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O. Univ.-Prof. Dr. Rudolph Jakob Schweyen (Univ. Wien)

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I. Introduction

1. G proteins [1,2]

Guanine-nucleotide-binding (G) proteins are regulators of a wide variety of cellular functions. They receive signals from the cellular surface and elicit a specific response in the cell. G proteins are divided into the two groups of heterotrimeric GTPases and small GTPases. While heterotrimeric GTPases consist of three subunits, the alpha subunit and a complex of beta and gamma subunits, small GTPases consist of only one protein. In order to to carry out regulatory functions all G proteins can be switched between an active and an inactive form ***(Figure 1)***. While activation is performed by GEFs (guanine-nucleotide-exchange factors, also known as GDP dissociation stimulators (GDSs)) which induce an exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP), inactivation happens through GTP hydrolysis via GAPs (GTPase activating proteins) but also on a lower extent by the small G protein's intrinsic GTP hydrolysing domain.

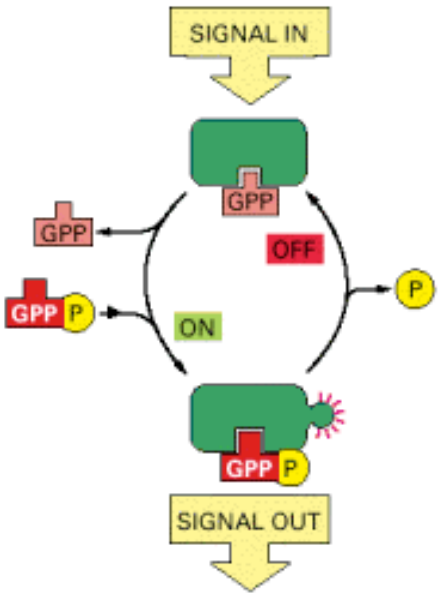


Figure 1. Signaling by GTP-binding protein *From Alberts et al. (2002)* [1]

<u>Small GTPases</u>

Major regulatory functions of small GTPases comprise cell growth, differentiation, cell motility, cytokinesis, axonal guidance and intracellular trafficking through the Golgi, nucleus and the endosomes. The molecular mass of these small monomeric proteins averages around 20 – 25 kDa. After the discovery of the first small GTPase Ras, a multitude of additional small GTPases has been identified. All of them can be assigned to one of the five subfamilies Ras, Rho, ARF (ADP-ribosylation factors), Rab or Ran.[3]

Rho GTPases

The mammalian Rho GTPases belong to the superfamily of Ras and are currently devided into eight subgroups comprising 22 members ***(Figure 2)***: Cdc42 (Cdc42, TC10, TCL, Chp, Wrch-1), Rac (Rac 1-3, RhoG), Rho (RhoA, RhoB, RhoC), Rnd

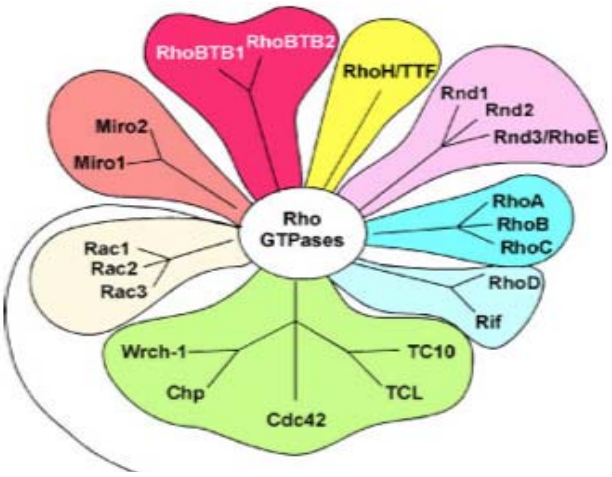


Figure 2. Members of the Rho GTPase family
From E. M. Sorokina and J. Chernoff (2005)[4]

(Rnd1, Rnd2 and Rnd3/RhoE), RhoD (RhoD and Rif), RhoH/TTF, ThoBTB (RhoBTB1 and RhoBTB2) and Miro (Miro-1 and Miro-2).[4] Rho GTPases are anchored within the plasma membrane or other membranes of the cell by post-translationally added lipid anchors. Those are either prenylations with farnesyl phosphate or geranygeranyl pyrophosphate or in few cases also palmitoylation. These lipid anchors are necessary to transmit signals at the plasma membrane.

Rho-GTPases act on the actin cytoskeleton

Proteins of the Rho-GTPase family play an important role in regulating the organization of the actin cytoskeleton and can thereby dramatically influence the morphology of a cell, as well as a cells ability to migrate and invade into surrounding tissues. For example Rho and Rnd have most dramatic and opposed effects on stress fibre formation. Rho being responsible for fibre formation and Rnd for their loss. Other Rho-GTPases like Rac1-3, Cdc42 or RhoD and Rif induce formation of lamellipodia or filopodia respectively. Rho-GTPases from the groups of RhoBTB and Micro, however, don't show any influence on the cytoskeleton at all.[4]

Functions besides acting on actin polymerization

Although Rho-GTPases are predominantly seen in context of modifying functions of the actin cytoskeleton they also have important regulatory effects on other cellular processes like gene transcription, cell cycle control, vesicle transport and enzyme activation ***(Figure 3)***. [5]

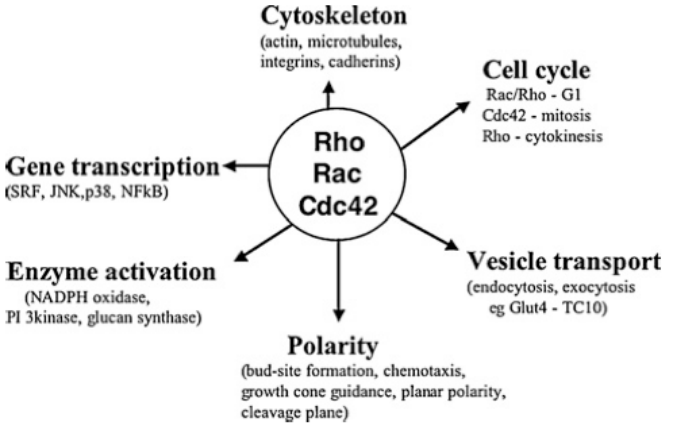


Figure 3. Cellular actions of Rho GTPases
From A. Hall (2005)

One of the first functions described was the regulation of an NADPH enzyme complex by Rac in phagocytic cells, to

produce reactive oxygen species.[6] This is interesting because it is a host response paralleled by actin driven phagocytosis of bacterial pathogens and led to the idea that Rho-GTPases might coordinately regulate multiple biochemical pathways to promote complex changes in cell behaviour[7].

An influence on gene transcription was first shown in 1995. Two groups described activation of JNK (c-Jun N-terminal kinase) and p38 MAPK (mitogen-activated protein kinase) cascades by Rac and Cdc42[8,9]. Also activation of the transcription factors SRF (serum-response factor)[10] and NFκB (Nuclear Factor κB)[11] were revealed.

Overall, yeast two-hybrid and affinity chromatography screening techniques have revealed at least 45 targets of Rho, Rac and Cdc42.[Error! Bookmark not defined.]

Very interestingly, many human malignancies show alterations of Rho GTPases and/or their subsequent pathways.

Regulation of Rho GTPase activity by GEFs and GAPs

In order to perform time and site specific regulation of GTPase activity as it is for example essential for migratory and axon guiding functions, it is necessary for the cell to control GTPase activation by regulating GEFs and GAPs ***(Figure 4)*** Although GEF regulation is not very well known at this time, it is of central importance for the understanding of complex GTPase regulated responses. For Rho GTPases there have been more than 85 GEFs and at least 70 GAPs identified. In the complex network of GEFs there are some that act either specifically on one GTPase (e.g. Lbc for Rho[12], or Tiam1 for Rac[13]) and others that act on several GTPases at once (e.g. Vav and Dbl[14]). Interestingly the Rho-GEF family is much larger than GEF families of other GTPases which again underlines the complexity of the pathways regulated by Rho-GTPases.

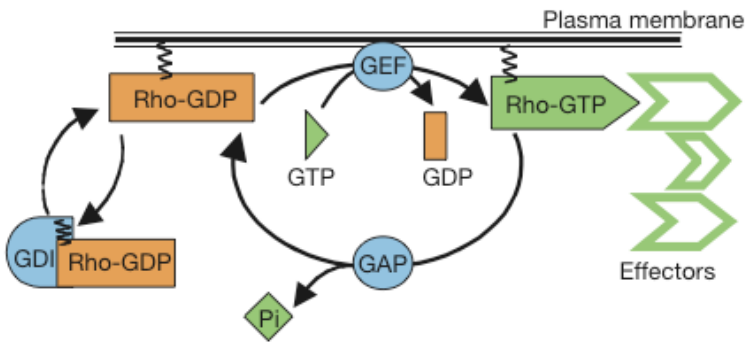


Figure 4. De/activation of Rho-GTPases by GEFs and GAPs. Rho-GDP blocking by GDIs. *From Etienne-Manneville et al. (2002)* [15]

Regulation by Rho-GDIs

Another mechanism by which Rho GTPase activity is controlled, is complexation of Rho-GDP with Rho-GDP dissociation inhibitors (Rho-GDI). Mammals have three known Rho-GDIs: Rho-GDIα, β and γ.[16] Active Rho-GTP acts on effector molecules until its GTP is hydrolysed to GDP. Consequently a Rho-GDI will bind to Rho-GDP and create an inactive Rho-GDP-Rho-GDI complex. This complex can be reactivated by an external signal that will cause the Rho-GDI to dissociate from the complex and enables Rho-GEFs to activate the enzyme again.[17]

Regulation on a transcriptional level

Although GDP/GTP exchange is the prevalent regulatory mechanism for the „common" GTPases RhoA, Rac1 and Cdc42, whose mRNA levels are more or less constant in all tissues, other Rho-GTPases are under transcriptional regulation.[18] An example for timely controlled gene expression is given by RhoG, which is a growth-stimulus-induced early response gene[19]. Rac2 however is a Rho-GTPase that shows tissue specificty for hematopoietic cells.[20] Members of the Rnd subfamily, who don't seem to be regulated by GTP/GDP exchange at all, show upregulated levels on mitogenic stimuli.[21]

Protein stability is also favoring importance of transcriptional regulation, since some Rho-GTPases (e.g. RhoB) are very unstable and therefore have short half-lifes in the cell.[22]

Genetic assembly of Rho GTPases

All Rho-GTPases possess several conserved domains as shown in ***Figure 5***. Some Rho GTPases have a longer N-terminal extension (left) and some (also) a longer C-terminal hypervariable region. RhoBTB and Miro proteins house additional domains which will not be discussed here. Conformational changes between GTP- and GDP-bound Rho-GTPases occur inside the effector domain which is also required for downstream target binding. Rho and Rac isoforms have very different hypervariable regions that can include a polybasic regions and/or sites for palmitoylation. Farnesylation and geranylgeranylation (both post-translational prenylations) happen at the cysteine of the CAAX box on the C-terminal end of the protein. Subsequently the remaining amino acids AAX (two aliphatic and one variable amino acid) are removed and replaced by a methyl group.

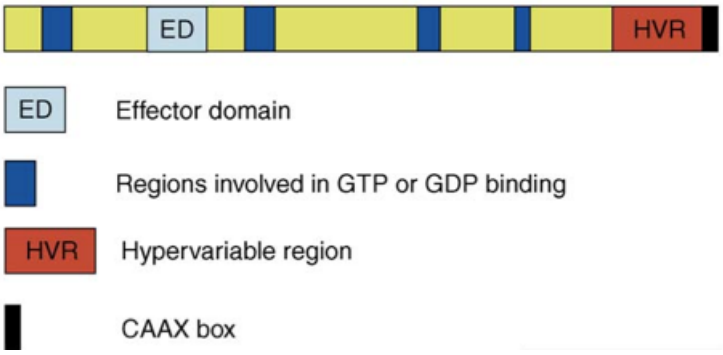


Figure 5. Domain structure of Rho GTPases *From Ridley et al. (2006)*[23]

2. Membrane proteins[1]

Proteins are associated with membranes in various different ways ***(Figure 6)***. They are divided into integral membrane proteins and peripheral membrane proteins. Members of the latter are tethered to the membrane by noncovalent interactions. These membrane proteins can be released from the membrane by strong ionic forces or extreme pH values while integral membrane proteins cannot.

