* uptake is not affected by VCL knockdown. In addition, the group of C.Hauck previously published that VCL is recruited to the site of *S. aureus* invasion, which is in line with data shown in this work (Agerer et al., 2005).

The knockdown of the following genes reduced the amount of intracellular bacteria significantly and at least down to 80%: CAPN4, GRIK5, RYR3, PRKAR1A, PIK3CG, PCSK9, RYR2 and PKN1. The standard assay for monitoring bacterial invasion is a colony forming unit (CFU) assay. Here, the number of bacteria isolated from all cells is counted and an average of bacteria per cell is determined. The amount of uninfected cells cannot be determined with this assay. For the comparison of the results of a CFU assay and the FACS assay, the parameter "rel. invasion" was introduced. Here, the percentage of infected cells was multiplied with the amount of intracellular bacteria. Rel. invasion correlates roughly with a CFU assay. The rel. invasion of all the discussed knockdown experiments (above and below), was less than 60% compared to control infection.

4.1.1.3 Glutamate Receptor, Ionotropic, Kainate (GRIK5) also known as KA2

GRIK5 is a glutamate receptor, which is predominantly expressed in the human brain. It can form heterodimers with GlR5-7 but stays in the ER when it is expressed alone (Hayes et al., 2003). About its precise function or role in other than brain cells nothing is known so far. Hence, the role of GRIK5 during *S. aureus* invasion is indeed interesting and has to be investigated in more detail. In a first step, the expression of GRIK5 during *S. aureus* infection should be monitored. Then possible interaction partners can be determined with the help of a pull-down assay.

4.1.1.4 Ryanodine receptor 2 (RYR2) and ryanodine receptor 3 (RYR3)

RYRs are, like IP3 receptors, receptors leading to calcium release from the endoplasmic reticulum (ER). Their role in heart and other muscle cells as well as in neuronal cells was investigated in detail; therefore it was long believed that they are solely expressed here. But functional RYR2 and RYR3 were also found in gut epithelial cells (Verma et al., 1996). In addition, RYR2 was found in a non-excitable kidney cell line (LLC-RK1) (Tunwell and Lai, 1996). However, the function in epithelial cells is not clear and a connection to bacterial infections has not been made shown so far. A Dual RNA-Seq experiment performed in our lab (cf. Dissertation M.Grosz) showed that the expression of RYR1 and RYR3 is low in 293T cells, but upregulated after *S. aureus* infection. In addition, RYR2 is highly expressed and also upregulated during *S. aureus* 6850 infection (cf. Dissertation M.Grosz).

The involvement of RYRs during *S. aureus* infection is, to my knowledge, completely new and has to be investigated in more detail. The localization of RYRs during infection should be investigated. They are normally located at the ER (Bhat and Ma, 2002) but may also have another localization during infection. The expression of GFP-RYR fusion proteins could be helpful here, but also more modern tags like SNAP or CLIP could be used.

4.1.1.5 Protein kinase, cAMP-dependent, regulatory, type I, alpha (PRKAR1A)

PKAs play a role in cytoskeleton rearrangement and integrin signalling (Manganello et al., 2003; Shan et al., 2006). In addition, PKAs have been shown to be antagonists of Ras /ERK signalling (Pursiheimo et al., 2002). Interestingly, the proteomics data mentioned above showed that a PKA is activated

during *S. aureus* infection (Miller et al., 2011). But no further function during *S. aureus* infection has been identified. The siRNA knockdown experiments performed in this study, showed that a HRAS knockdown reduces the intracellular bacterial replication, whereas a PRKAR1A knockdown increases it. This is an evidence for an antagonistic behaviour of HRAS and PKA during *S. aureus* infection. To ascertain this, the downstream effectors of PKA during *S. aureus* infection should be determined.

4.1.1.6 Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Gamma (PIK3CG) also known as p110γ

PIK3CG is a catalytic subunit of the PI3 kinase gamma (PI3Kγ). It has an exceptional position since it is the only catalytic subunit of the class IB PI3 kinases and thus is activated by G-protein coupled receptors (GPCR) (Foster et al., 2003; Stoyanov et al., 1995). The role of p110γ is heavily investigated in immune cells. Here, it plays an important role in innate immunity (Costa et al., 2011). An involvement of PI3-kinases during *S. aureus* invasion was already proposed, but here, the class IA PI3-kinases were investigated (Oviedo-Boyso et al., 2011). PI3Kγ has been shown to be involved in actin reorganization downstream of GPCRs and it can therefore activate Rac. (Ma et al., 1998). Thus, the involvement of PI3Kγ during *S. aureus* infection is worth to be investigated into more detail. Especially interesting is the identification of the GPCR, which activates PI3Kγ.

4.1.1.7 Proprotein convertase subtilisin/kexin type 9 (PCSK9)

PCSK9 is secreted by cells and extracellularly binds to the low-density lipoprotein receptor (LDLR) resulting in its endocytosis. Subsequently, it inhibits the recycling from the endosome and thus leads to a lysososmal degradation of the LDLR. Accordingly, PCSK9 induces a reduction of LDLR uptake and thus lipid uptake (e.g. cholesterol) (Maxwell et al., 2005; Qian et al., 2007). The hepatitis C virus (HCV) takes advantage of this central role of PCSK9 during cholesterol uptake. During an infection, it negatively regulates the expression of PCSK9 to accelerate lipid uptake, which is necessary for viral development (Syed et al., 2014). In addition, PCSK9 plays a role during apoptosis induction in neurones. Here, PCSK9 promotes the degradation of ApoER2, resulting in a higher sensitivity of the cell to apoptotic stimuli. In epithelia, PCSK9 increases the proteasomal degradation of the epithelial Na^+ channel (ENaC) and as a consequence modulates Na^+ uptake (Sharotri et al., 2012). The role of PCSK9 during *S. aureus* invasion could be diverse. On the one hand, the experiments with the cholesterol trap MβC showed that accurate cholesterol concentrations are essential for bacterial invasion (**Fig. 13A**). On the other hand, ion concentrations and/or fluxes are important during *S. aureus* invasion into host cells and thus the degradation of ENaC by PCSK9 could be important. A control experiment showed that the knockdown of PCSK9 does not result in a downregulation of ITGB1 or ITGA5 and thus the reduced invasion rate is very likely dependent on the downregulation of PCSK9 (**app. Fig. 45A**). But its precise role during *S. aureus* invasion has to be investigated in more detail.

4.1.1.8 Protein kinase N1 (PKN1) also known as PRK1

PKN1 belongs to the PKC superfamily (Mukai and Ono, 1994). It is activated via three different mechanisms; first, by unsaturated fatty acids like arachidonic acid, second by limited proteolysis (Mukai et al., 1994) and thirdly, by RhoGTPases. One of these RhoGTPases is RhoA, which can bind PKN1 and thus activating it by phosphorylation (Amano et al., 1996; Watanabe et al., 1996). In addition, PKN1 binds to Rac1 and then interacts with another Rho GTPase namely RhoB (Owen et al., 2003). The binding to activated RhoB targets PKN1 to the endosome (Mellor et al., 1998). Besides, it can directly interact with α-actinin (Mukai et al., 1997). All these interactions suggest that PKN1 is involved in actin dynamics but a direct involvement (e.g. in the endocytosis process) has never been shown. The shRNA screen identified PKN1 as an invasion linked gene; it was 17.7 fold enriched in the uninfected sample compared to control (input). The following siRNA experiments confirmed that a knockdown of PKN1 reduces *S. aureus* invasion into HeLa cells. Here, the interaction with Rho proteins could play a fundamental role, since the Rho inhibitor C3-transferase has been shown to reduce *S. aureus* invasion down to 60% (Schroder et al., 2006).

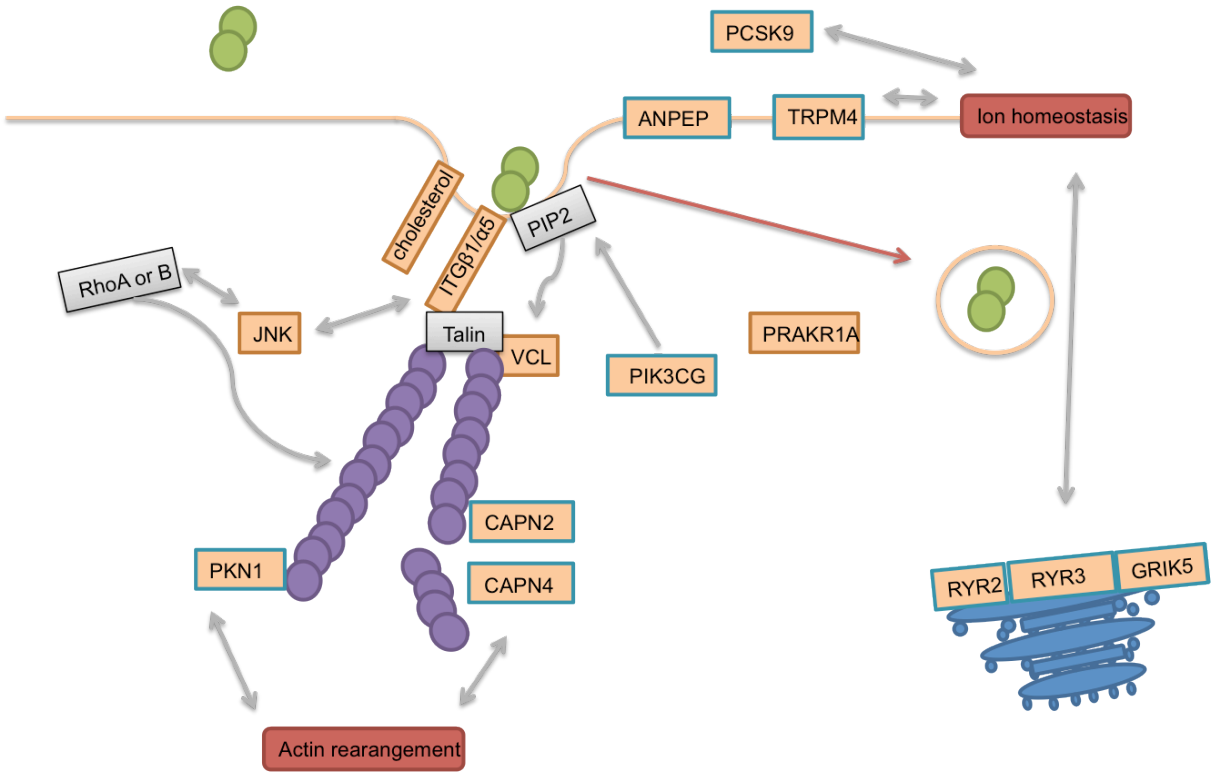

Figure 42 Model of *S. aureus* invasion into HeLa cells, based on data generated within this study. Ion homeostasis and actin rearrangement are the two main processes involved in *S. aureus* invasion. PCSK9, TRPM4 and the ER channels RYR2, RYR3 and GRIK5 can influence ion homeostasis. PI3Kγ, CAPN2, CAPN4, PKN1 and JNK can influence the actin cytoskeleton. Cholesterol influences the receptor activation and/or clustering at the plasma membrane. Integrins play a fundamental role since they initiate signalling and are the indirect (via fibronectin) binding partners for *S. aureus*. The role of ANPEP is uncertain. Proteins in petrol-framed boxes were identified in this study for the first time. Grey boxes show proteins that were not investigated in this study. For further information refer to the text above.

Calpain small subunit 1 (CAPNS1) also known as CAPN4

Calpains will be discussed in a separate chapter below.

4.1.2 Host cell factors promoting the intracellular replication of *S. aureus* 6850 in HeLa cells

The importance of the bacterial invasion process for host cell death induction was already discussed above. Further studies in our lab suggested in addition, that bacterial phagosomal escape is a prerequisite step for intracellular replication of *S. aureus* (Grosz et al., 2014). The inhibitor studies performed in this work are a further indicator for this and since we were not able to identify one single inhibitor, which reduced intracellular replication and showed regular escape rates, we concluded that the inhibition of phagosomal escape leads to the inhibition of intracellular replication. Therefore, and since the FACS-based replication assay established in this work is very efficient, we measured intracellular bacterial replication after siRNA knockdowns in order to identify proteins that are important for the steps following the host cell invasion.

The siRNA that reduced bacterial intracellular replication most is the one against FPR2. This is especially interesting, since it is believed to be solely expressed in immune cells and was already linked to *S. aureus* infection. Further factors reducing intracellular replication are: CAPN4, JUN, LYN, HRAS, AKT1, ITGB1, ITGA5 and ITGAM. Since the FACS-based assay was developed in this study I performed a CFU assay with some of the siRNAs to verify the FACS results. Here, all tested siRNAs (targeted against HRAS, AKT1, FPR2, JUN) as well as HMGCS1 (which showed a reduction down to 70% but was not significant in the FACS-based assay) showed a significant reduction of intracellular replication of *S. aureus*.

Some of the siRNAs, which reduced intracellular bacterial replication, had already an effect on bacterial invasion; therefore the question arose, if the reduction of intracellular replication is a result of the smaller amount of intracellular bacteria or of a decreased replication rate. An argument against the second possibility is that the replication was measured on single cell level. The means of fluorescence levels 3hrs and 1h p.i. were compared. Consequently, the duplication from one to two bacteria was rated equally as the duplication from 4 to 8 bacteria. In addition, not all siRNAs that led to a decreased invasion rate decreased intracellular replication. As a consequence, it is very likely that some siRNAs have an effect on both, invasion and intracellular bacterial replication.

4.1.2.1 Formyl peptide receptor 2 (FPR2) also known as FPRL1

FPR2 is a GPCR, which was originally identified as an immune cell receptor, but recent studies showed that it is expressed in a lot of different cell lines, also in HUVECs (Babbin et al., 2006; Becker et al., 1998; Koczulla et al., 2003; Rescher et al., 2002). So beside their function on neutrophils, where they sense chemokines, FP-receptors play a role in epithelial cells during wound healing. Here, they activate the PI3Kγ, which then activates RAC and CDC42. This induces the cytoskeleton

rearrangements, which are necessary for wound closure (Babbin et al., 2007). FP-receptors have already been linked to *S. aureus* infections. *S. aureus* expresses an inhibitor against FPR1, which is the chemotaxis inhibitory protein (CHIPS). In addition, it expresses an inhibitor against FPR2, which is called formyl peptide receptor-like 1 inhibitor (FLIPr) and one against both receptors, called FLIPr-like (Prat et al., 2006; Prat et al., 2009). The inhibition of FPR signalling via these proteins protects *S. aureus* against the immune system. In contrast, PSMs have been shown to activate FPR2 on neutrophils (Kretschmer et al., 2010). That is why an involvement of FPR2 during intracellular *S. aureus* replication is especially interesting. To ensure that FPR2 is expressed in HeLa cells, we performed qRT-PCR experiments. FPR2 was upregulated during *S. aureus* infection of HUVEC (data not shown) and HeLa cells (**app. Fig. 45C**). The upregulation was reduced after an siRNA knockdown. The downregulation of FPR2 by siRNA resulted in reduced intracellular bacterial replication. In addition, the knockdown of FPR2 resulted in reduced phagosomal escape rates (data not shown). This is especially interesting, since we already showed that PSMα is responsible for phagosomal escape in HeLa cells (Grosz et al., 2014). These data suggest that *S. aureus* infection induces the expression of FPR2, which then contributes (maybe as the direct receptor of PSMα) to the phagosomal escape of *S. aureus*.

CAPN4

The role of CAPN4 during *S. aureus* infection will be discussed in a separate chapter below.

4.1.2.2 Jun proto-oncogene (JUN)

JUN is a transcription factor, which can be activated by the Ras/MEK/ERK signalling (Pulverer et al., 1991). It is a proto-oncogene and its targets are pro-survival factors. One of them is cyclinD1, which is important for cell cycle progression. In addition, JUN can inhibit apoptosis after UV exposure. Both processes are ensured by different phosphorylation sites of JUN (Wisdom et al., 1999). During *S. aureus* infection of conjunctival epithelial cells JUN is phosphorylated and thus activated in a JNK dependent manner. The result is the expression of IL-8 (Venza et al., 2007). Interestingly, the expression of IL-8 during *S. aureus* infection of airway epithelial cells has been shown to be calcium dependent (Ratner et al., 2001). This suggests an involvement of calcium signalling during JUN activation. The data provided in this study showed that a JUN knockdown reduces intracellular bacterial replication (**Fig. 18+19**). To sum up, one could speculate that JUN is activated by HRAS signalling during *S. aureus* infection and subsequently activates the transcription of its targets.

4.1.2.3 V-Yes-1 Yamaguchi Sarcoma Viral Related Oncogene Homolog (LYN)

LYN is a member of the Src family and a tyrosine protein kinase (Yamanashi et al., 1987). It is activated by DNA damage and subsequently leads to the activation of a signalling cascade including PKCδ, MEKK1, MKK7 and JNK. The result is an apoptosis induction (Yoshida et al., 2002; Yoshida et al., 2000). In addition, LYN can activate the SH2 Domain-Containing Inositol 5'-Phosphatase (SHIP)

and therefore inhibits the AKT1 pro-survival pathway in neutrophils (Baran et al., 2003; Gardai et al., 2002). Even more, LYN knockout mice show manifestations like SHIP knockout mice, suggesting a direct and important activation of SHIP by LYN (Harder et al., 2004). SHIP blocks AKT1 in *Francisella tularensis* infections and thereby inhibits the phagosome-lysosome fusion, which is normally induced by AKT1 (Rajaram et al., 2009). For *S. aureus* infection this might mean that LYN is indirectly (via SHIP) responsible for the dephosphorylation of AKT1 in *S. aureus* infected cells. We currently investigate this in more detail and want to find out if a LYN knockdown (via siRNA) reduces AKT1 dephosphorylation.

4.1.2.4 Harvey Rat Sarcoma Viral Oncogene Homolog (HRAS)

An involvement of HRAS during *S. aureus* infection is especially interesting since HRAS is an important signalling molecule. The general role of HRAS is already described in the introduction. A linkage to *S. aureus* infection is not made so far. But HRAS seems to be important for different virus infections e.g. for internalization of the hepatitis c virus (Zona et al., 2013). The central role of HRAS during *S. aureus* infection is discussed below and displayed in **Fig. 43**.

4.1.2.5 v-akt murine thymoma viral oncogene homolog 1 (AKT1)

AKT1 is a serine-threonine protein kinase, which is activated by phosphorylation. An important activator is the PI3 kinase, which generates either PIP2 or PIP3 to activate AKT1. This happens either directly or indirectly via PDK1. AKT1 itself can phosphorylate a huge number of cellular proteins, all more or less directly leading to cell survival. Therefore, bacterial infections, e.g. with *Salmonella typhimurium*, normally induce AKT1 phosphorylation and thus induce prolonged host cell survival (Steele-Mortimer et al., 2000). The dephoshorylation of AKT1 during *S. aureus* infection (**Fig. 28**) could be responsible for host cell death. However, the mechanism how the dephosphorylation takes place, has to be investigated in more detail. In addition, a knockdown of AKT1 resulted in decreased intracellular bacterial replication, consequently indicating an involvement of AKT1 during this process (**Fig. 18+19**). It can only be speculated how these two results can fit together.

4.1.2.6 integrin, alpha M, complement component 3 receptor 3 subunit (ITGAM) also known as CD11b

ITGAM is, like FPR2, a receptor predominantly found on immune cells. It has already been linked to *S. aureus* infections since it is the cellular receptor of leukocidin A/B (LukAB). LukAB is a bi-component leukotoxin, which kills human phagocytes through membrane disruption (Dumont et al., 2011; DuMont et al., 2013; Ventura et al., 2010). The expression of ITGAM is upregulated in *S. aureus* infected HUVEC (data not shown) and HeLa cells (**app. Fig. 45D**). In addition, the knockdown of ITGAM reduced intracellular replication. So the knockdown of ITGAM and FPR showed the same effect on *S. aureus* infection. Hence, it is possible that ITGAM also acts in epithelial and endothelial cells as a receptor for LukAB and so contributes to phagosomal escape.