Integral membrane proteins are either attached to the membrane by being extended across it once or multiple times, with a sequence of hydrophobic amino acids interacting with the hydrophobic parts of the membrane. Or they are attached by being anchored with either a GPI (glycosylphosphatidylinositol) anchor or a lipid chain which can again be a fatty acid chain or a prenyl group. Some proteins (so called ß-barrels) are even attached by being completely integrated into the membrane and thus serving as a channel.

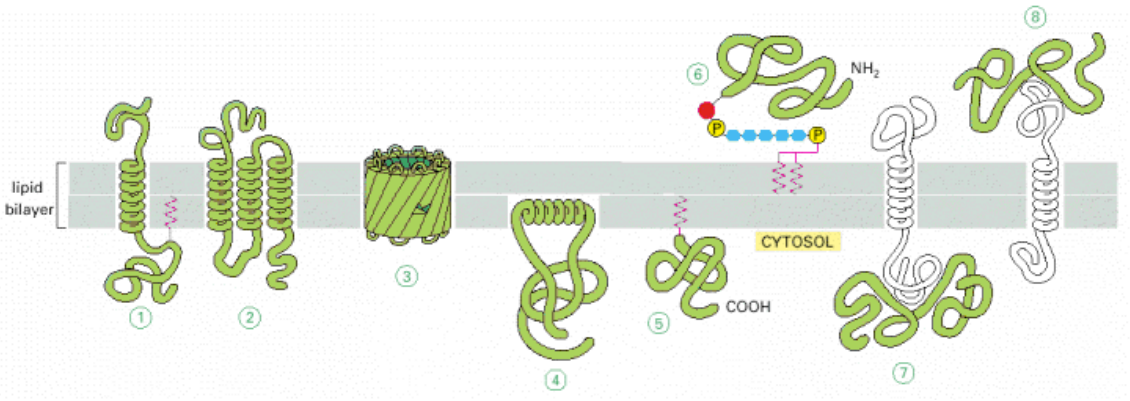


Figure 6. Various ways in which membrane proteins associate with the lipid bilayer. From Alberts et al. (*2002*)[1]

a. Prenylations

Farnesylation and geranylgeranylation are prenylations that can help localize a water-soluble protein to a membrane after its synthesis in the cytosol. The prenyl groups are covalently bound to a protein by a thioether linkage () with a proteins cysteine. The cysteine residue is part of the CAAX motif which is located at the C terminus of the protein ***(Figure 5)***. After successful attachment of the prenyl group, the three C-terminal residues are cleaved and the C-terminus is subsequently methylated before the protein is attached to a membrane ***(Figure 7)***. Farnesyl lipid anchors are 15-carbon unsaturated hydrocarbon chains while geranygeranyl lipid anchors consist of 20 carbon atoms.

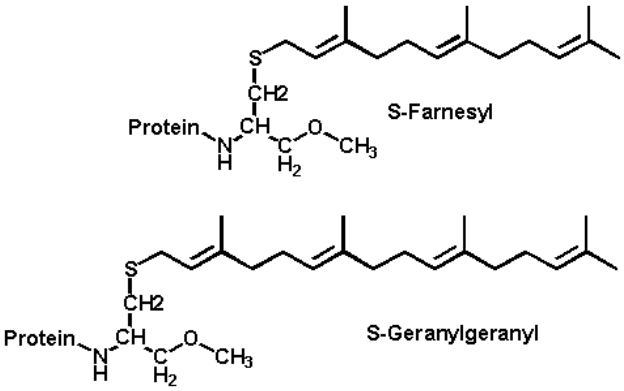


Figure 7. Prenyl groups attached to cystein residues of a protein. The three C-terminal amino acids of the CAAX motif have been substituted by a methyl group.

b. Prenyl transferases

Mammalian cells express three protein prenyltransferases[16]: Farnesyl protein transferase (FTase), Geranylgeranyl prenyltransferase-1 (GGTase-1) and GGTase-2. Farnesyl diphosphate and geranylgeranyl diphosphate are the substrates for those enzymes. The isoprenyl lipid residues are covalently linked to a cystein residue within a CAAX motif. Each protein prenyltransferase recognizes this specific four amino acid sequence at the C-terminus of its target ***(Table 1)***. The terminal amino acid is thereby an important structural determinant for controlling the specificity of prenylation.[24]

Enzyme	**Recognized motifs** C = cysteine a = aliphatic amino acid X = C-terminal amino acid	**Recognized proteins**
FTase	CaaX with X being: serine, methionine, glutamine or alanine	H-Ras, N-Ras, K-Ras, prelamin A, HDJ2, PTP-CAAS/PRL tyrosine phosphatases, **RhoB** and other Rho family GTPases, Rheb, CENP-E, and CENP-F
GGTase1	CaaL	
GGTase2	XXCC, XCXC, or CCXX	Rab family of small GTPases

Table 1 Targets of the three mammalian protein prenyltransferases. *Table assembled from* [25,26,27,28,29,30,31,32]

c. Localization of GTPases depends on prenylations

Proper cellular localization of human Rho-GTPases is dependent on their type of post-translational modification. While most Rho-GTPases can only be modified by one specific prenylation, RhoB can have two prenylations at once ***(Table 2)***. Additionally RhoB can receive a third modification, a palmitoylation.[30] TC10 and TCL are the only other Rho proteins who have predicted palmitoylation sites. Palmitoylation supports membrane localization and compartment specificity, which is also promoted by a polybasic region close to the C-terminus.[18,33] Another post-translational modification that occurs in combination with prenylations is phosphorylation, although it is only known for RhoA and Rnd3[34,35]. RhoBTB and Miro proteins seem to become not modificated at all.

Dependent on their post-translational modifications Rho-GTPases are localized to different compartment-membranes of the cell. While most of them are detectable on the plasma membrane[30,36,37,38,39,40], some Rho GTPases are also directed to endosomes / endomembranes[41,42] , the perinucleus[39] and the golgi[38,43]. The groups of not modified Rho-GTPases Miro and RhoBTB are prevalent in mitochondria[44] and predicted to be vesicularily localized[45], respectively. Rho-GTPases have also been detected in the cytosol[30,43]. ***Table 2*** shows a summary of the currently known modifications and localizations of human GTPases.

Rho GTPase	Localization	Post-translational modifications
RhoA	PM and cytosol [16]	GG and PH [23,105]
RhoB	PM and endosomes [16]	GG, F and P [105]
RhoC	PM and cytosol [16]	GG [105]
RhoD	PM and endosomes [97]	GG[c]
Rif	PM [8]	GG[c]
Rnd1	PM [98]	F[c]
Rnd2	endosomes and cytosol? [99]	F[c]
Rnd3/RhoE	PM, Golgi and cytosol [13]	F and PH [14,106]
RhoH/TTF	?	GG[c]
Rac1	PM [18]	GG [107]
Rac2	PM and cytosol (Knaus, 1991)	GG [107]
Rac3	PM and endomembranes [100]	GG [100]
RhoG	PM and endosomes [101]	GG[c]
Cdc42	PM and Golgi [63]	GG[c]
TC10	PM and perinuclear [9]	F and P[c]
TCL	PM and endosomes [76]	F and P[c]
Wrch1	PM and endomembranes [102]	P [102]
Chp/Wrch2	PM and endomembranes [103]	P [103]
RhoBTB1	Vesicular[d] [3]	None known
RhoBTB2	Vesicular[d] [3]	None known
Miro1	Mitochondria [104]	None known
Miro2	Mitochondria [104]	None known

Table 2 Localization and modifications of mammalian Rho GTP-binding proteins.

Abbreviations: PM = plasma membrane; GG = geranylgeranylation; F = farnesylation, P = palmitoylation, PH = phosphorylation; [c] = predicted; [d] = localization not defined

Modified from Ridley et al. (2006)[23]. Reference numbers in brackets do not apply.

d. Prenylation inhibitors

Farnesyl transferase inhibitors

Farnesyl transferase inhibitors (FTI) are competitive inhibitors of farnesyl transferases (FT) that are developed since the late 1980's.[46] The aim of targeting FTs is to reduce active Ras which plays an important role in many human malignancies and cancer and needs farnesylation for its proper cellular localization to and thus its function. [47]

FTIs were first developed as competitive CaaX peptides[48] some of which served as a substrate for FTs (e.g. Cys-Val-Phe-Met) and some of which were real inhibitors that blocked FTs catalytic function[25,48,49,50]. New inhibitors had to be invented because these peptides were poorly cell permeable and targeted by intracellular degradation. At first the two aliphatic residues of the CaaX sequence were changed for benzodiazepine or aminomethylbenzoic acid, which resulted in more stable and also effective FT inhibitors.[51,52,53,54] Additionally to the development of peptide inhibitors, screenings for small molecule inhibitors were performed and followed in development of two potential drugs that have made their way to clinical trials: Lonafarnib[55,56] and tipifarnib[57]. These drugs are highly specific in inhibition of FTases ***(Table 3)***.

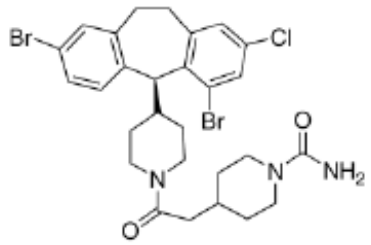
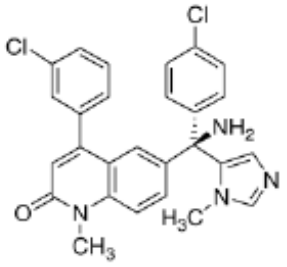
FTI	FTase IC_{50}	GGTase-1 IC_{50}
Lonafarnib 	1,9nM	> 50.000nM
Tipifarnib 	7,9nM	IC_{40} = 50.000nM

Table 3 Specificity and efficiency of Farnesyl transferase inhibitors. The IC-50 value indicates the amount of inhibitor needed to reduce an enzymes catalytical activity to 50% in vitro. *Values from [55,56,57]. Molecular structures from [46].*

Geranylgeranyl transferase inhibitors

Recently Sjögren et al. reported the development of a mouse strain homozygous for a conditional allele of the gene that encodes GGTase-I. They found that ablation of the GGTase-I-encoding gene in cells destined to produce lung tumors driven by oncogenic K-Ras resulted in delayed onset and decreased severity of disease. Thus this animal model confirms that GGTase-I is a good target for anti-cancer drug development. [58],[59] First GGTase-I inhibitors have already been developed.

FTIs and GGTase-I inhibitors are of course also used for research purposes, to investigate localization and function of prenylated proteins like many of the Rho-GTPases.

3. Apoptosis [1]

1. Apoptosis

Apoptosis, also known as programmed cell death, is a common process in all kinds of multicellular organisms. Although it might at first glance be incomprehensible that a healthy cell would kill itself, it soon becomes clear that programmed cell death is an absolutely indispensable mechanism for multi cellular life forms.

Apoptosis is necessary during development of organs and organisms. An example that shows necessarity of apoptosis very clearly is the embryonic development of mouse paws: they begin to grow as spadelike structures without apparent digits until apoptosis removes cells between them.
Around 50% of neurons die during development to adjust their number to the number of target cell that require innervation. Cells of the liver, the intestine and the bone marrow constantly kill themselves to make place for follower cells. This happens a billion times a hour in every human and enables renewal. Without the interplay of proliferation and apoptosis there could not be development in many parts of an organism.

When a cell dies of apoptosis, it dies neatly without destroying its plasmamembrane or damaging its neighbors. It shrinks and condenses, the cytoskeleton collapses, the nuclear envelove disassembles and the nuclear DNA breaks up into fragments.
Necrosis however is the type of cell death that results of acute injury. As a result of that they swell and burst leading to a potentially damaging inflammatory resopnse.

2. Other types of programmed cell death

The two common types of programmed cell death are called „Type I cell-death“ which is apoptosis and „Type II cell-death“ which is also known as autophagy. The latter is characterized by the formation of autophagosomes which later fuse with lysosomes to remove organelles. It does only show few of the characteristic apoptotic features like membrane blebbing, chromatin condensation and DNA fragmentation.[60] But autophagy does not have to end in cell death. It mainly describes the removal of damaged or obsolete cellular compartments by lysosomes as it happens in hepathic cells in huge amounts as part of the cellular function. If autophagy is supposed to lead to cell death, cellular organelles are digested in a specific sequence prior to destruction of the nucleus. [61]

Besides Type I and II cell-death, other pathways named „non-apoptotic“ or „caspase-independent“ or „necrosis-like programmed cell death“ have been described.[62] These effective, alternative death mechanisms may serve as backup for apoptosis but can also be the main type of programmed cell death. Their existence has been proofed mainly with the tool of caspase inhibitors.

3. Caspases – triggering and executing apoptosis

The intracellular machinery executing apoptotic events seems to be conserved in all animal cells. The main group of enzymes which is responsible for the execution of apoptotic events is a large family of cysteine proteases which cleave peptide bonds at aspartic acids.[63] They are called caspases (**C**ystein + **ASP**artate) and are expressed in virtually all animal cells.

Caspase activation was long described as the „point of no return“ commitment to cell death[64], but evidence for nonapoptotic caspase activity is growing. Caspase activation with nonapoptotic effects has been ascribed a role in cytokine processing during inflammatory response, terminal differentiation of specific cell types and T and B lymphocyte proliferation.[65]

Caspase activation and the caspase cascade[1]

Caspases are expressed as inactive zymogens, so called procaspases, which are composed of a prodomain followed by p20 large and p10 small subunits ***(Figure*** 9***)***.[66] Activation of these precursors is mediated by a series of cleavage events, first separating the large and small subunits, followed by the removal of the prodomain ***(Figure 8)***.[67]

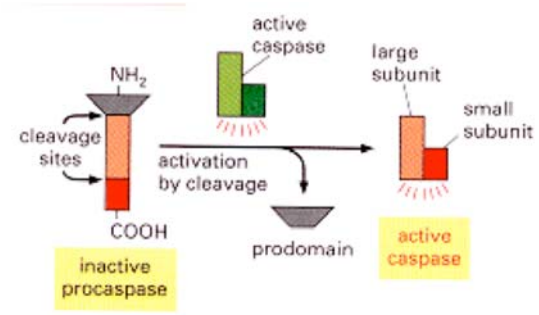


Figure 8. Procaspase activation *From Alberts et al. (2002)*[1]

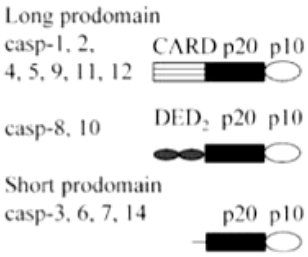


Figure 9. Prodomain length based caspase subfamilies. *From Degterev et al. (2003)*[70]

Once acivated, caspases will cleave other procaspases, which leads to an amplifying cascade by which all existing procaspases will be activated in a short time. Among caspases are some (so called effector caspases) which will cleave key proteins of the cell, like nuclear lamins and DNAse inhibitors. As soon as effector caspases are activated the cell will immediately develop morphological hallmarks of apoptosis.

Although it is known for executioner caspases-3 and -7 that the activation event is proteolytic cleavage, recent studies conducted on caspases-2, -8 and -9 have challenged this tenet of caspase activation. [64]

Caspase activating processes

An important question is of course how the first caspases are activated so that the caspase cascade can be initiated. Adaptor proteins play an important role in this regard. They aggregate multiple initiator procaspases, which possess some amount of proteolytic activity even in an unprocessed form. When brought together, they begin to cleave each other and thereby start the death cascade.

There are two distinct ways by which adaptor proteins themselves can be recruited and aggregated: An extrinsic pathway via activation of death receptors and an intrinsic pathway like the release of mitochondrial cytochrome c.

Extrinsic activation of apoptosis requires activation of death receptors on the surface of a cell. An example for that is the Fas system which is used by killer lymphocytes. They express the protein „Fas ligand“ on their surface which interacts with the death receptor protein „Fas“ on a target cells surface. This interaction causes aggregation of adaptor proteins on the inside of the cell and thus binding and subsequent cleavage of procaspase-8 molecules and activation of the caspase cascade as described above. Interestingly this process can be triggered by stressed cells themselves. Therefore they produce Fas ligand and Fas protein at the same time.

Intrinsic activation often happens when cells are damaged or undergo stress. These mechanisms are of central importance for mitochondrial transition pore formation, which is accomplished by the release of mitochondrial cytochrome c into the cytosol. Cytochrome c binds to the adaptor protein Apaf-1 which subsequently leads to cleavage of procaspase-9 and thus activation of the so called apoptosome.[68]

Caspase subfamilies

Evolution has greatly increased the number of caspases over time. From four caspases in *C. elegans* to seven in *Drosophila melanogaster* up to eleven in *mus musculus* and *homo sapiens*.[69]

Classification of caspases by different rubrics leads to very similar results, suggesting a strong correlation between structure and function. Categorization by function leads to three functinally differnt groups ***(Table 4).*** Initiator caspases link distinct upstream signaling pathways with the downstream execution steps. They possess long prodomains containing one of two characteristic protein protein interaction motifs: „death effector domain“ (DED) (caspase-8 and -10) or „caspase activation and recruitment domain“ (CARD) (caspase-1, -2, -4, -5, -9, -11, and -12) ***(***

Figure 9***).*** Initiator caspases-1, -5 and -11 control apoptosis as well as inflammatory responses. Caspases-3, -6 and -7 are „effector“ or „executioner caspases“ of apoptosis and characterized by only a short prodomain sequence. [70]

Initiator **Apoptosis / Inflammation**	**Initiator** **Apoptosis**	**Effector** **Apoptosis**
Caspase-1	Caspase-2	Caspase-3
Caspase-5	Caspase-8	Caspase-6
Caspase-11	Caspase-9	Caspase-7
	Caspase-10	
	Caspase-12	

Table 4. Function based caspase subfamilies From Degterev et al. (2003)[70]

Caspase-2

Caspase-2 has a variety of features that suggest a role as initiator caspase. A long CARD-containing prodomain for example or its early activation in response to a variety of apoptotic stimuly.[71] But caspase-2 is unique in that it includes features of initiator caspases and effector caspase at the same time.[72] The latter being VDVAD substrate specificity as it is common for effector caspases caspase-3 and -7.[73] Above that it may be activated by downstream executioner caspases such as caspase-3[74.]It is therefore at this moment the most tantalizing member of the caspase family.[75]

Intracellular localization of caspase-2 has been assigned to nuclei and the Golgi.[76,77] Nuclear localization is promoted by two nuclear localization sequence (NLS) on its prodomain. Interestingly mutations in this domain don't cause any reductions of the proteases capability to induce apoptosis.[76,78] In the Golgi, caspase-2 may contribute to the fragmentation of this organelle which suggests a role of caspase-2 in organelle-specific apoptotic events.[77]

The interesting feature of caspase-2 is that it might primarily act on noncaspase substrates which makes them differ from other initiator caspases. In this regard it was shown that caspase-2 releases apoptogenic factors from mitochondria directly which might be induced by caspases-2 mediated cleavage of Bid.[79,80,81]

Regulation and inhibition of apoptosis

Activation of apoptosis depends on central regulators like p53. Those are transcription factors which control transcription of other downstream regulators. The Bcl-2 family of proteins for example is responsible for regulation of the release of cytochrome c and subsequent procaspase activation.[82] Some members of the Bcl-2 family prevent the release of cyt. C (death inhibitors, e.g. Bcl-2, Bcl-XL) while others promote it either by inhibiting death inhibitors (e.g. Bad) or by actually stimulating cyt c release from mitochondria (e.g. Bax and Bak).

Another important process in regulating apoptosis is its inhibition by a protein family called IAP (inhibitors of apoptosis). They act either by blocking procaspases from being activated or by inhibiting active caspases.[1]

4. RhoB

As described in I.1.b RhoB (ras homolog gene family, member B) belongs to the family of the Rho-GTPases. Therefore it can be switched from an inactive into an active state by GDP-GTP exchange (c.f. I.1). RhoB has also recently been shown to be epigenetically regulated in an age- and tissue-specific manner by histone modification.[83]

The three members of the Rho GTPase family, RhoA, RhoB and RhoC show about 85% sequence homology. Nevertheless, each of them fulfills different and distinct functions in the cell. All of them act on the cytoskeleton: RhoA plays a key role in the regulation of actomyosin contractility. RhoB, which is localized primarily on endosomes, has been shown to regulate cytokine trafficking, while RhoC may be more important in cell locomotion.[84] But only RhoB seems to play an additional important regulative role in other aspects like apoptosis or tumor suppression[85]. Since RhoB has only been subject of research during the last few years there is still much work to be done on this diverging Rho-GTPase.

a) RhoB DNA, protein sequence, sequence features and crystal structure

The human RhoB gene (RHOB) consists of 2.367 bases in DNA, 2384 bases in mRNA, 591 bases in spliced mRNA ***(Figure 10)*** and of 196 amino acids as a protein ***(Table 5)***. RhoB was previously also reffered to as „Aplysia RAS-related homolog 6“ (ARH6) and is located on chromosome 2, 2p24. Known features targeted to the primary sequence and amino acid modifications are listed in ***Table 6***. Recently, also the crystal structure of RhoB in a GDP bound state was resolved: ***Figure 11***.

Figure 10. RhoB open reading frame. *Grey*: all 2384 bases of RhoB mRNA. *Blue*: Open reading frame from position 393 to 983 (591 base pairs). *Figure made with „EnzymeX 3“ from Alexander Griekspoor and Tom Groothuis.*

```
1   maairkklvv vgdgacgktc llivfskdef pevyvptvfe nyvadievdg kqvelalwdt
61  agqedydrlr plsypdtdvi lmcfsvdspd slenipekwv pevkhfcpnv piilvankkd
121 lrsdehvrte larmkqepvr tddgramavr iqaydylecs aktkegvrev fetatraalq
181 krygsqngci ncckvl
```

Table 5. RhoB amino acid sequence (Homo sapiens). *From www.pubmed.com*

Key		From	To	Description
FT	CHAIN	1	193	Rho-related GTP-binding protein RhoB.
FT	PROPEP	194	196	Removed in mature form.
FT	NP_BIND	12	19	GTP (By similarity).
FT	NP_BIND	59	63	GTP (By similarity).
FT	NP_BIND	117	120	GTP (By similarity).
FT	MOTIF	34	42	Effector region (Potential).
FT	MOD_RES	41	41	ADP-ribosylasparagine; by botulinum toxin
FT				(By similarity).
FT	MOD_RES	193	193	Cysteine methyl ester.
FT	LIPID	189	189	S-palmitoyl cysteine.
FT	LIPID	192	192	S-palmitoyl cysteine.
FT	LIPID	193	193	S-farnesyl cysteine; in plasma membrane
FT				form.
FT	LIPID	193	193	S-geranylgeranyl cysteine; in endosomal
FT				form.
FT	MUTAGEN	14	14	G->V: No effect on internalization of EGF
FT				receptor but decreases trafficking of
FT				receptor to the lysosome with associated
FT				accumulation in late endosomes.
FT	MUTAGEN	39	39	F->G: Abolishes binding to PKN1 and
FT				trafficking of EGF receptor.
FT	MUTAGEN	189	189	C->S: No effect on prenylation. Reduced
FT				palmitoylation. Abolishes palmitoylation;
FT				when associated with S-192.
FT	MUTAGEN	192	192	C->S: Reduced geranylgeranylation but no
FT				effect on farnesylation. Reduced
FT				palmitoylation. Abolishes palmitoylation;
FT				when associated with S-189.
FT	MUTAGEN	193	193	C->S: Abolishes methylation,
FT				palmitoylation and prenylation.
FT	MUTAGEN	194	194	K->L: No effect on palmitoylation or
FT				prenylation.
FT	STRAND	4	12	
FT	HELIX	18	27	
FT	STRAND	41	48	
FT	STRAND	51	59	
FT	TURN	67	69	
FT	HELIX	70	73	
FT	STRAND	79	85	
FT	HELIX	89	97	
FT	HELIX	99	106	
FT	STRAND	112	117	
FT	HELIX	119	123	
FT	HELIX	125	133	
FT	HELIX	141	150	
FT	STRAND	154	158	
FT	TURN	161	163	
FT	HELIX	167	179	