4.1.2.7 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase 1, soluble (HMGCS1)

HMGCS1 catalyses the synthesis of Acetyl-CoA with Acetoacetyl-CoA. This fusion results in HMG-CoA, which is the first product of the cholesterol biosynthesis. Thus, HMGCS1 is the rate-limiting factor in this process (Ramachandran et al., 1978). Since bacterial invasion is reduced in cells treated with the cholesterol trap MβC, an appropriate cholesterol concentration is necessary for invasion (**Fig. 13A**). The experiment with an siRNA targeted against HMGCS1, showed that appropriate cholesterol concentrations are also crucial for bacterial replication (**Fig. 19**). Nevertheless – in this case like in all other cases –, I do not think that the bacterial replication itself is inhibited, but rather the phagosomal escape. To prove this, an escape assay in the presence of an siRNA targeted against HMGCS1 must be performed.

4.2 Summary of the screening results

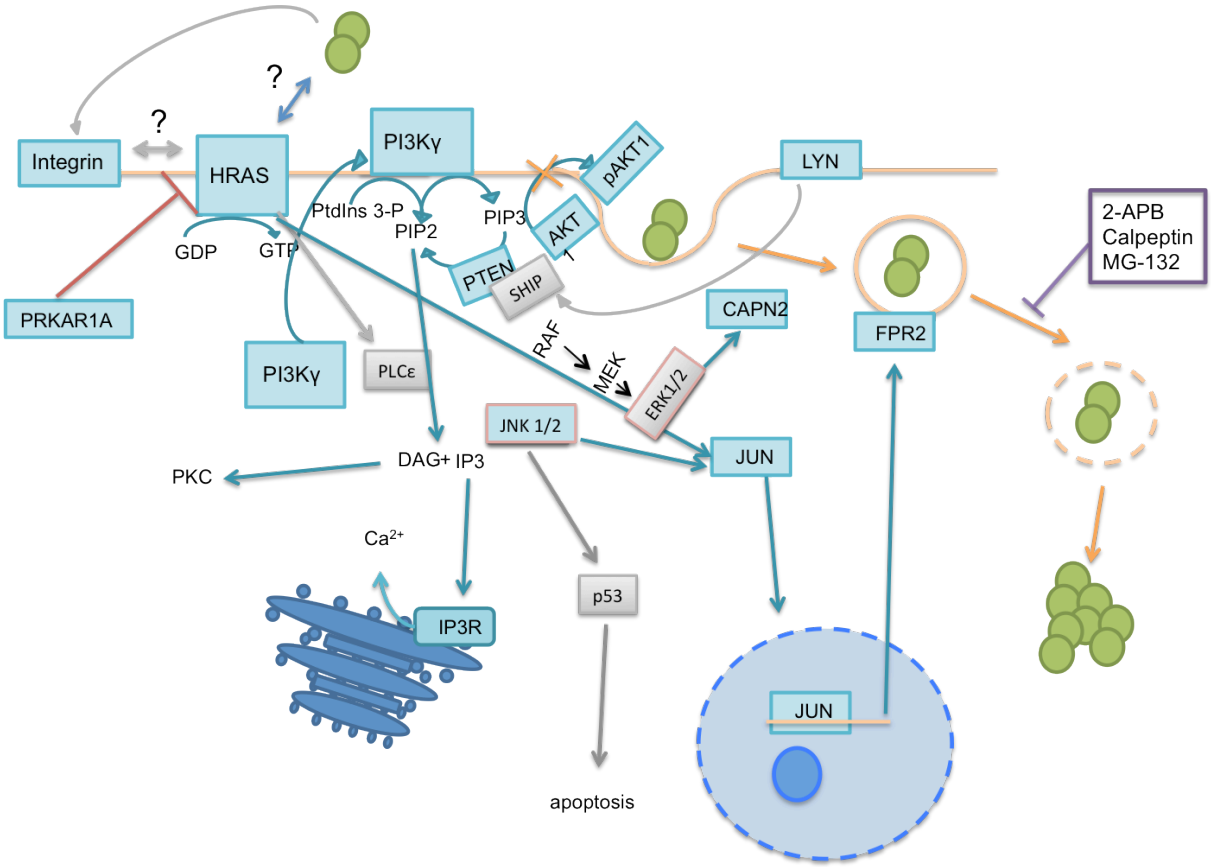

Figure 43 Model of intracellular signalling induced by *S. aureus* infection. Proteins in petrol boxes have been investigated in this study and shown to be involved in *S. aureus* infection since a knockdown via siRNA reduced invasion rate and/or intracellular replication. For ERK1/2 and JNK1/2 phosphorylation during *S. aureus* infection has already been shown. HRAS can induce RAF/MEK/ERK signalling resulting in JUN activation. JUN is shown to be important for bacterial replication, but as I assume, rather for phagosomal escape. In addition, ERK can phosphorylate and activate CAPN2, necessary for bacterial invasion. HRAS in addition activates PI3Kγ, which produces PIP2 and PIP3. This can lead to pAKT1, which is surprisingly not induced during *S. aureus* infection but might be the reason for host cell death. PIP2 can be cleaved by PLC to DAG and IP3, whereby IP3 can activate the IP3-receptors at the ER. The effects of 2-APB indicate a further involvement of the IP3-receptors. A direct activation of PLCε by HRAS has been shown earlier. JUN, presumably in combination with additional factors, could induce FPR2 and ITGAM expression. Beside the dephosphorylation of AKT, also JNK activation can induce host cell death. PRKAR1A has been shown to inhibit HRAS signalling and seems to be the antagonist of HRAS signalling, necessary for bacterial intracellular replication. A knockdown of PRKAR1A increases intracellular bacterial replication, whereas a HRAS knockdown decreases it. See further information in the text.

The data presented in this study suggest a direct or indirect (via integrins) interaction between *S. aureus* and HRAS, which results in the activation of HRAS. The importance of HRAS during *S. aureus* infection was shown by the siRNA studies (**Fig. 18+19**). An involvement of HRAS is further supported by the observation that a lot of downstream effectors of HRAS were identified with the shRNA and siRNA screens. A well-established signalling cascade is the HRAS/Raf/MEK/ERK cascade. ERK 1 and 2 are both phosphorylated during *S. aureus* infection. (Ellington et al., 2001) And they can phosphorylate JUN and therefore activate it (Pulverer et al., 1991). ERK can also activate CAPN2

(Meng et al., 2009). This study shows that both factors, JUN and CAPN2, are involved in *S. aureus* infection (**Fig. 15-19**). In addition, JUN can be activated by JNK and activated JUN might be involved in the induction of FPR2 expression. This work shows that a JNK inhibitor reduces bacterial invasion (**Fig. 13B**). Further, a knockdown of JNK1 (MAPK8) seems to reduce host cell death, since the annexin assays performed with the siRNA knockdown cells showed that of all siRNAs used, only the one against JNK1 was able to show less annexin staining than control infection. Since none of the experiments showed a significant reduction of annexin binding, data are not shown here. However, the fact that only JNK was identified is an interesting observation since JNK can induce apoptosis via p53 (Oleinik et al., 2007). In addition, JNK activates cytochrome c release of mitochondria in ultraviolet (UV) rays induced apoptosis (Tournier et al., 2000). Therefore JNK1 could be responsible for host cell death induced by *S. aureus*. However, the data shown here, suggest that not only one single factor induces cell death, but rather a complex signalling cascade including JNK1.

Coming back to HRAS, a second target of it is PI3Kγ, which can generate PIP2 and PIP3. PIP2 can be hydrolysed to IP3 and DAG via PLCs and induces calcium signalling. PIP3 is an anchor for AKT1, which is phosphorylated at the plasma membrane (cf. introduction). Unexpectedly, AKT phosphorylation decreased during *S. aureus* infection (**Fig. 28**). And since this is an anti pro-survival signal, this might be one process involved in cell death induction.

Intracellular replication is increased in cells with an siRNA knockdown of PRKAR1A and decreased during a knockdown of HRAS. In contrast, both knockdowns showed reduced bacterial invasion. Therefore it is likely that HRAS plays a role during both important steps: invasion and intracellular replication. And the second step is antagonised by PKA.

The shRNA screen generated a large quantity of data and thus could give only initial indications. Therefore it is a work-intensive method, with moderate output. For the subsequent siRNA screen, robust screening methods were critical for the success. In addition, not only factors directly identified with the shRNA screen but also factors connected with them had to be examined. Consequently, an shRNA screen should be repeated at least 3-4 times and needs an exhaustive validation. But is a good method to get a first impression of factors involved in the investigated process. Nevertheless, RNASeq experiments or phosphoproteomics could be valuable alternative methods, which can also be continued by siRNA knockdown experiments.

4.3 2-APB inhibits *S. aureus* induced host cell death

The in silico analysis of the shRNA screen was done with three different methods, first a KEGG pathway analysis (data not shown), second a GO classification of the hits with WebGestalt (**Fig. 11+12**) and third a STRING pathway analysis (**Fig. 9+10**).

All three methods, even tough very different in their approach, suggested an important role of calcium during *S. aureus* infection. Calcium is an important second messenger in eukaryotic cells, and the pore-forming toxins of *S. aureus* have already been linked to calcium signalling. One of these toxins is the γ-leucotoxin HlgC/HlgB (Jover et al., 2013). It triggers a multi-step process in which initially CD38 is activated and NAADP is synthesised. NAAPD thereafter activates the calcium channels of the endolysosomal compartments resulting in an increase of the cytosolic calcium concentration. This activates RYR and IP3 receptors, which further increase the cytosolic calcium concentration. Finally, the STIM-Orai complex is activated. This whole process is however independent of the pore formation of the HlgC/HlgB leucotoxin. Instead, a complex host cell process in neurone cells is activated by leucotoxin (Jover et al., 2013).