Table 6. Features of RhoB (Homo sapiens) as predicted by Swiss-Prot database.

http://expasy.org/uniprot/P62745

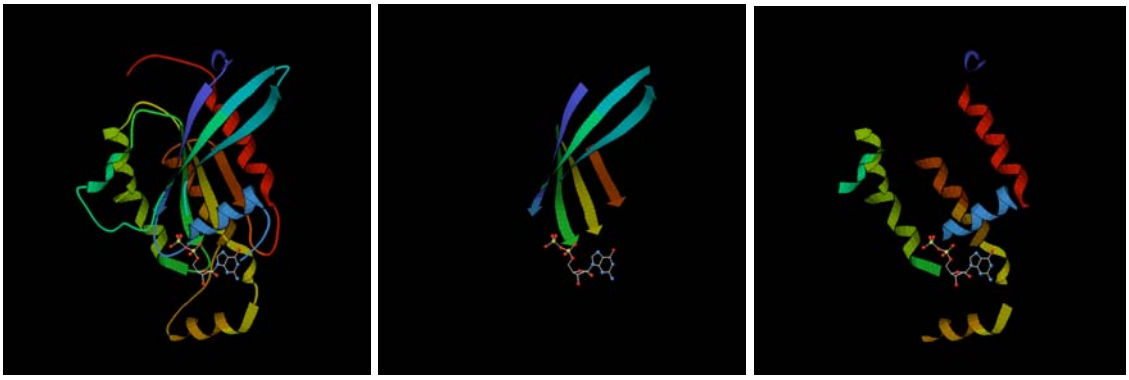


Figure 11. The crystal structure of RhoB in the GDP-bound state , visualized by Java-Software „King“ version 2.12 (Kinetic Image, Next Generation). (a) complete stucture, (b) ß-folds only, (c) alpha helixes only. *Unpublished results by Turnbull et al.*[86]

a) Modifications and subcellular localization

RhoB has several sites for post-translational modification ***(Figure 12)***: Farnesylation (F) and geranylgeranylation (GG) take place as well as palmitoylation (P). (c.f. chapter I.2). Anchorage in late endosome membranes and cell membrane is dependent on prenylation. The farnesylated form is localized to the plasma membrane while the geranylgeranylated form is localized to the endosome. RhoB localizes to the plasma membrane (PM), Golgi, and motile peri-Golgi vesicles. Inhibition of palmitoylation mislocalizes RhoB to the endoplasmic reticulum.[87]

Furthermore isoprenylation of RhoB is necessary for its degradation[88] and palmitoylated cysteine 192 is required for RhoB tumor-suppressive and apoptotic activities[89],[90].

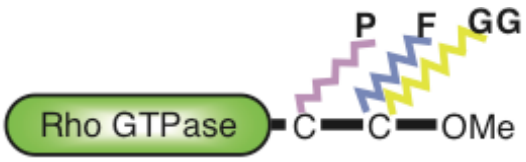


Figure 12 Posttranslational modifications of RhoB *From Wennerberg et al. (2004)*[18]

b) Described functions of RhoB

<u>Regulator of protein signaling and trafficking</u>

RhoB plays a pivotal role in the dynamic regulation of the actin cytoskeleton. It is involved in intracellular protein trafficking of a number of proteins, targets PRK1 to endosomes[91] and is involved in trafficking of the EGF receptor from late endosomes to lysosomes[92]. It is also required for stability and nuclear trafficking of Akt which

promotes endothelial cell survival during vascular development.[93] Additionally RhoB was identified as a component of outside-in signaling pathways that coordinate Src activation with its translocation to transmembrane receptors.[94]

Negative modifier of cancer prograssion

Interestingly RhoB is described as an anti-tumor-progressing protein in several types of tumors. It limits the proliferation of transformed cells by facilitating turnover of the oncogene c-Myc.[95] RhoB expression levels are dramatically decreased in lung[96,97], head and neck, and brain cancer[98], when tumors become more aggressive.

Modulator of cancer cell apoptosis

Another role which could be of great importance for understanding initiation of yet unclear apoptotic events is RhoB's promotion of proapoptotic signaling of regulators involved in cell cycle checkpoints, cell adhesion, vesicle trafficking, MAPK signaling, transcription, and immunity.[85] RhoB was furthermore shown to mediate apoptosis in neoplastically transformed cells after DNA damage.[99]

Effects of Farnesyltransferase inhibitors

RhoB is one of the targets of farnesyltransferase inhibitors which are currently under investigation as cancer therapeutics (see chapter I.2.d "Prenylation inhibitors"). RhoB is essential for apoptosis and antineoplastic activity of farnesyltransferase inhibitors in a mouse model.[100] These elevate the levels of geranylgeranylated RhoB and cause mislocalization, leading to apoptosis and antineoplastic effects.[99,89]

c) RhoB modifying toxins C3 + lethal toxin

Bacterial toxins act on the largest variety of cellular processes. One of them is for sure phagocytosis which is inhibited by several extracellular bacterial pathogens. In this regard the family of Rho-GTPases plays a highly important role.[101,102] Following the two exotoxins „C3" from *Clostridium botulinum* and „lethal toxin" (also „LT" or „TcsL") from *Clostridium Sordellii* will be described.

RhoB inactivation by C3

C3 exotoxin of *C. botulinum* catalyses ADP ribosylation and inactivation of rho family GTPases ***(Figure 13)***. This chemical modification is performed by an ADP-ribosyltransferase which attaches an ADP molecule to RhoB and thereby prevents its activation through GDP-GTP exchange.

Phenotypes of rat hepatoma FAO and human glioma U333 cells treated with C3 toxin for 24h to 48h (3-30 micrograms/ml) are disappearance and collapse of the intermediate filament network and the actin microfilaments while microtubules remain intact. This causes rounding up of FAO cells.[103]

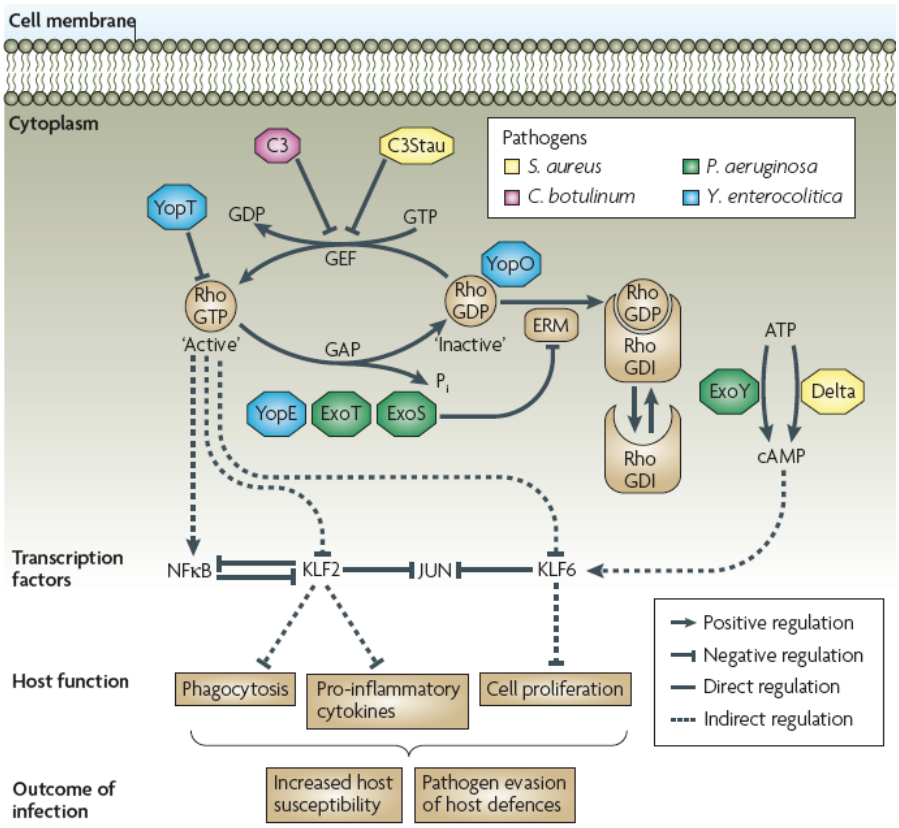


Figure 13. Regulation of Rho proteins by microbial pathogens. Note that at the moment no Rho-GDIs are known for RhoB. *From O'Grady et al. (2007)*[102]

RhoB upregulation by inhibition of Ras via a lethal toxin

C. sordelliis „lethal toxin" (LT) belongs to the group of large clostridial cytotoxins which are a family of structurally and functionally related exotoxins from *Clostridium difficile* (toxins A and B), *C. sordellii* (lethal and hemorrhagic toxin) and *C. novyi* (alpha-toxin). The exotoxins are major pathogenicity factors which in addition to their in vivo effects are cytotoxic to cultured cell lines causing reorganization of the cytoskeleton accompanied by morphological changes. The exotoxins are single-chain protein toxins, which contain of three domains: receptor-binding, translocation and catalytic domain. These domains reflect the self-mediated cell entry via receptor-mediated endocytosis, translocation into the cytoplasm, and execution of their cytotoxic activity by an inherent enzyme activity.[104]

Enzymatically, LT catalyzes the transfer of a glucosyl moiety from UDP-glucose to Rac and (R-,H-,K-,N-)Ras but neither to Rho nor Cdc42.[105,106] The covalent attachment of

the glucose moiety to a conserved threonine within the effector region of the GTPases renders them functionally inactive. [107]

For the investigation of RhoB, LT could serve as a useful tool in that it activates RhoB transcription and thus RhoB protein levels. RhoB upregulation was also shown for C3 from *C. limosum* in murine fibroblasts and could also help in the investigation of RhoB functions.[108]

5. Statins

Statins inhibit a rate-limiting step in mevalonate synthesis which is an early part of cholesterol synthesis. They are reversible and competitive inhibitors of the microsomal enzyme 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase which is responsible for catalyzing the reduction of HMG-CoA to mevalonate ***(Figure 14)***.

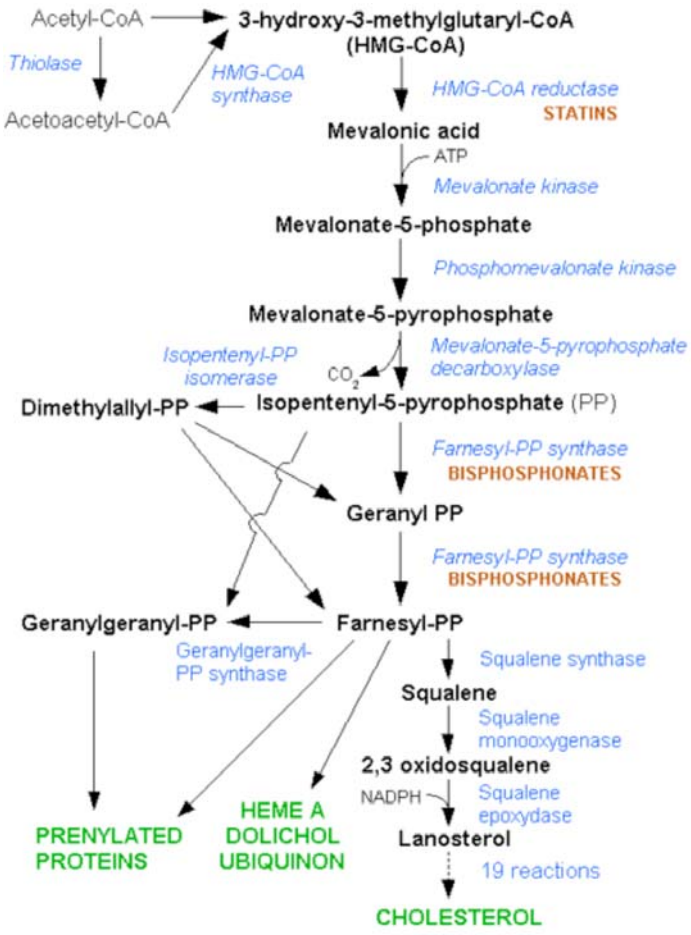


Figure 14. Cholesterol synthesis via the mevalonate pathway.

Statin mediated depletion of mevalonate prevents endogenous de novo synthesis of cholesterol so that low-density-lipoprotein (LDL) cholesterol uptake into cells is promoted and LDL serum levels sink by 30-50% after statin use for four to six weaks.[109,110] The efficacy of this therapeutic principle has been confirmed in clinical trials, which showed reduced risk of a recurrent coronary event in patients with established coronary disease treated with Simvastatin.[111] Statins are therefore widely used and well tolerated cholesterol-lowering drugs.[111,112]

However, side effects may arise in skeletal muscle. These may range from transient increases in creatine kinase (CK), muscle pain, and cramps to myositis and potentially life-threatening rhabdomyolysis.[113,112,114] As a consequence of a series of reported cases of death Bayer AG voluntarily withdrew its Cerivastatin based drug Baycol or Lipobay from the U.S. market in August 2001. Unfortunately the molecular mechanisms leading to those muscle-specific side effects are not well understood yet.

Molecular mechanism of statin action

A variety of at least twelve statins exists ***(Table 7)***, some of which were synthetically derived („type I" statins, e.g. lovastatin, Simvastatin, pravastatin). The others have been purified by fermentation („type II" statins, e.g. atorvastatin, fluvastatin, rosuvastatin) and were originally found in microorganisms which use HMG-CoA inhibitors as a self-defense mechanism.[115]

However, all statins possess a molecular structure referred to as HMG-module. This structure mimics 3-hydroxy-3-methyl-glutaryl and thus acts as a comparative inhibitor at the active site of HMG-CoA reductase.[116] In order to be active this HMG-module has to be available as an hydroxy acid. This is important as some statins exist in an inactive form of a lactone until they are activated in the liver.

While type I statins have an additional hydrophobic part, type II statins exhibit longer side chains that are more or less hydrophobic. Those side chains establish hydrogen- and ionic-bonds with HMG-CoA reductase which is the reason for nano-molar K_i values of statins.

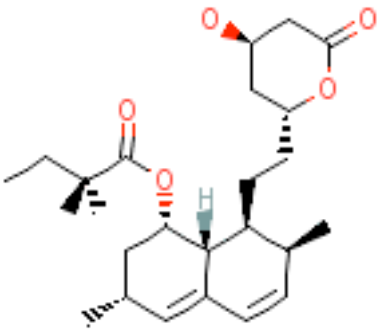
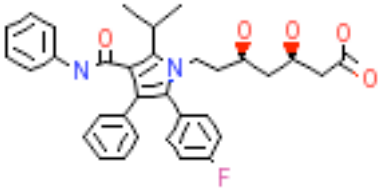
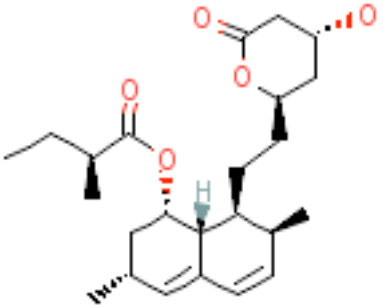
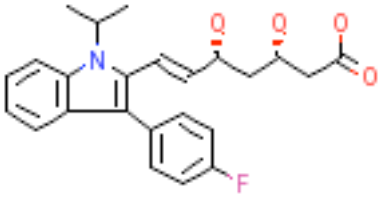
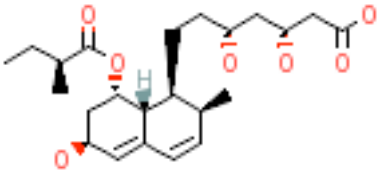
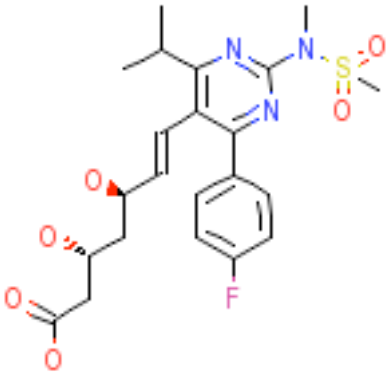
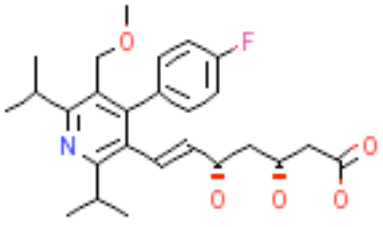
Type I statins	Type II statins
Simvastatin (Zocor, Lipex) 	Atorvastatin (Lipitor) 
Lovastatin (Mevacor, Altocor) 	Fluvastatin (Lescol) 
Pravastatin (Pravachol, Selektine, Lipostat) 	Rosuvastatin (Crestor) 
	Cerivastatin (Baycol, Lipobay) 

Table 7. Important members of the statin family. Type I statins are fermentation-derived, type II statins are synthetic compounds. Brand names given in brackets.

Effects of statin action

At first and most important, inhibition of HMG-CoA reductase leads to reduced LDL serum levels. This is due to depletion of intracellular cholesterol which is made up for by an elevated expression of LDL receptors on the cell surface which promotes LDL uptake from blood.
Four independent studies have shown, that Statins can reduce the cardio vascular risk and prevent cardiac events by about 30% versus a standard therapy without a Statin.

But statins do not exclusively lead to lower LDL levels. As can be seen in ***Figure 14*** mevalonate is a very early intermediate product in cholesterol synthesis. Many more intermediate products are following until cholesterol is finally obtained. Isoprenoids are necessary for GTPase modification, dolichol is needed in protein glycosylation and ubiquinone as an electron donor in the respiratory chain. Thus, functional consequences of Statin action may involve misguided signalling of small G proteins, ER-stress or unfolded protein response and impairment of mitochondrial energy production. Other important cellular processes which might be influenced by these intermediates involve regulation of the cytoskeleton, calcium homöostasis, protease inhibition, cellular signalling and apoptosis.

Statins also act on the regulation of important biochemical pathways like gluconeogenesis or the pentosephosphate pathway. Enzymes influenced by Statins stimulate HMG-CoA-Synthase and Isopentenyl-Diphosphat-delta-Isomerase and reduce Fructose-1,6-diphosphatase, Ketohexokinase and Glucose-6-phosphat-1-dehydrogenase activity.

Clinically relevant are also the so called pleiotropic effects of Statins which are ascribed anti-inflammatory, anti-proliferatory, anti-oxidative, anti-thrombotic, vasculo protective and pro-angiogenic effects. Moreover, they also show a positive influence on contractility of cardiomyocytes by modulating their sarcolemmal Na+/K+ pump.[117,118]

Statins cause apoptosis in dhSKM cells

It has been shown that treatment of differentiated human skeletal muscle cells (dhSKM) with elevated doses of Simvastatin activates caspase-3 and -9. This effect was in part reversible by addition of mevalonic acid.[119]

6. Skeletal muscle

Skeletal muscle is a type of striated muscle which can be arbitrarily moved. Skeletal muscles are responsible for movements of our limbs among others and are controlled via somatic nerve stimulation. Movement is thereby always performed via muscle contraction. The second type of striated muscle would be the heart muscle which is involuntarily controlled. Smooth muscles (found in arteries, the gastrointestinal tract, etc.) can also not be arbitrarily controlled but their structure and function is fundamentally different from skeletal and heart muscle.

Skeletal muscles can be devided into red and white musculature, the red type (slow-twitch) being responsible for endurance and the white type (fast-twitch) for fast and strong movements. Muscles are wrapped in a connective tissue called epimysium, and are attached to the skeleton via tendons.

Skeletal muscle assembly

Skeletal muscles are built of muscle fibres which can be up to 40cm long in the thigh ***(Figure 15)***. Several connective tissues keep the structure of the muscle well defined. A septum is wrapped around each muscle fibre and joins them to muscle fibre bundles (fascicles) building up the muscle which is again wrapped into the so called epimysium.

Muscle fibres consists of thousends of myofibrils which are long and thin structures made of myofilaments. These protein structures are responsible for muscle contraction.

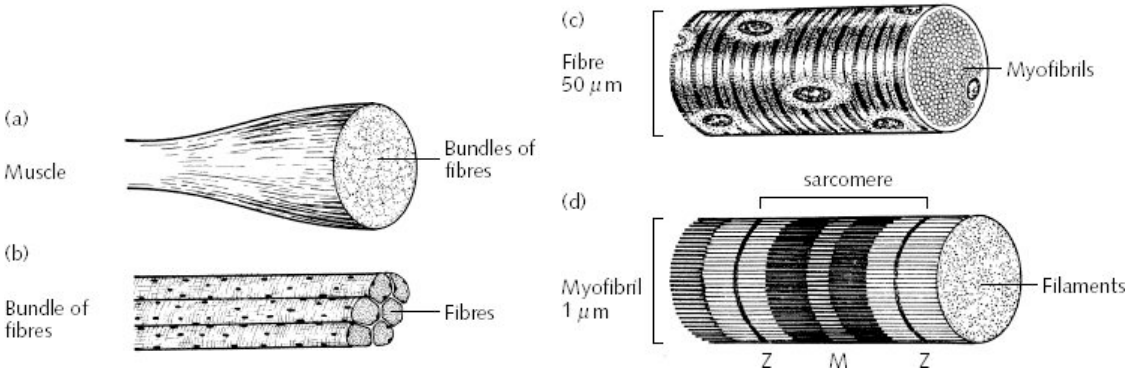


Figure 15. Assembly of skeletal muscle. *Adapted from Jennett, S. (1989). Human physiology. Churchill Livingstone, Edinburgh).*

Myofibrils themselfes are devided into segments called sarcomeres which primarily exist of actin and myosin filaments that move along each other in an opposite direction causing the contraction. Under the microscope myofibrils can be devided into segments of different thikness called I, A and H segment which are bordered by Z discs.

Interestingly muscle cells have multiple nuclei which are located at the border of muscle fibres. During embryonic development muscle cells align to each other and fuse to a mutinuclear cell called myotube or syncytium. Thus, it is not possible for a myotube to divide. Instead they grow by producing new organelles like the energy providing mitochondria.

Muscle fibres are surrounded by satellite cells. These can be isolated from muscle biopsies and kept in cell culture. These cells will be used as a source for differentiated muscle cells in this study.

Diseases of skeletal muscle

Myopathy is a neuromuscular disease resulting in muscular weakness. But it is a very general term including cramps, stiffness and spasm as well as dystrophies, rhabdomyolysis and many other myopathies.

Muscle inflammation is referred to as myositis. Many such conditions are considered likely to be caused by autoimmune conditions, rather than directly due to infection. Elevation of creatine kinase in blood is indicative of myositis.

Lysis due to injury of skeletal muscle is reffered to as rhabdomyolysis. Thereby it is not important if the injury was caused by physical, chemical or biological factors.

Since destruction leads to release of the content of damaged muscle cells into the bloodstream rhabdomyolysis is a risk for acute renal failure. Myoglobin for example is an important component of muscles injurious to the kidney. Accumulation of such products might be treated with dialysis, intravenous fluids or hemofiltration.