Since the screen suggested an important role of calcium, I was interested if a process like this could also take place in HeLa cells. For the prevention of calcium release from the endoplasmic reticulum (ER) I used 2-APB and was able to inhibit host cell death normally induced by *S. aureus* infection (**Fig. 24**). HeLa cells did neither round up nor detach from the surface and intracellular replication was inhibited (**Fig. 24+25**). Phagosomal escape was also inhibited, which confirms the hypothesis that phagosomal escape is a prerequisite for intracellular replication (**Fig. 26**). Therefore indeed, the prevention of the ER calcium release has a crucial effect on *S. aureus* infection. The bacterial invasion rate was reduced concentration dependent, but this reduction did not have any effect on the following processes. Even if cells were infected with two fold bacteria and consequently were higher infected than control cells, downstream events like phagosomal escape, intracellular replication and cell death induction were inhibited (**Fig. 22**). These data suggest that calcium plays a role during the two important steps of infection, invasion and phagosomal escape. Since these processes are distinctly separated in time, the question is, if both signals are connected or if two independent events cause two different calcium signals. One possibility could be, that one bacterial toxin, like the γ-leucotoxin HlgC/HlgB is responsible for the first calcium signal resulting in the internalization of bacteria, whereas another bacterial toxin induces a second calcium signal from within the cell. This seems very likely since bacterial gene expression alters greatly 2hrs and 6hrs p.i. and therefore different toxins are expressed (Garzoni et al., 2007). In addition, studies with the calpain inhibitor ALLN (see next chapter) showed that calpains are active approximately 2hrs p.i. (**Fig. 30**). This also indicates that two distinct calcium signals are generated, since it is unlikely that the calcium signal inhibiting invasion is still active more than 1h later. One possible mechanism for the second calcium

signal might be cytochrome c. It can bind to IP3 receptors after release from the mitochondria and increase their activity (Boehning et al., 2003). The release of cytochrome c during *S. aureus* infection is not unlikely since the mitochondrial membrane potential decreases during infection (**Fig. 39A**). The inhibition of *S. aureus* induced cell death with 2-APB reveals similarities between *S. aureus* and group A Streptococcus (GAS) infections, since also GAS induced cell death was preventable with 2-APB (Cywes Bentley et al., 2005). As 2-APB inhibits all important steps of *S. aureus* infection we used it in a sepsis mouse model to determine the influence in vivo (**Fig. 27**). Although the rescued CFU did not show a significant difference between control and 2-APB treated group, some of the mice treated with 2-APB do show a drastically reduced bacterial load. Hence, calcium signalling is a promising new target for drug development.

4.4 Calpains play opposing roles during *S. aureus* invasion and further infection

Of all the factors involved in *S. aureus* infection and were identified in this study, calpains were investigated most extensively. Beside experiments with three different calpain inhibitors (ALLN, calpeptin and PD150606) several different siRNA knockdown experiments were performed. In all experiments performed with inhibitors, bacterial invasion rate was not changed (data not shown). In contrast, siRNA knockdown of CAPN2 or CAPN4 reduced the relative *S. aureus* invasion (rel. invasion) significantly (**Fig. 17**). Interestingly, the knockdown of CAPN1 increased the invasion rate compared to control infection and a knockdown of both calpains restored invasion back to control level (**Fig. 35**). At a first glance this seems plausible, since all calpain inhibitors inhibit both calpains and therefore they do not alter the invasion rate. However, a knockdown of CAPN4 should in principle have the same effect than a double knockout of CAPN1 and CAPN2 since both large subunits use the small subunit CAPN4 to form the active heterodimer. One reason for the different results could be an additional role of CAPN4 beside the interaction with CAPN1 and CAPN2. If this is the case, the calpain inhibitors might not be able to inhibit this additional role of CAPN4, since they bind to the catalytic and not the regulatory subunit. Another reason could be that CAPN1 has a stronger affinity to CAPN4 than CAPN2 and therefore binds the small amount of CAPN4 that is available in siRNA knockdown cells. This would result in a phenotype of a CAPN2 single knockdown. Other explanations could be, that the calpain inhibitors have grave side effects or cannot reduce calpain activity down to 0% and therefore do not show the same result than the siRNA experiments.

As for the second important step – phagosomal escape – calpeptin was the only inhibitor, which was able to prevent it (**Fig. 34**). The same was true for intracellular replication (**Fig. 33**). The knockdown of CAPN4 by siRNAs reduced replication significantly. In contrast, CAPN1 and CAPN2 knockdown increased intracellular replication. Here, the CAPN4 knockdown showed the same results like the calpeptin inhibitor experiments, confirming the idea that the inhibitor treatment has the same effect than a double knockdown, since all inhibitors inhibit CAPN1 and CAPN2. However, the double

knockdown of CAPN1 and CAPN2 did not show a decrease in intracellular replication but rather a slight increase compared to the control infection (data not shown).

Therefore, again, the double knockdown of CAPN1 and CAPN2 has different effects than a CAPN4 knockdown. A CAPN4 knockdown has the opposite effect than the calpain inhibitors in case of invasion but has the same effect like calpeptin in case of intracellular replication. The double knockdown of CAPN1 and CAPN2 shows in both cases a different phenotype than CAPN4 knockdown does.

But not only the siRNA knockdowns show contradictory results, also the inhibitors have different effects. This is partly explainable by the different affinities for their targets. Calpeptin has the highest affinity for both CAPN1 and CAPN2. This is the inhibitor, which showed the strongest effect on *S. aureus* infection. It inhibits all important steps of the infection. ALLN showed less affinity but still a higher one than PD150606. Including the observation that calpeptin and ALLN both inhibited rounding up and host cell detachment it seems very likely that the affinity of the different inhibitors plays a role for the different potentials inhibiting the important steps of *S. aureus* infection. Interestingly, none of the inhibitors was able to prevent annexin staining. This is a big difference to 2-APB, which was able to prevent this hallmark of *S. aureus* infection, too.

Table 13 Summary of calpain results. "n.t." means not tested. "="means unaltered. "-" means decreased. "+" means increased.

	Calpeptin	ALLN	PD150606	siCAPN1	siCAPN2	siCAPN4	siCAPN1+2
Invasion	=	=	=	+	-	-	=
Escape	-	=	=	n.t.	n.t.	n.t.	n.t.
Replication	-	=	=	+	+	-	+
Cell detachment	-	-	=	n.t.	n.t.	n.t.	n.t.

To sum up, CAPN2 was identified as an additional, so far unknown factor, supporting *S. aureus* invasion into HeLa cells. Furthermore, the knockdown of CAPN4 resulted in the reduction of intracellular bacterial replication. The inhibitor studies confirmed this additional role of calpains during *S. aureus* infection. However, different affinities of the inhibitors made it difficult to clearly demonstrate the exact role of calpains during *S. aureus* infection. The next steps in this direction could be the creation of stable shRNA knockdown cell lines targeting all different calpains. Then, pull down experiments for the identification of direct calpain targets should be performed.

4.5 Involvement of the proteasome during *S. aureus* infection

Calpain inhibitors can, to some extent, inhibit the proteasome as well. Furthermore, PSMA1, a subunit of the proteasome, was a top hit in the shRNA screen, for invasion as well as for cell death relevant factors. Therefore, the effect of the proteasome inhibitor MG-132 was investigated. Like 2-APB and calpeptin the proteasome inhibitor MG-132 inhibited intracellular replication but had no direct bacteriostatic or bacteriotoxic effect (**Fig. 36B**). In addition, the escape rate was reduced in the

presence of MG-132. It is important to mention that ethanol accelerates escape drastically, since as early as 1h p.i. a marked escape signal is already detectable. Since MG-132 is solved in ethanol, here, an earlier escape signal is measurable comparing to the other inhibitors inhibiting escape. The comparison of ethanol to MG-132 treated samples however clearly shows that escape is slowed down (**Fig. 37**). Nevertheless, in contrast to all other inhibitors, which inhibited phagosomal escape, in case of MG-132 it cannot be excluded that bacterial replication is slowed down. Live cell imaging has to be performed to investigate if the intracellular replication is decelerated or not. If this is the case, one explanation could be that *S. aureus* infection increases proteasomal degradation to have access to small peptides, which serve as an amino acid source for the bacteria.

MG-132 can also block host cell death, indicated by the lack of cell rounding and detachment of cells (**Fig. 37**). Since MG-132 also inhibits, to some extent, calpains (**Table 12**), the ubiquitination level during infection was monitored, in order to find out, if the ubiquitination pattern changes during *S. aureus* infection. 0.5h and 1h p.i. the over-all ubiquitination is clearly increased (**Fig. 38A**). A control sample, which was treated with MG-132 showed that blocking the proteasome has the same effect (**Fig. 38B**). The proteasome degrades proteins, which were tagged by ubiquitin. Since ubiquitination is increased 1h p.i. , it could either be that protein ubiquitination is elevated or that proteasomal degradation decreased. Moreover, the ubiquitination can also have other functions than proteasomal degradation, but since the proteasome inhibitor shows a clear effect on *S. aureus* infection, the proteasome function is definitively important for *S. aureus* infection. Therefore the increased ubiquitination signal is very likely a signal for increased proteasomal degradation.

To sum up, *S. aureus* infection needs an intact proteasomal degradation process and seems to increase ubiquitination of different host cell proteins. Here, a pull down assay with subsequent mass spectrometry analysis of the isolated proteins (or a western blot analysis for investigation of proteins like IκB), would be the next step in order to specifically identify ubiquitinated host cell proteins.

4.6 *S. aureus* infection induces mitochondrial membrane potential loss

Mitochondria play an important role during cell death. In addition, they are, like the endoplasmic reticulum (ER), major players in the calcium homeostasis of the cell. An increase of cytosolic calcium can among others lead to a reversible mitochondrial membrane potential loss (MMPL) (Carafoli, 2002).

The shRNA screen identified mitochondrial proteins and thus proposed an involvement of mitochondria during *S. aureus* infection (**Table 15+16**). Therefore, I was interested if the mitochondrial membrane potential (MMP) changes during *S. aureus* infection. The MMP of HeLa cells decreased substantially after *S. aureus* infection (**Fig. 39**). This decrease is comparable to the one induced by the uncoupler CCCP (Carbonyl cyanide *m*-chlorophenyl hydrazone). In contrast, the non-cytotoxic *S. aureus* strain CowanI does not reduce the MMP. Interestingly, the two MOMP

blockers cyclosporinA and bongkrek acid were not able to rescue the loss (Connern and Halestrap, 1992; Crompton et al., 1988; Klingenberg et al., 1970). In contrast, CGP37157 an inhibitor of the mitochondrial Na^{+}/Ca^{2+} exchanger (mNCX), rescued MMPL significantly (**Fig. 40B**), indicating an involvement of ion flows in MMPL.

Another interesting finding is that cells treated with MG-132 can rescue their MMP 4hrs p.i. (**Fig. 41**). This suggests, that the MMPL is reversible and not the reason for *S. aureus* induced host cell death. This hypothesis is also supported by the fact, that infected cells treated with CGP37157 die, too. The next question, which has to be answered, is, whether the MMPL is a result of mitochondrial calcium overload. In order to find this out, measurements of mitochondrial calcium concentrations during *S. aureus* infections should be performed. In addition, the question whether *S. aureus* proteins are involved in this process should be answered. Since *S. aureus* produces a lot of pore-forming toxins, a direct pore-formation in the mitochondrial membrane is conceivable. I already started to work on the second question and used the Nebraska library to test some *S. aureus* USA300 Lac mutants, but none of them was able to prevent the MMPL (data not shown).

4.7 Conclusions and outlook

The effects of the different inhibitors identified in this study strongly confirm that *S. aureus* invades host cells, escapes the phagosome and then replicates within the cytosol. Finally, the host cells die.

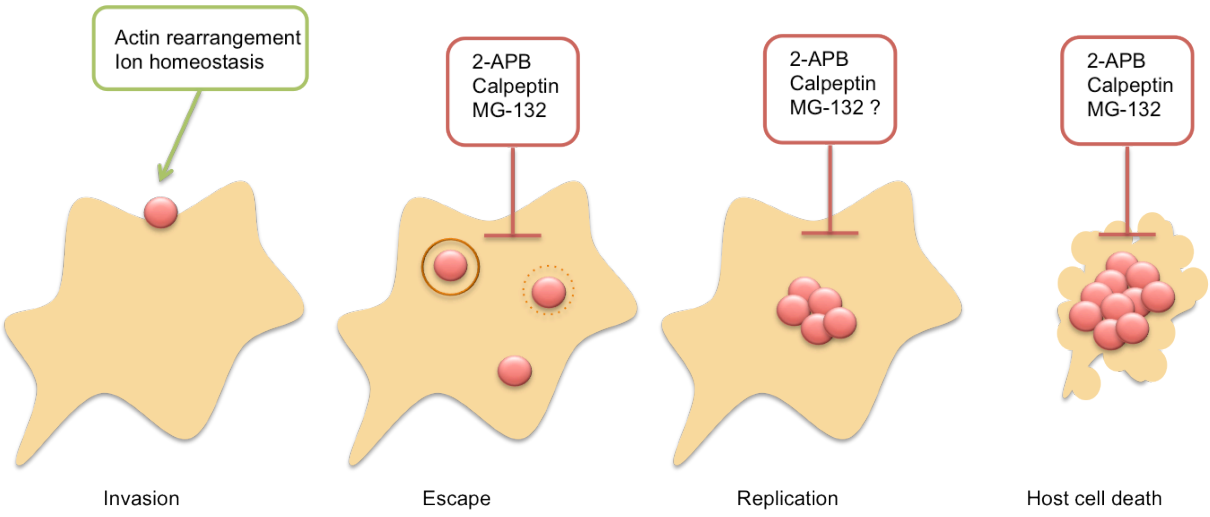

Figure 44 The four steps of a *S. aureus* 6850 infection of HeLa cells. First, *S. aureus* invades the human host cell with the help of the actin cytoskeleton. The ion homeostasis of the cell is also involved. The second step is phagosomal escape. This step can be inhibited by an IP3R inhibitor (2-APB), a calpain inhibitor (calpeptin) and the proteasome inhibitor MG-132. The third and fourth steps are bacterial replication and host cell death, which both are also inhibited by 2-APB, calpeptin and MG-132.

Every single step of this *S. aureus* infection is a complex, highly regulated, process. The invasion needs more than fibronectin to integrin binding. It needs a signalling process, in which numerous different host cell proteins are involved. This was revealed by the siRNA experiments performed in this study.

Phagosomal escape, the second step, mandatorily depends on the presence of specific host cell proteins, since different inhibitors (2-APB, MG-132 and calpeptin) reduced phagosomal escape rate. Phagosomal escape is not just dependent on PSMAα (Grosz et al., 2014), but it is also dependent on LukAB, another bacterial factor already linked to bacterial escape and subsequent killing of neutrophils (DuMont et al., 2013). In addition, this study shows that also in HeLa cells, host cell factors are involved in escape. The two known receptors ITGAM (CD11b) for LukAB and FPR2 for PSMα are involved. These receptors are slightly or not expressed in HeLa and HUVEC cells under normal conditions but are upregulated after *S. aureus* infection. The importance of this upregulation was demonstrated by the knockdown of FPR2 with siRNAs. Here, intracellular bacterial replication is significantly reduced (**Fig. 18+19**). In addition, the treatment of cells with 2-APB inhibits the upregulation of the two receptors (**app. Fig. 45C**), raising the question, if the effect of 2-APB is predominantly based on the inhibition of the upregulation of these genes. A second question in this context is, which processes, responsible for the upregulation of FPR2 and ITGAM, are inhibited by 2-APB.

As a consequence, phagosomal escape is not just a result of ordinary perforation of the phagosomal membrane, but rather a highly regulated process in which the eukaryotic host cell plays a crucial role as well. Further studies are necessary to understand the transcriptional regulation of ITGAM and FPR2 as well as the involvement of other host cell factors involved in this process. Here of course, the transcription factor JUN needs to be investigated in more detail, since the siRNA screen identified it to be a crucial player during infection.

Since intracellular replication was never reliably inhibited after bacteria have escaped, it seems that once *S. aureus* is in the cytosol, bacterial replication is not preventable anymore and therefore the bacteria can spread at ease. This is precisely why the inhibitors identified in this study are so precious; they inhibit phagosomal escape as the crucial step of *S. aureus* infection, which normally allows *S. aureus* to replicate within the host.

The factors identified in this study have to be investigated in more detail, as it is mentioned in the corresponding chapters. The screens revealed totally new insights into the host pathogen interactions between *S. aureus* and human cells and thus are an excellent fundament upon which the *S. aureus* infection can be investigated into more detail from a new perspective.

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5 Appendix

5.1 Control qRT-PCR results

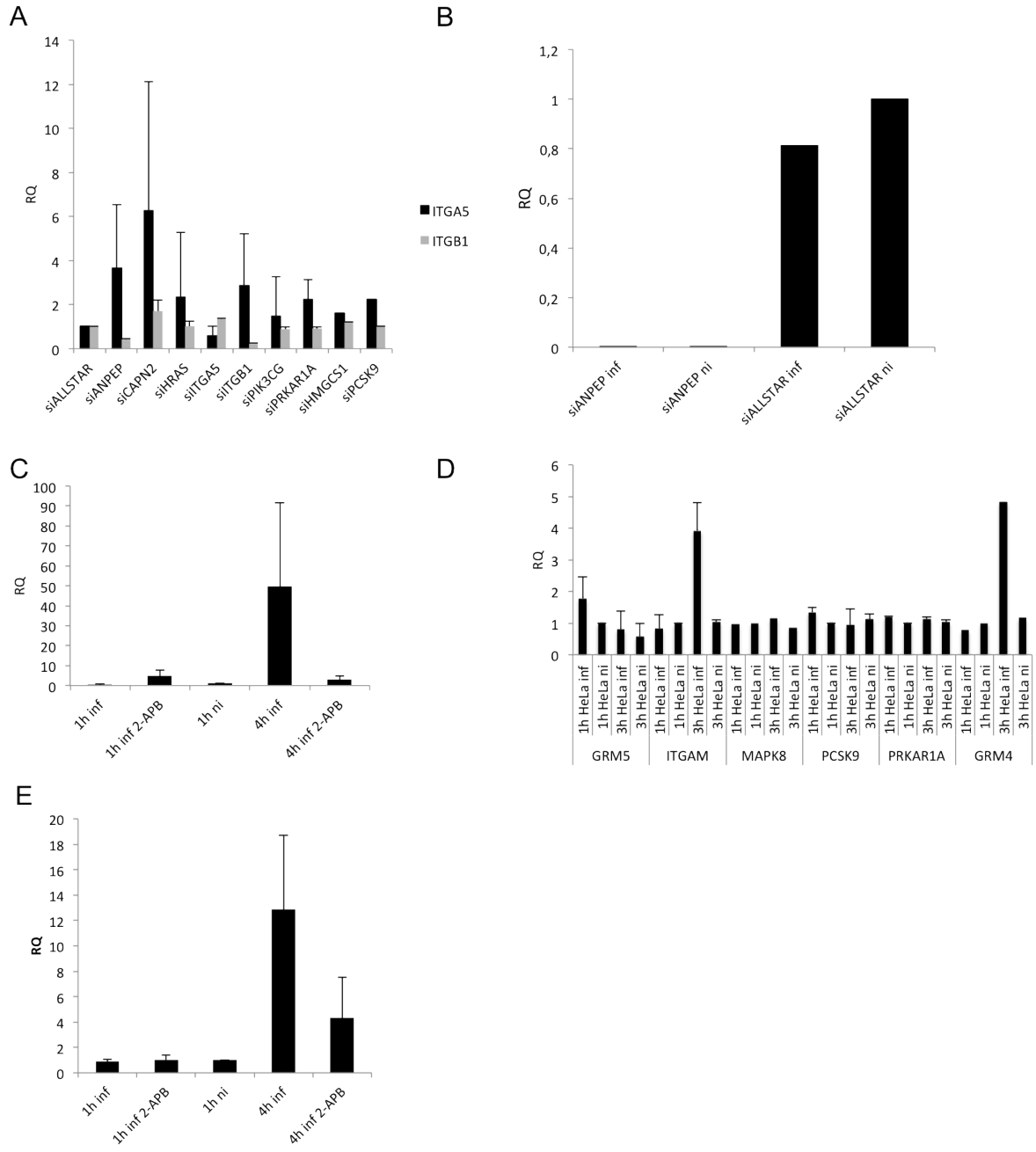

Figure 45 qRT-PCR results. (A) siRNA knockdown cells were tested for ITGB1 and ITGA5 expression. Two independent experiments were performed. (B) Knockdown of ANPEP after siRNA transfection was monitored. One experiment was performed. (C) FPR2 expression after *S. aureus* infection ± 2-APB was measured. Three independent experiments were performed. (D) Expression of different genes, which were hits of the shRNA screen, was measured. Two independent experiments were performed. (E) ITGAM expression after *S. aureus* infection ± 2-APB was measured. Three independent experiments were performed.