The most common cause of rhabdomyolysis is muscular trauma. Less common causes include muscle enzyme deficiencies, electrolyte abnormalities, infectious causes, drugs, toxins and endocrinopathies. As in myositis, the most sensitive laboratory finding of muscle injury is an elevated plasma creatine kinase level.[120]

7. Aims of this diploma thesis

As demonstrated in previous work from our lab *(Sacher et al. 2004)*[119] Statins are able to trigger apoptosis in skeletal muscle (SKM) cells. A genome wide gene chip analysis was performed and will provide unbiased information about the transcriptional response of Statin treated differentiated human SKM cells. In order to further elucidate the molecular action of Statin induced myotoxicity the following questions are raised:

- Which proteins are involved in Statin induced apoptosis and which pathways are relevant?

- Are prenylated proteins like RhoB among the strongest regulated genes upon Simvastatin treatment?

- Is artificial upregulation of RhoB enough to trigger apoptosis and can apoptosis be prohibited by deactivating RhoB via C3 toxin?

- The bacterial toxin LT can elicit a very similar myotoxic phenotype as Statins. How far is the molecular mechanism of LT and Statin induced apoptosis comparable?

II. Materials and methods

Cell culture of primary cells

All experiments were carried out with differentiated primary human skeletal muscle cells obtained from skeletal muscle biopsies of healthy individuals who underwent diagnostic testings. The leftover material (100–400 mg) was used to isolate satellite cells. The procedure was approved by the local ethic committee
of the Medical University of Vienna (Dr. Weigl; Department of Anaesthesiology, General Hospital, Vienna, Austria). Satellite cells were kept in growth medium and differentiation was initiated by switching to differentiating medium as described previously.[121]

Growth medium

Ham's F-12, 15% fetal calf serum, 50ng / 500ml epidermal growth factor, 10mg / 500ml insulin, 200μg / 50ml dexomethasone, 250mg / 500ml fetuin and bovine serum albumin, 78g / 500ml glucose, 200mM L-glutamine, 5000 units / ml penicillin G, 5mg / ml streptomycin, and 250μg / 500ml amphotericine B

Differentiating medium

Dulbecco's modified Eagle's medium supplemented with 5% horse serum, and 4mM L-glutamine, 100ng / ml insulin, and 0.1g / ml gentamicin.

Caspase assays

Differentiated human skeletal muscle cells were maintained in the absence and presence of statins, toxins, mevalonic acid, lipids (GGPP, FPP) and caspase inhibitors at concentrations and incubation times indicated in the respective figures. Thereafter, the cells were washed with phosphate-buffered saline (PBS) and lysed with ice-cold caspase-lysis buffer supplemented with protease inhibitors (1.4μg/ml aprotinin, 10μg/ml leupeptin, and 100μM pefablock). Microsomal fractions and cytosol were separated by centrifugation at 45,000*g* at 4°C for 20 min. The pellet was resuspended in caspase lysis buffer, and samples were stored at –80°C. Protein concentration was determined by Bio-Rad Protein Assay. All steps starting with cell lysis were carried out on ice. Aliquots of the supernatant (20–50μg protein) were incubated in reaction buffer and 50 μM 7-amino-4-trifluoro-methylcoumarin (AFC)-conjugated substrate at 37°C for 90 min in the dark. Cleavage of caspase substrates was measured at an excitation wavelength

of 405 nm and an emission wavelength of 535 nm by a fluorescence plate reader (Wallac 1420 multilabel counter VICTOR-2; PerkinElmer Life and Analytical Sciences, Wellesley, MA). As a negative control, the AFC-conjugated substrates were diluted in lysis buffer and reaction buffer in the absence of protein. Blanks were substracted from each probe and the fluorescence signal expresses as au/mg/min.

Caspase-lysis buffer

25mM HEPES, pH 7.4, 5mM EDTA, 1mM EGTA, 5mM $MgCl_2$, 5mM dithiothreitol

Caspase reaction buffer

25mM HEPES, pH7.4, 6.6% sucrose, 1,4mM CHAPS (non-denaturating detergent), and 5mM dithiothreitol

Caspase-specific agents

Caspase-3 substrate: Ac-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC)

Caspase-2 substrate: Ac-Val-Asp-Val-Ala-Asp-AFC (Ac-VDVAD-AFC)

Western Blot

Pellets and supernatants were prepared from differentiated human skeletal muscle (dhSKM) cells as described above and used for Western blot analysis. Protein samples (5–15g) were diluted in 4x sample buffer, heated for 5 minutes at 95°C prior to use.

RhoB western blots from whole cell lysates:

The dhSKM cells from 3,5cm cell culture dishes were washed twice with PBS. Cells were lysed by freezing with liquid nitrogen and subsequent thawing. 200μL of lysis buffer [1ml 5x Laemmli + 50μL PMSF (100 mM) + 4ml HS buffer (150mM NaCl, 50mM TRIS pH 7,4, 5mM $MgCl_2$)] was added and incubated for 5 minutes on ice. Cells were scratched off and incubated for 10 minutes at 37°C before destruction of DNA by brief and weak sonication (5'', 50% cycle, 15-20% power).

Gel electrophoresis

For RhoB detection denaturating 12% SDS-polyacrylamide (PAA) gels ***(Table 8)*** were loaded with highest possible volumes of probes (20-30 μL). Running buffer (0,025 M Tris; 0,192 M Glycin, 0,15% SDS) was precooled to 4°C. Gels were run at 100V (stacking gel) / 130V (separating gel) for approx. 120 minutes. Gels were stopped shortly before the blue dye was running out. Electrophoresis was carried out on BIO-

RAD apperatures and protein diffusion was lowered by constant cooling of the apperature with ice.

	separating gel 12%		***stacking gel 5%***	
H2O	3,24	ml	2,785	ml
30%-0.8% Acryl-Bisacryl	4	ml	0,835	ml
1,5M Tris pH 8.8	2,5	ml	---	
0,5M Tris pH 6.8	---		1,25	ml
10% SDS	100	µL	50	µL
10% APS	150	µL	75	µL
TEMED	10	µL	5	µL
final	**10**	**ml**	**5**	**ml**

Table 8. Gel composition for polyacrylamide gels.

Protein transfer

Proteins were transferred to nitrocellulose membranes (pore size, 0.45m; Schleicher & Schuell, Dassel, Germany) in precooled transfer buffer (Tris (50mM), Methanol (20-30%), Glycin (40mM)) at 100V for 60-80 minutes.

Preliminary protein staining was done by incubating the membrane in Ponceau S for 10 minutes and coloring was removed with MilliQ water. Remaining protein in PAA gels was stained with coomassie blue solution (0,5% coomassie brilliant blue R250, 50% methanol, 7% acetic acid) and destained with 20% methanol / 7% acetic acid in order to control for efficient protein transfer.

Immunodetection

Membranes were blocked with 5% BSA for 30-60 minutes. Primary antibodies were incubated for 1 hour at room temperature (Actin, Tubulin), at least 4 hours at room temperature (RhoB) or over night at 4°C (RhoB) respectively. Between antibody incubations, membranes were rinsed and washed 3 times for 10 minutes with volumes of at least 50ml of Tris-buffered saline including 0,2% Tween (0,2% TBS-T) (pH 7,4; 1 liter: 8,8g NaCl, 0,2g KCl, 3g Tris, 2ml Tween-20). After secondary antibody incubation, membranes were also rinsed and washed with TBS-T two times, followed by at least 10 minutes equilibration in TBS.

Immunodetection was carried out by the use of the following antibodies:

Primary antibodies:

Human RhoB (119): Polyclonal rabbit IgG antibody; diluted 1:250; Santa Cruz Biotechnology, Inc., Santa Cruz, CA

Human RhoB (C-5, sc-8048): Monoclonal mouse antibody; diluted 1:250; Santa Cruz Biotechnology, Inc., Santa Cruz, CA
Human Caspase-2 (ICH-1$_L$): Monoclonal mouse IgG1; diluted 1:1000; BD Biosciences Pharmingen (Becton, Dickinson and Company)
Human Actin (AC-40): Monoclonal mouse IgG antibody from Sigma Chemical Co. (St. Louis, MO) (1:2500)
Human Tubulin: Monoclonal mouse antibody; diluted 1:250; Santa Cruz Biotechnology, Inc., Santa Cruz, CA

Secondary antibodies:
Anti-mouse (derived from sheep, diluted 1:10.000) and anti-rabbit (derived from donkey, diluted 1:20.000) antibodies from Amersham Biosciences conjugated with horseradish peroxidase.e

Signal detection was carried out with enhanced chemiluminescence (ECL) detection system from Pierce Chemical (Rockford, IL) or for weak signals with ECL-plus from GE Healthcare (formerly Amersham Biosciences). Films (Kodak, BioMax X-Omat AR) were developed with a Kodak developer machine.

Reuse of membranes (stripping)
Membranes were incubated in stripping buffer (100mM mercaptoethanol, 2% SDS and 62,5mM Tris-HCl buffer pH 6,7) for 20-30 minutes. After stripping, membranes were reequilibrated in TBS for 3 x 5 minutes and blocked with 5% BSA for 30 minutes. Immuno detection was described as described above.

Band analysis
Protein standards obtained from Amersham Biosciences, UK were used for proper band assignment:
Low molecular weight: 14,4 / 20,1 / 30 / 45 / 66 / 97 kDa
The intensity of the bands of interest was quantified with Image J gel analyzer plugin (http://rsb.info.nih.gov/ij) and compared with the corresponding loading control.

Immunohistochemistry

For immunohistochemistry, differentiated human skeletal muscle cells were fixed with 4% paraformaldehyde for 30 min. The cells were washed with 50mM NH_4Cl and subsequently permeabilized for 5 min with a solution containing 0.1% Triton X-100 and 0.1% citrate in phosphate-buffered saline. The cells were blocked with 2% bovine serum albumin.

All antibodies were diluted 1:200 and incubated for 1 h at 37°C. Immunostaining of cells was visualized with a goat anti-rabbit Cy3-conjugated (diluted 1:500; Amersham Biosciences, Inc., Vienna, Austria) or goat antimouse Alexa 488-conjugated secondary antibody (diluted 1:500; Molecular Probes, Leiden, The Netherlands).

As a negative control, cells were treated under identical conditions without applying a first antibody. Images were collected using a confocal microscope from Carl Zeiss (Jena, Germany) equipped with an argon laser system (LSM 410). The digitized pictures were stored and analyzed off-line using Photoshop (Adobe Systems GmbH, Munich, Germany) and ImageJ software.

Plasmid extension in E. coli

The Rho binding domain of Rhotekin (GST-Rhotekin RBD pulldown assay) was expressed as a recombinant fusion with GST in *Escherichia coli*. The *E. coli* strain containing the plasmid with the Rhotekin Rho binding domain (RBD) was a kind gift from Prof. Dr. Christian Nanoff (Inst. of Pharmacology, Vienna Medical University, Austria).

An overnight culture was grown in 240ml LB medium containing 100µg / ml Ampicillin (Amp). The next day 6 liters of 100µg/ml Amp LB medium were inoculated and grown to an optical density of 0,5 - 0,8 (at 600nm) at 37°C. Induction of RBD expression was then induced with 0,5mM IPTG for 2 hours at 37°C. Bacteria were spun down at 5000rpm for 20 min at 4°C and resuspended in 60 ml bacteria lysis buffer. Lysates were then sonicated (6 x 15 sec, 25% power, 90% cycle) on ice and cleared by centrifugation (20.000g, 20 min). Supernatants were then rotated with 2ml (wet volume, 50:50) glutathione beads (Sigma) for 2 hours at 4°C. Following, beads were washed six times with 10ml washing buffer (1 sec spin down at ca. 1000rpm) and then stored in 4ml washing buffer plus 10% glycerol at -80°C.

<u>1 liter LB-Medium (pH 7,4):</u>

10g NaCl, 10g Trypton (Applichem A1553), 5g yeast extract (Applichem A3732).