5.2 Abbreviations

Table 14 Abbreviations. Gene names are not listed here.

2-APB	2-aminoethoxydiphenyl borate
AC	Adenylate cyclase
ALLN	Acetyl-L-leucyl-L-leucyl-L-norleucine
ANT	Adenine nucleotide translocator
APC	Allophycocyanin
ATP	Adenosine triphosphate
B. acid	Bongkrek acid
CA	Community-acquired
CAV1	Caveolin 1
CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
CFU	Colony forming unit
CsA	CyclosporinA
CWT	C-terminal cell wall-targeting domain
CyP-D	CyclophilinD
CytC	Cytochrome c
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
Eap	Extracellular adherence protein
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FN	Fibronectin
FnBP	Fibronectin binding proteins
FSC	Forward scatter
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
h	Hour
HA	Hospital-acquired
HeLa	Henrietta Lacks
HMC	Human mesothelial cells
hrs	Hours
IP3	Inositol triphosphate
IP3R	IP3 receptors
LB	Lysogeny broth
LTA	Lipoteichoic acid
MAC	Memebrane attack complex of the complement
MAC-T cells	Mammary alveolar cells
Mcl-1	myeloid leukemia cell differentiation protein
MCU	Mitochondrial calcium uniporter
min	Minutes
MMP	Mitochondrial membrane potential
MMPL	Mitochondrial membrane potential loss
mNCX	Mitochondrial Na^+/Ca^{2+} exchanger
MOI	Multiplicity of infection
MOMP	Mitochondrial outer membrane permeabilisation
mRFP	Monomeric red fluorescent protein
MRSA	Methicillin resistant Staphylococcus aureus
MβC	Methyl-β-cyclodextrin
N. meningitidis	*Neisseria meningitidis*
NFκB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
nm	Nanometre

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PGN	Peptidoglycan
PI	Phosphatidylinositol
PI3K	Phosphoinositide-3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PLC	Phospholipase c
PT	Permeability transition
PtdIns	Phosphatidylinositol
PTK	Protein tyrosine kinase
qRT-PCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RYR	Ryanodine receptors
S. aureus	*Staphylococcus aureus*
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SSC	Side scatter
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TLR2	Toll like receptor 2
TSB	Tryptic soy broth
VDAC	Voltage-dependent anion channel
WebGestalt	WEB-based GEne SeT AnaLysis Toolkit
WHO	World health organization
YFP	Yellow fluorescent protein

5.3 Tables containing shRNAscreen results

Table 15 Invasion relevant genes at least 3 fold enriched and part of the STRING pathway.

gene symbol	gene name	fold change
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	7.1
ABCB7	ATP-binding cassette, sub-family B (MDR/TAP), member 7	3.3
ABCD3	ATP-binding cassette, sub-family D (ALD), member 3	3.5
ACTR1A	ARP1 actin-related protein 1 homolog A, centractin alpha (yeast)	3.1
ADM	Adrenomedullin	3.2
ANTXR1	anthrax toxin receptor 1	3.1
AP2S1	adaptor-related protein complex 2, sigma 1 subunit	18.4
ARHGEF17	Rho guanine nucleotide exchange factor (GEF) 17	3.1
AZIN1	antizyme inhibitor 1	4.3
BAZ1B	bromodomain adjacent to zinc finger domain, 1B	*
C14orf166	chromosome 14 open reading frame 166	4.7
CASP3	caspase 3, apoptosis-related cysteine peptidase	4.3
CASP4	caspase 4, apoptosis-related cysteine peptidase	3.2
CASP7	caspase 7, apoptosis-related cysteine peptidase	5.1
CASP8AP2	caspase 8 associated protein 2	22.7
CCNT2	cyclin T2	3.3
CCR1	chemokine (C-C motif) receptor 1	4.2
CD1D	CD1d molecule	10.0
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	4.0
CDC26	cell division cycle 26	8.9
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	9.7
CETP	cholesteryl ester transfer protein, plasma	3.9
CREG1	cellular repressor of E1A-stimulated genes 1	3.7
CYSLTR1	cysteinyl leukotriene receptor 1	3.9
DBF4	DBF4 homolog (S. cerevisiae)	3.7

DDX47	DEAD (Asp-Glu-Ala-Asp) box polypeptide 47	5.1
DFFA	DNA fragmentation factor, 45kDa, alpha polypeptide	3.5
DLG1	discs, large homolog 1 (Drosophila)	4.2
DUSP12	dual specificity phosphatase 12	3.1
EBNA1BP2	EBNA1 binding protein 2	3.6
ECHDC1	enoyl CoA hydratase domain containing 1	*
ELP2	elongator acetyltransferase complex subunit 2	3.4
ENSG00000085231	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kDa	3.1
FBXL7	F-box and leucine-rich repeat protein 7	3.9
FBXW11	F-box and WD repeat domain containing 11	9.5 + 5.8
FBXW8	F-box and WD repeat domain containing 8	14.8
FTL	ferritin, light polypeptide	3.2
FZD4	frizzled family receptor 4	4.5
GGPS1	geranylgeranyl diphosphate synthase 1	5.8
GNA14	guanine nucleotide binding protein (G protein), alpha 14	4.4
GPER	G protein-coupled estrogen receptor 1	3.5
GRIK1	glutamate receptor, ionotropic, kainate 1	4.0
GRM5	glutamate receptor, metabotropic 5	3.9
HAP1	huntingtin-associated protein 1	3.4
IDI1	isopentenyl-diphosphate delta isomerase 1	3.1
IFNAR1	interferon (alpha, beta and omega) receptor 1	*
IKBKB	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta	4.3
ITK	IL2-inducible T-cell kinase	5.6
KIF17	kinesin family member 17	*
KIF20B	kinesin family member 20B	7.1
KLF8	Kruppel-like factor 8	5.4
LDLCQ3	HMGCR 3-hydroxy-3-methylglutaryl-CoA reductase	4.2
LNPEP	leucyl/cystinyl aminopeptidase	3.4
LYZ	lysozyme	3.1
MAP3K5	mitogen-activated protein kinase kinase kinase 5	8.6
MAPK9	mitogen-activated protein kinase 9	3.1
MED11	mediator complex subunit 11	5.7
MED13L	mediator complex subunit 13-like	3.9
MED4	mediator complex subunit 4	5.9
MIER3	mesoderm induction early response 1, family member 3	10.7
MSH6	mutS homolog 6 (E. coli)	3.4
MYF5	myogenic factor 5	5.0
NEDD4L	neural precursor cell expressed, developmentally down-regulated 4-like, E3 ubiquitin protein ligase	3.2
NOLC1	nucleolar and coiled-body phosphoprotein 1	3.6
NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	3.5
OXCT2	3-oxoacid CoA transferase 2	9.1
P2RY12	purinergic receptor P2Y, G-protein coupled, 12	8.9
PANX1	pannexin 1	3.4
PKN1	protein kinase N1	17.7
PLXNB3	plexin B3	3.8
POLR2G	polymerase (RNA) II (DNA directed) polypeptide G	3.8
PSMA1	proteasome subunit alpha type-1 isoform 3	20.7 + 15.0
PTHLH	parathyroid hormone-like hormone	3.2
PUS7	pseudouridylate synthase 7 homolog (S. cerevisiae)	4.3
RAB1A	RAB1A, member RAS oncogene family	3.4
RNF11	ring finger protein 11	3.0
RNF138	ring finger protein 138, E3 ubiquitin protein ligase	*
RPL27	ribosomal protein L27	3.7
RPS6KA3	ribosomal protein S6 kinase, 90kDa, polypeptide 3	3.2

RSL1D1	ribosomal L1 domain containing 1	*
SCP2	sterol carrier protein 2	6.4
SHMT2	serine hydroxymethyltransferase 2 (mitochondrial)	16.5
SMC2	structural maintenance of chromosomes 2	3.4
SRF	serum response factor (c-fos serum response element-binding transcription factor)	3.3
SRPRB	signal recognition particle receptor, B subunit	7.3
STRAP	serine/threonine kinase receptor associated protein	3.8
SUMF2	sulfatase modifying factor 2	3.1
TAF1A	TATA box binding protein (TBP)-associated factor, RNA polymerase I, A, 48kDa	3.1
TAF2	TAF2 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 150kDa	6.4
TBP	TATA box binding protein	5.6
TFR2	transferrin receptor 2	7.3
THOC7	THO complex 7 homolog (Drosophila)	3.8
TIA1	TIA1 cytotoxic granule-associated RNA binding protein	18.5
TIMM9	translocase of inner mitochondrial membrane 9 homolog (yeast)	3.2
TNFSF13	tumour necrosis factor (ligand) superfamily, member 13	7.3
TOX3	TOX high mobility group box family member 3	*
URM1	ubiquitin related modifier 1	3.1
USP1	ubiquitin specific peptidase 1	3.1
UTRN	Utrophin	4.7
VIP	vasoactive intestinal peptide	12.7
WDR3	WD repeat domain 3	5.3
WIPF2	WAS/WASL interacting protein family, member 2	3.2
XPO1	exportin 1 (CRM1 homolog, yeast)	6.3
ZAP70	zeta-chain (TCR) associated protein kinase 70kDa	3.2

*bioinformatics analysis found this gene name for the corresponding hit but detailed analysis could not confirm this attribution

Table 16 Cell death associated genes at least 4 fold enriched and not enriched (>4fold) in invasion pathway. Genes which are not connected in the STRING pathway are not shown.

gene symbol	gene name	fold change
ACCN3	Acid-Sensing (Proton-Gated) Ion Channel 3	35.5
ADCY9	adenylate cyclase 9	15.2
ALDH1B1	aldehyde dehydrogenase 1 family, member B1	4.7
ARHGAP15	Rho GTPase activating protein 15	4.2
ARHGEF17	Rho guanine nucleotide exchange factor (GEF) 17	4.3
ARHGEF9	Cdc42 guanine nucleotide exchange factor (GEF) 9	11.5
BANF2	barrier to autointegration factor 2	8.3
BCL2	B-cell CLL/lymphoma 2	4.5
BTG2	BTG family, member 2	4.6
C12orf10	chromosome 12 open reading frame 10	4.9
CD27	CD27 molecule	4.2
CDCA2	cell division cycle associated 2	10.0
CDCA7	cell division cycle associated 7	4.5
CENPL	centromere protein L	*
CETP	cholesteryl ester transfer protein, plasma	14.9
CHD1	chromodomain helicase DNA binding protein 1	4.9
CLEC4E	C-type lectin domain family 4, member E	20.0
CNOT8	CCR4-NOT transcription complex, subunit 8	8.0
CPNE1	copine I	4.9
CRABP1	cellular retinoic acid binding protein 1	5.4
CXCL5	chemokine (C-X-C motif) ligand 5	5.0

CYP4A11	cytochrome P450, family 4, subfamily A, polypeptide 11	5.6
DDX18	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	5.8
DHX38	DEAH (Asp-Glu-Ala-His) box polypeptide 38	12.8
DOCK9	dedicator of cytokinesis 9	12.9
DPYD	dihydropyrimidine dehydrogenase	5.0
ELP2	elongator acetyltransferase complex subunit 2	10.0
ELP3	elongator acetyltransferase complex subunit 3	5.0
EXPH5	exophilin 5	15.5
FPR1	formyl peptide receptor 1	6.2
GALR2	galanin receptor 2	5.6
GIMAP4	GTPase, IMAP family member 4	7.3
GLUL	glutamate-ammonia ligase	5.1
GPC1	glypican 1	4.8
GPER	G protein-coupled estrogen receptor 1	4.4
GRHL1	grainyhead-like 1 (Drosophila)	5.1
GZMK	granzyme K (granzyme 3; tryptase II)	4.6
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)	7.3
HSPA1L	heat shock 70kDa protein 1-like	6.1
HSPD1	heat shock 60kDa protein 1 (chaperonin)	7.3
IDI1	isopentenyl-diphosphate delta isomerase 1	4.2
IL13RA2	interleukin 13 receptor, alpha 2	4.0
IREB2	iron-responsive element binding protein 2	4.2
ITM2B	integral membrane protein 2B	5.5
ITPRIP	inositol 1,4,5-trisphosphate receptor interacting protein	6.1
JAK1	Janus kinase 1	24.1
KEAP1	kelch-like ECH-associated protein 1	*
MAP3K11	mitogen-activated protein kinase kinase kinase 11	6.7
MAPK8	mitogen-activated protein kinase 8	4.3
MARCH1	membrane-associated ring finger (C3HC4) 1, E3 ubiquitin protein ligase	14.4
MPEG1	macrophage expressed 1	
MPO	myeloperoxidase	4.3
MRPL23	mitochondrial ribosomal protein L23	6.3
NARS	asparaginyl-tRNA synthetase	5.3
NCOR1	nuclear receptor corepressor 1	*
NOLC1	nucleolar and coiled-body phosphoprotein 1	11.4
NOTCH1	notch 1	4.4
NPY1R	neuropeptide Y receptor Y1	17.8
NUDT9	nudix (nucleoside diphosphate linked moiety X)-type motif 9	4.6
NUPL2	nucleoporin like 2	5.4
OGDH	oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	5.0
OR4L1	olfactory receptor, family 4, subfamily L, member 1	5.2
OR5F1	olfactory receptor, family 5, subfamily F, member 1	6.2
OR5M8	olfactory receptor, family 5, subfamily M, member 8	4.7
PCSK9	proprotein convertase subtilisin/kexin type 9	18.0
PDE6B	phosphodiesterase 6B, cGMP-specific, rod, beta	7.0
PIK3CG	phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma	4.5
PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)	5.0
POLR3GL	polymerase (RNA) III (DNA directed) polypeptide G (32kD)-like	7.7
PPARA	peroxisome proliferator-activated receptor alpha	8.2
PPP5C	PPP5C protein phosphatase 5, catalytic subunit	4.8
PRKAR1A	protein kinase, cAMP-dependent, regulatory, type I, alpha	5.6
PRPF18	PRP18 pre-mRNA processing factor 18 homolog (S. cerevisiae)	4.9
RAB3A	RAB3A, member RAS oncogene family	7.5
RALBP1	ralA binding protein 1	4.4
RARS	arginyl-tRNA synthetase	5.6
RGS6	regulator of G-protein signaling 6	4.2

RPH3AL	rabphilin 3A-like (without C2 domains)	4.9
RPL23	ribosomal protein L23	5.6
RPL37A	ribosomal protein L37a	4.1
RPL6	ribosomal protein L6	4.2
RUNX2	runt-related transcription factor 2	10.4
RXRA	retinoid X receptor, alpha	5.8
SEPHS1	selenophosphate synthetase 1	4.7
SKIL	SKI-like oncogene	5.8
SMAD3	SMAD family member 3	4.3
SNRNP40	small nuclear ribonucleoprotein 40kDa (U5)	5.1
SYNJ2BP	synaptojanin 2 binding protein	5.3
SYTL4	synaptotagmin-like 4	4.9
TFCP2	transcription factor CP2	7.4
TRAM1	translocation associated membrane protein 1	5.0
TRIM33	tripartite motif containing 33	15.0
TRPM3	transient receptor potential cation channel, subfamily M, member 3	4.6
TRPM4	transient receptor potential cation channel, subfamily M, member 4	20.1
TRPM8	transient receptor potential cation channel, subfamily M, member 8	4.2
UBE2H	ubiquitin-conjugating enzyme E2H	4.1
URM1	ubiquitin related modifier 1	5.9
WWP1	WW domain containing E3 ubiquitin protein ligase 1	4.8
ZAP70	zeta-chain (TCR) associated protein kinase 70kDa	4.0
ZFYVE27	zinc finger, FYVE domain containing 27	5.3

Table 17 Top hits of the shRNA screen. Gene involved in invasion.

fold change	ID	gene symbol	gene name
77	XM_291375	LOC343018	similar to farnesoid X receptor beta splice variant 1
44	NM_005530	IDH3A	isocitrate dehydrogenase 3 (NAD+) alpha
41	AL832069		DKFZp313K0918
31	AK058070		
31	XM_303632		LOC349917
25	AB002330		KIAA0332
23	NM_012115	CASP8AP2	caspase 8 associated protein 2
23	NM_012068	ATF5	activating transcription factor 5
21	XM_064191	LOC124555	similar to multi-PDZ-domain-containing protein
21	NM_002012	FHIT	fragile histidine triad
21	NM_002786	PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1
20	XM_298465		LOC344989
20	XM_370613		similar to RIKEN, LOC387755
19	NM_025160	WDR26	WD repeat domain 26
19	NM_015705	SGSM3	small G protein signaling modulator 3
19	NM_022037	TIA1	TIA1 cytotoxic granule-associated RNA binding protein
18	NM_021575	AP2S1	adaptor-related protein complex 2, sigma 1 subunit
18	NM_213560	PKN1	protein kinase N1
18	NM_144693	ZNF558	zinc finger protein 558
18	AL832753		DKFZp686A1627
17	XM_296215		LOC341487
17	XM_211900		LOC285447
17	NM_016257	HPCAL4	hippocalcin like 4
16	NM_005412	SHMT2	serine hydroxymethyltransferase 2 (mitochondrial)
16	NM_015910	WDPCP	WD repeat containing planar cell polarity effector
16	XM_301471		similar to hypothetical protein FLJ22611, LOC350045
16	NM_024708	ASB7	ankyrin repeat and SOCS box containing 7
15	NM_032333	FAM213A	family with sequence similarity 213, member A
15	NM_002786	PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1
15	AB023191		KIAA0974

15	NM_012174	FBXW8	F-box and WD repeat domain containing 8
15	NM_152772	TCP11L2	t-complex 11, testis-specific-like 2
15	NM_022083	C1orf24	chromosome 1 open reading frame 24
14	XM_212349		LOC284518
14	XM_298199		LOC344659
14	NM_175570	C21orf32	chromosome 21 open reading frame 32
14	NM_024081	PRRG4	proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)
14	XM_212086		hypothetical protein LOC285941
13	XM_295992		LOC341108
13	AL049999		DKFZp564M182
13	NM_024781	CCDC102B	coiled-coil domain containing 102B
13	NM_015343	CTDNEP1	CTD nuclear envelope phosphatase 1
13	XM_305478		LOC349072
13	NM_003381	VIP	vasoactive intestinal peptide
13	NM_001862	COX5B	cytochrome c oxidase subunit Vb
13	XM_210292		similar to golgi autoantigen, golgin subfamily a, 2; golgin-95, LOC283783
13	NM_015419	MXRA5	matrix-remodelling associated 5
12	NM_145027	KIF6	kinesin family member 6
12	XM_303972		LOC350297
12	NM_031305	ARHGAP24	Rho GTPase activating protein 24
12	XM_297257		LOC343934
12	XM_087298		hypothetical LOC151766
12	NR_001286		processed pseudogene mtTFA 3, LOC260339
12	NM_032842	TMEM209	transmembrane protein 209
12	NM_153711	FAM26E	family with sequence similarity 26, member E
12	XM_305213		LOC351892
12	S77547		potassium ion channel gene
11	XM_103682		LOC150876
11	NM_033412	SLC25A51	solute carrier family 25, member 51
11	XM_302222		similar to ribosomal protein L13a; 60S ribosomal protein L13a; 23 kD highly basic protein, LOC351967
11	NM_022489	INF2	inverted formin, FH2 and WH2 domain containing
11	NM_004390	CTSH	cathepsin H
11	NM_012382	TTC33	tetratricopeptide repeat domain 33
11	NM_152622	MIER3	mesoderm induction early response 1, family member 3
10	XM_305449		LOC352155
10	XM_299667		LOC346696
10	NM_207458	FLJ46026	FLJ46026 protein
10	NM_153447	NLRP5	NLR family, pyrin domain containing 5
10	NM_001766	CD1D	CD1d molecule

Table 18 Top hits of the shRNA screen. Genes involved in cell death.