Bacterial lysis buffer:

50mM Tris, pH 7,5; 1% Triton X-100; 150mM NaCl; 5mM MgCl2; 1mM DTT; 10µg/ml leupeptin; 10µg/ml aprotinin, 0,1mM PMSF

Washing buffer:

50mM Tris, pH 7,5; 0,5% Triton X-100; 80mM NaCl; 5mM MgCl2; 1mM DTT; 10µg/ml leupeptin; 10µg/ml aprotinin, 0,1mM PMSF

GST-Rhotekin RBD binding assay

The dhSKM cells were lysed as described for the caspase assays. Approximately 70% of the dhSKM supernatants was incubated with GST-RBD beads on 4°C for 90 minutes (volumes specified for each experiment). After incubation, supernatants were completely removed and beads were washed once with 500µL washing buffer which was then also completely removed. 30-90µL of 1 x sample buffer was then added to the beads and heated to 95°C for 10 minutes. Beads were spun down and the supernatant put on a 12% PAA gel for electrophoresis and a complete western blot procedure for detection of activated RhoB.

DNA-chip analysis

A concentration series with 1µM and 10µM Simvastatin (Sim) and a time series with 10µM Sim with incubation times of 2h, 12h and 24h were performed on dhSKM cells in two separate experiments as listed in the table below. Additionally cells were (simultaneously) treated with 1mM mevalonic acid (MA).

	2h (Exp. 2)	**12h (Exp. 2)**	**24h (Exp. 2)**	**24h (Exp. 1)**
Control	√	√	√	√
10µM Sim	√	√	√	√
1µM Sim	---	---	---	√
10µM Sim + 1mM MA	√	√	√	√
1mM MA	√	√	√	√

RNA purification

RNA was purified from dhSKM cells using Qiagen RNeasy® Mini kit. Therefore cells were grown and differentiated on 5cm cell culture dishes. Cells were harvested via trypsinisation for 5 minutes. Plasma membranes were lysed with provided buffer RLN. RNA wasm first precipitated with 96% ethanol and then purified using the provided spin columns as instructed. Purified RNA was kept in 40µL RNase-free water and stored at -80°C.

DNA-chip analysis

Human Genome U133 Plus 2.0 Array GeneChips® from Affymetrix, Inc. were used to hybridize our probes with the whole human genome. Reverse transcription of purified RNA, gene chip hybridization and the readout were done by Prof. Dr. Martin Bilban (Medical University Vienna). Data was analyzed using the following software tools (all freeware) under the guidance of Jacques Colinge, PhD (CEMM, Vienna):

RMA express 0.5 (Robust Multichip Average)

Converting raw data (CDF files) into Excel readable files and at the same time assigning Gene IDs to Affymetrix labels (CEL file). RMA express operates three steps: background adjustment, quantile normalization and summarization.

http://rmaexpress.bmbolstad.com/

Multi Experiment Viewer (MEV) 4.0

Statistical analysis like cluster building and significance tests.

http://www.tm4.org/mev.html

GenMAPP 2

Visual display of pathway regulations.

http://www.genmapp.org/

III. Results

DNA-Chip analysis

Affymetrix whole genome DNA-chips were used to obtain expression values of all human genes in differentiated human skeletal muscle cells (hSKM) under different treatment conditions. In a first experiment a concentration series of Simvastatin was performed after 24 hours of treatment. A time series over 2h, 12h and 24h was carried out in a second experiment for 10μM Simvastatin. Cells treated with the same conditions as described were additinally treated with mevalonic acid. This allows to filter for lipid independently regulated genes.

We used RMA Express software to obtain expression values from raw data files. An intensity threshold of 20 was used to filter weak signals. In a next step we filtered for fold changes of at least 2,3 in any condition. These conditions generated a list of 1007 genes which we feeded into „MultiExperiment Viewer“ software for further analysis.

First, a direct search for specific regulation patterns was performed. Therefore three patterns were created:
Lipid dependent: Increasing upregulation with rising concentrations of Simvastatin. Abrogation of upregulation with 1mM mevalonic acid (MA).
Lipid independent: Increasing upregulation with rising concentrations of Simvastatin, but no effect after simultaneous treatment with 1mM MA.
Apoptotic regulation: Strong upregulation after 1μM Sim, but no regulation after 10μM Sim. 1mM MA has a rescuing effect together with 10μM Sim.
Results obtained for p-values < 0,1 are displayed in ***Table 9***.

Second, an algorithm which performs distance measuring in order to cluster similarily regulated genes was applied to the 1007 genes. The method used for distance measuring was pearson correlation. One of the obtained clusters showed the properties of lipid dependent regulation and all of its 36 genes were also contained in the manual search for lipid dependently regulated genes ***(Table 10)***. This underlines the importance of these candidates.

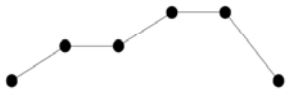
Lipid dependent	Lipid independent	Apoptotic
		
- 1 1 10 10 - **Sim** - - 1 - 1 1 **MA**	- 1 1 10 10 - **Sim** - - 1 - 1 1 **MA**	- 1 1 10 10 - **Sim** - - 1 - 1 1 **MA**
ACSS2	ACAT2	ANK3
ATP2B4	ACSS2	ATP2A1
C13orf33	ATP6V0E	C15orf5
C14orf78	C14orf78	CDK6
DREV1	DREV1	EHBP1L1
EMP3	FADS1	SEC31L1
FADS1	FCRLM2	
FCRLM2	HMGCS1	
GRK5	HMOX1	
HMOX1	KRT34	
KLF2	LOC284702	
KLF4	LOC641917 /// LOC644271	
KRT34	PDK4	
LOC221091	TM4SF1	
NCKAP1	TSC22D3	
OAS1		
PDK4		
PLA2G4A		
RAB27B		
RGC32		
RHEB		
RHOB		
RPTN		
SAMD9		
TM4SF1		
TMEM132B		
TNXA /// TNXB		
TNXB		
TSC22D3		
ZNF709		

Table 9. Results of direct cluster search (official gene symbols). The results displayed were obtained with „MultiExperiment Viewer“. 1007 strongly regulated genes were feeded into the software and matched for three different regulation patterns with a p-value of at least 0,1. The patterns are graphically displayed in the first column. Higher dots represent a relatively stronger regulation. Simvastatin (Sim) in μM, mevalonic acid (MA) in mM. *Lipid dependent*: Upregulation is prevented by simultaneous MA treatment. *Lipid independent*: MA has no preventing effect on Simvastatin induced upregulation. Apoptotic: Strong upregulation under 1μM Sim, no detection after 10μM Sim. MA partly prevents complete downregulation under 10μM Sim. *Some genes appeared more than once in the original list, since most genes are represented by three or more different DNA probes on the chip. These multiple hits were left out for reasons of clarity. Results are displayed in alphabetical order.*

Pearson correlation (lipid dependent)	
ATP2B4	PDK4
C13orf33	RAB27B
C14orf78	RGC32
EMP3	**RHOB**
FCRLM2	RPTN
GRK5	SAMD9
HMOX1	TM4SF1
KLF2	TMEM132B
KLF4	TNXA /// TNXB
KRT34	TNXB
LOC221091	TSC22D3
OAS1	ZNF709

Table 10. Results of pearson correlation (official gene symbols). Cluster analysis via pearson correlation led (among many other clusters) to this group of genes, which were regulated in a lipid dependent manner. All genes in this cluster have also been found after the direct search for a lipid dependent regulation pattern (**Table 9**). A p-values of < 0,1 was used for this clustering.

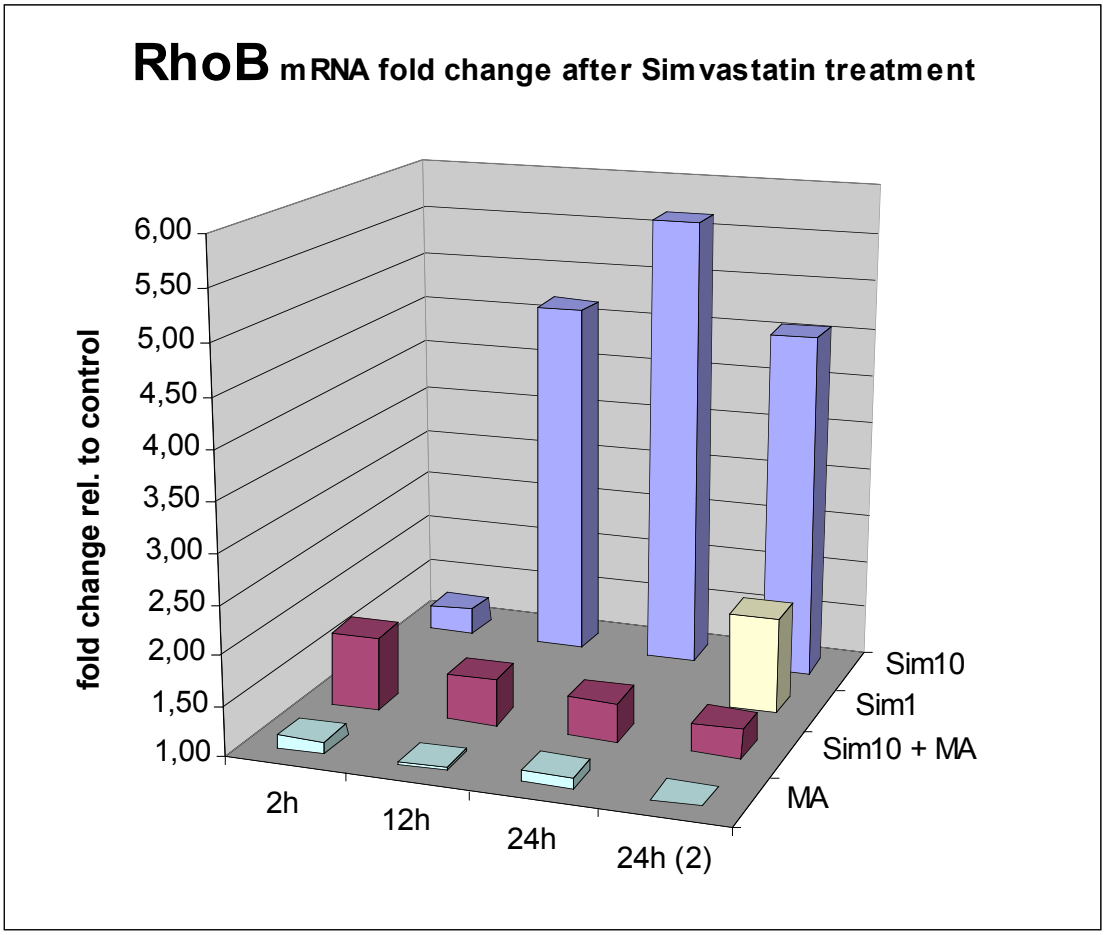
RhoB mRNA is strongly upregulated

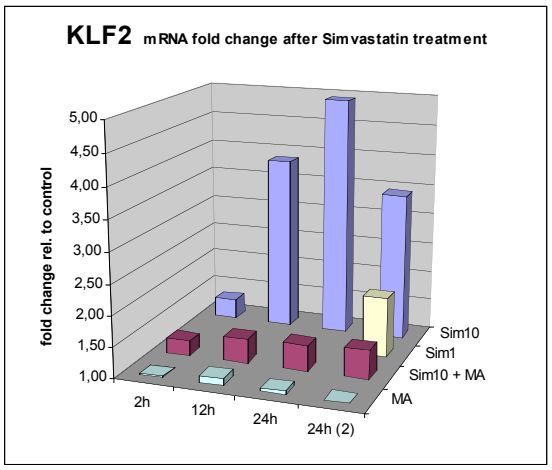
We chose to further investigate RhoB because of three reasons:

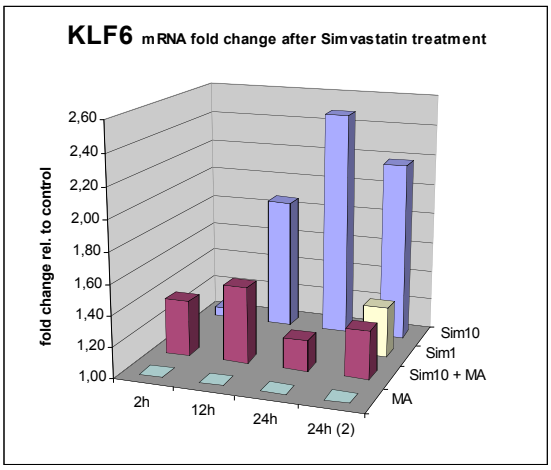
1. RhoB is among the top 10 regulated genes after 10μM Simvastatin treatment with two of its five representing probes on the chip.