fold change	ID	gene symbol	gene name
67	NM_016257	HPCAL4	hippocalcin like 4
53	NM_005108	XYLB	xylulokinase homolog (H. influenzae)
47	NM_004968	ICA1	islet cell autoantigen 1, 69kDa
46	XM_291947	HEPHL1	hephaestin-like 1
39	XM_292494		similar to hypothetical protein FLJ10656; cyclin-dependent kinase inhibitor-related protein, LOC339074
38	XM_060143		similar to KIAA1191 protein, LOC126729
37	NM_001862	COX5B	cytochrome c oxidase subunit Vb
36	NM_002138	HNRNPD	heterogeneous nuclear ribonucleoprotein D (AU-rich element RNA binding protein 1, 37kDa)
35	NM_020322	ASIC3	acid-sensing (proton-gated) ion channel 3
34	NM_021575	AP2S1	adaptor-related protein complex 2, sigma 1 subunit

34	XM_301265		similar to double homeobox, 4; double homeobox protein 4, LOC347792
32	XM_304750		LOC352865
30	NM_173503	EFCAB3	EF-hand calcium binding domain 3
29	XM_166432		hypothetical protein LOC221442
27	AK058070		FLJ25341
27	XM_378462		hypothetical gene supported by AK026100, transcript variant 1, FLJ22447
26	NM_024718	RABL6	RAB, member RAS oncogene family-like 6
25	NM_130770	HTR3C	5-hydroxytryptamine (serotonin) receptor 3C, ionotropic
25	NM_146573		Mus musculus olfactory receptor 1002
25	NM_033412	SLC25A51	solute carrier family 25, member 51
25	AK022563		FLJ12501
24	XM_296654		LOC342110
24	NM_002227	JAK1	Janus kinase 1
24	NM_144690	ZNF582	zinc finger protein 582
24	XM_297861		LOC343794
23	NM_015343	CTDNEP1	CTD nuclear envelope phosphatase 1
23	NM_153711	FAM26E	family with sequence similarity 26, member E
23	NM_032728	PPAPDC3	phosphatidic acid phosphatase type 2 domain containing 3
22	XM_297450		LOC344214
21	XM_304419		LOC351268
21	XM_293197		similar to 9930016O13 protein
21	NM_018038		hypothetical protein FLJ10246
21	NM_207336	ZNF467	zinc finger protein 467
21	NM_024081	PRRG4	proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)
21	XM_293982	CUL-1	similar to Cullin homolog 1, LOC345820
20	NM_017636	TRPM4	transient receptor potential cation channel, subfamily M, member 4
20	NM_014358	CLEC4E	C-type lectin domain family 4, member E
20	AL365412		
19	AF322907	NSD1	NSD1
19	XM_375838	TATDN3	TatD DNase domain containing 3, transcript variant 1
19	XM_210306	UDG	similar to Uracil-DNA glycosylase, mitochondrial precursor, LOC283853
19	XM_370613		similar to RIKEN cDNA 3830422K02, LOC387755
19	NM_031305	ARHGAP24	Rho GTPase activating protein 24
18	NM_152779	GLIPR1L1	GLI pathogenesis-related 1 like 1
18	NM_174936	PCSK9	proprotein convertase subtilisin/kexin type 9
18	L07615	NPYY1	Human neuropeptide Y receptor Y1
18	XM_379215		hypothetical protein LOC132241
18	XM_299429		LOC346332
17	NM_001046	SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporter), member 2
17	NM_014014	SNRNP200	small nuclear ribonucleoprotein 200kDa (U5)
17	NM_018503		hypothetical protein PRO1598
17	XM_498399		similar to hypothetical protein, LOC392557
16	NG_000899	VDAC2P1	voltage-dependent anion channel 2 pseudogene 1
16	XM_303336		LOC349542
16	NM_015705	SGSM3	small G protein signaling modulator 3
15	NM_015065	EXPH5	exophilin 5
15	XM_297907		LOC343827
15	NM_016620	ZNF644	zinc finger protein 644
15	NM_001116	ADCY9	adenylate cyclase 9
15	NM_015906	TRIM33	tripartite motif containing 33
15	NM_000078	CETP	cholesteryl ester transfer protein, plasma

15	XM_297247		LOC342997
15	XM_298268		LOC344754
15	NM_001001887	IFIT1	interferon-induced protein with tetratricopeptide repeats 1
14	NM_017923	MARCH1	membrane-associated ring finger (C3HC4) 1, E3 ubiquitin protein ligase
14	XM_293082		similar to mucin, LOC344533
14	NM_012287	ACAP2	ArfGAP with coiled-coil, ankyrin repeat and PH domains 2
14	XM_210397		similar to hypothetical protein XP_065662, LOC285027
14	XM_294960	C1orf70	chromosome 1 open reading frame 70
14	AK094662		
13	M33234	HUMTCRB22	Human T-cell receptor beta chain gene variable region.
13	NM_006783	GJB6	gap junction protein, beta 6, 30kDa
13	NM_024977		hypothetical protein FLJ12078
13	NM_199351	ILDR2	immunoglobulin-like domain containing receptor 2
13	XM_209554	FRG2C	FSHD region gene 2 family, member C
13	NM_015296	DOCK9	dedicator of cytokinesis 9
13	NM_172234	IL17RB	interleukin 17 receptor B
13	XM_297619		LOC344417
13	NM_022037	TIA1	TIA1 cytotoxic granule-associated RNA binding protein
13	NM_014003	DHX38	DEAH (Asp-Glu-Ala-His) box polypeptide 38
13	XM_304880		LOC351520
13	NM_018026	PACS1	phosphofurin acidic cluster sorting protein 1
12	NM_152302	C20orf158	chromosome 20 open reading frame 158
12	BC026055		Mus musculus serine hydroxymethyltransferase 1 (soluble)
12	XM_296829		LOC342373
12	NM_024667	VPS37B	vacuolar protein sorting 37 homolog B (S. cerevisiae)
12	XM_066948		similar to hypothetical protein BC011593, LOC139891
12	XM_114002	NY-REN-24	NY-REN-24 antigen
12	NM_182594	ZNF454	zinc finger protein 454
12	NM_207305	FOXD4	forkhead box D4
12	NM_015185	ARHGEF9	Cdc42 guanine nucleotide exchange factor (GEF) 9
11	R25699		
11	NM_004741	NOLC1	nucleolar and coiled-body phosphoprotein 1
11	XM_294143		similar to Bullous pemphigoid antigen 1 isoforms 1/2/3/4/5/8 (230 kDa bullous pemphigoid antigen) (BPA) (Hemidesmosomal plaque protein) (Dystonia musculorum protein), LOC346244
11	XM_103682		LOC150876
11	XM_291670		similar to High mobility group protein 4 (HMG-4) (High mobility group protein 2a) (HMG-2a), LOC340697
11	XM_298199		LOC344659
11	XM_095043		LOC154797
11	NM_152617	RNF168	ring finger protein 168, E3 ubiquitin protein ligase
11	NM_013292	MYLPF	myosin light chain, phosphorylatable, fast skeletal muscle
11	XM_304162		LOC350524
10	NM_004348	RUNX2	runt-related transcription factor 2
10	NM_033426		KIAA1737
10	XM_303167		LOC350781
10	NM_003656	CAMK1	calcium/calmodulin-dependent protein kinase I
10	NM_031490	LONP2	lon peptidase 2, peroxisomal
10	NM_012193	FZD4	frizzled family receptor 4
10	NM_178499	CCDC60	coiled-coil domain containing 60
10	NM_152562	CDCA2	cell division cycle associated 2
10	NM_016139	CHCHD2	coiled-coil-helix-coiled-coil-helix domain containing 2
10	XM_302939		LOC349027
10	NM_002786	PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1

5.4 Publications and presentations

5.4.1 Publications

Grosz M, Kolter J, Paprotka K, **Winkler AC**, Schäfer D, Chatterjee SS, Geiger T, Wolz C, Ohlsen K, Otto M, Rudel T, Sinha B, Fraunholz M. Cytoplasmic replication of *Staphylococcus aureus* upon phagosomal escape triggered by phenol-soluble modulin α. Cell Microbiol. 2014 Apr 16(4):451-65. doi: 10.1111/cmi.12233.

Faulstich M, Hagen F, Avota E, Kozjak-Pavlovic V, **Winkler AC**, Xian Y, Schneider-Schaulies S, Rudel T. Neutral sphingomyelinase 2 is a key factor for PorB-dependent invasion of *N. gonorrhoeae* Cell Microbiol. 2014 Sep 16. doi: 10.1111/cmi.12361.

Subbarayal P, Karunakaran K, **Winkler AC**, Rother M, Gonzalez E, Meyer TF and Rudel T. EphrinA2 receptor (EphA2) is an Invasion and Intracellular Signaling receptor for *Chlamydia trachomatis* (in revision, PLOS Pathogens)

Grosz M[+] and **Winkler AC**[+], Oesterreich B, Fraunholz M, Ohlsen K, Otto M, Wolz C, Rudel T. 2-APB inhibits host cell death and the extraordinary up-regulation of FPR2 during *S. aureus* infection of epithelial cells (in preparation)
[+]These authors contributed equally to this work.

Winkler AC[+] and Grosz M[+], Ade C, Onyango M, Chunguang L, Rudel T. An shRNA screen revealed that the AKT1 survival pathway is crucial for enhanced invasion and intracellular replication of *S. aureus* (in preparation)
[+]These authors contributed equally to this work.

5.4.2 Poster presentations

Ann-Cathrin Winkler, Magdalena Grosz, Carsten Ade, Thomas Rudel. Identification of eukaryotic host cell factors involved in Staphylococcus aureus induced cytotoxicity. 16th International Symposium on Staphylococci and Staphylococcal Infections (ISSSI) August 2014 (Chicago, USA).

Ann-Cathrin Winkler, Carsten Ade, Martin Fraunholz, Thomas Rudel. Host cell death induced by *Staphylococcus aureus*. 2nd International Comference on the Pathophysiology of Staphylococci in the Post-Genomic Era. November 2012 (Kloster Banz, Germany).

5.5 Danksagung

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Ein ganz besonderer Dank hier an die, die mich die Jahre über begleitet haben:
Christine Siegl und Christine Ott, Annette Huber und Michaela Faulstich.

5.6 Selbsständigkeitserklärung

Ich erkläre ehrenwörtlich, dass die vorliegende Arbeit von mir selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde.
Diese Dissertation hat weder in gleicher, noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen.
Ich habe früher, außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden, keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den