2. RhoB is contained in both lists of lipid dependently regulated genes which were obtained from pattern search and from distance measuring. All RhoB associating GEF, GAP and GDI proteins are only slightly upregulated (< 1,5 fold). Thus, RhoB is causally linked to statin action (probably because of its farnesyl and geranylgeranyl membrane anchors).

3. RhoB was previously ascribed a role in regulation of apoptosis[99,100] and in indirect regulation of members of the Kruppel Like Factor (KLF) proteins (see *Figure 13* and [102]) which are also among the most significantly regulated genes. Regulation of RhoB, KLF2 and KLF6 is shown in ***Figure 13***. All these proteins are regulated in a very similar pattern.







RhoB	CTL	1µM Sim	10µM Sim	1mM MA	1µM Sim + 1mM MA	10µM Sim + 1mM MA
2h	364		462	409		642
12h	601		2809	622		886
24h (2 Exp)	774 ±7	1561	3948 ±261	734 ±77	767	1037 ±18

Figure 16. mRNA regulation of RhoB, KLF6 and KLF2. Y-Values are fold changes relative to control (CTL) in a logarithmic scale. X axis shows incubation times. „(2)“ marks an independent experiment of 24 hours. Z axis abbreviations: Sim = Simvastatin, concentrations in μM; MA = 1mM mevalonic acid. Original chip values of RhoB listed in the table below (± values represent std. deviation of two experiments).

Simvastatin leads to RhoB upregulation on protein level

To show upregulation of RhoB protein in Simvastatin treated differentiated human skeletal muscle (dhSKM) cells, we performed western blot analysis with total cell lysates. Therefore two commercially available antibodies (one monoclonal and one polyclonal) were used to detect RhoB. Unfortunately both of them show intense unspecific binding. For that reason, the mostly very weak band of RhoB at 25kDa must not be confused with the stronger lanes nearby, especially the band at approx. 28kDa.

Elevated RhoB levels and a Simvastatin concentration dependency could be approved ***(Figure 17)***. A noticeable upregulation occurs already after treatment with 1μM Simvastatin for 24h. 10μM Simvastatin remarkably induce RhoB protein expression. RhoB controls show almost no signal, even when large amounts of protein lysates are used. Moreover, Simvastatin induced RhoB levels are abrogated with mevalonic acid as shown in ***Figure 18***.

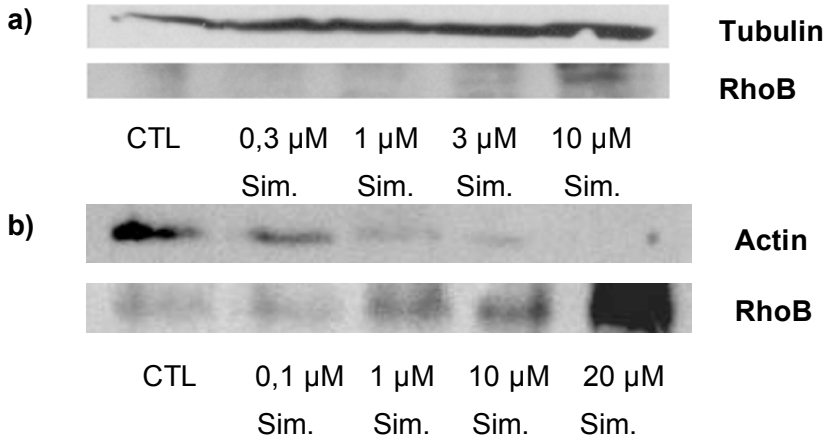


Figure 17. Simvastatin dependent RhoB upregulation in dhSKM cells. Experiments a) and b) were both treated for 16 hours with rising concentrations but different loading controls were used. RhoB is almost undetectable in untreated control cells (CTL) but increases with rising Simvastatin concentrations. Strong RhoB upregulation is visible with concentrations above 1μM. Membranes were cut for RhoB and Tubulin detection. **a)** The bands of the tubulin loading control are not clearly separated due to the large protein amounts necessary to detect RhoB. **b)** Actin has been used as an alternative loading control, but apparently actin levels are inversely proportional to RhoB levels and thus not practical as a loading control.

Ubiquitous proteins tubulin and actin were used as loading controls, what turned out to be complicated because of two reasons. First, they both make up for large parts of the whole protein in skeletal muscle cells. In order to detect the small amounts of RhoB,

large amounts of protein had to be used. Thus the bands of the loading controls are often not clearly separated (see Tubulin blot in ***Figure 17 a).***

Second, actin levels are sometimes inversely proportional to RhoB levels (see Actin blot in ***Figure 17 b)***. A reason for this phenomenon could be caspase activation in Simvastatin treated dhSKM cells[119] which we also show later on. Actin was one of the first substrates described for caspases[122]. To circumvent this problem, the cells have been lysed in lysis buffer prior to the addition of 4x Laemmli buffer and protein concentrations have been determined via Bradford. Equal amounts of protein were then loaded onto the gels.

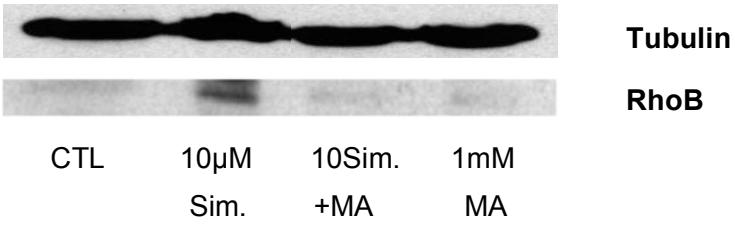


Figure 18. Reversibility of Simvastatin (Sim.) induced RhoB upregulation with mevalonic acid (MA). Control cells and cells treated with MA only show very low levels of RhoB. 10μM Sim. strongly induces RhoB. Simultaneous treatment with 10μM Sim. and 1mM MA leads to no RhoB upregulation.

Simvastatin induced RhoB is active

In order to perform its cellular functions, RhoB needs to be in an active, GTP-bound state. Therefore it is essential to know if the observed Simvastatin induced RhoB is GTP bound or not.

To check for active RhoB, we used a Rhotekin binding domain (RBD) pulldown assay. In order to perform this assay protein amounts of dhSKM cell lysate supernatants were determined via Bradford assay. Equal amounts of protein were incubated with equal amounts of RBD-GST-sepharose for one hour.

Unfortunately the Rhotekin binding domain precipitates also several other proteins besides RhoB, which, together with the low RhoB specificity of the available antibodies, explains why there is more than one protein visible on the nitrocellulose membrane after immuno detection.

A concentration dependent increase in active RhoB after Simvastatin treatment can clearly be seen in ***Figure 19 a)***. Moreover the upregulation and activation was abrogated to some degree by mevalonic acid. Mevalonic acid alone completely abrogated RhoB activation.

Several controls were performed to exclude an unspecific pulldown ***(Figure 19 c))***. First GST-sepharose without the GST-coupled RBD was incubated with control and Simvastatin probes and precipitated RhoB levels were compared with a simultaneously performed RBD-pulldown via westernblot. Unfortunately RhoB was also pulled down to some amount by GST-sepharose alone.

The second control was performed for unincubated RBD coupled GST-sepharose. In this case only one band at around 35kDa becomes visible. Since all RBD-pulldown blots show this band, it is most probably the Rhotekin binding domain which was detached from sepharose beads during probe heating in sample buffer.

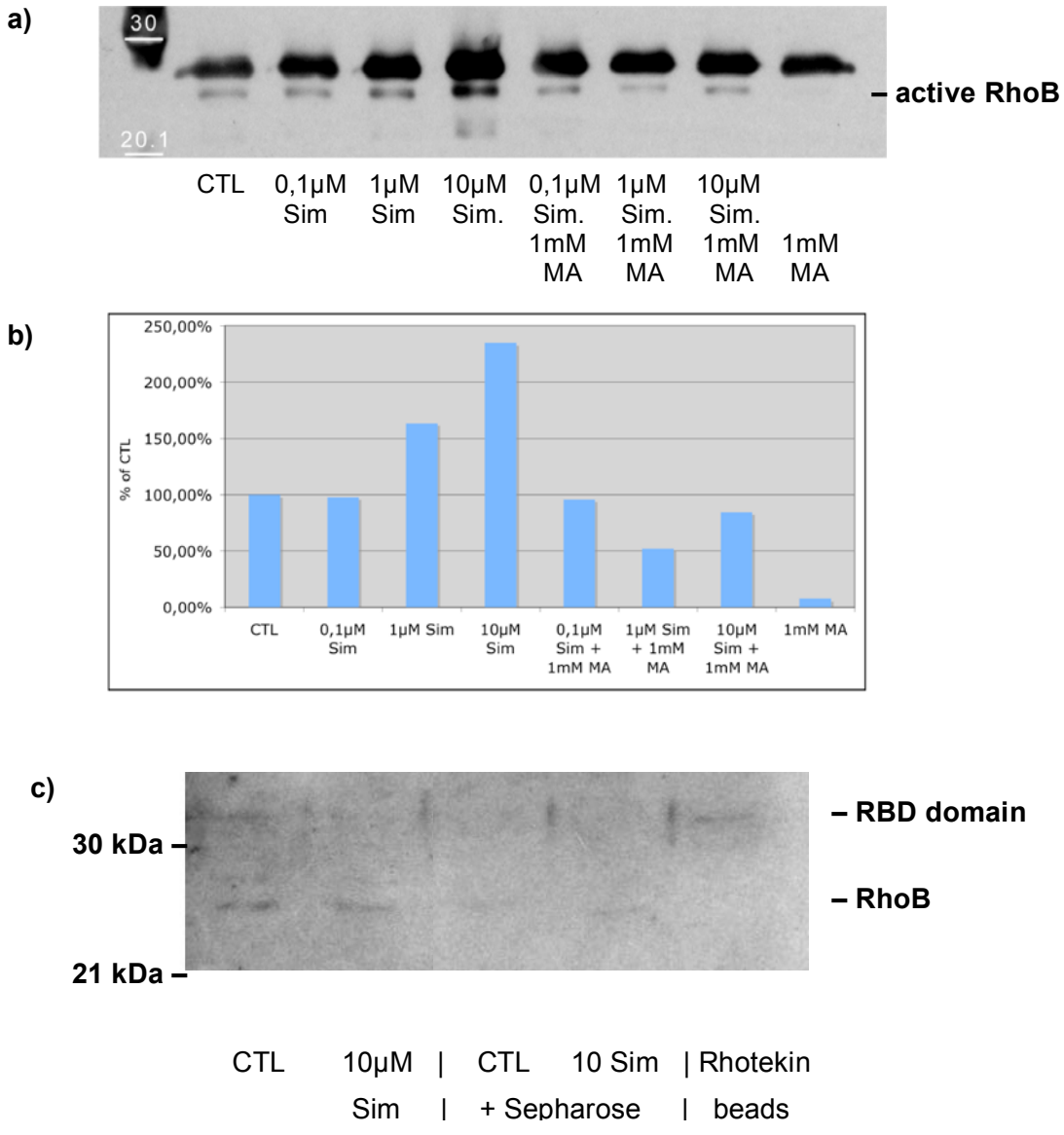


Figure 19. Rhotekin activation assay. dhSKM cells were treated with Simvastatin (Sim.) and/or mevalonic acid (MA) for 16 hours. Equal amounts of incubated cell lysates (130µg protein) were incubated with 120µL wet volume (ca. 55µL dry volume) RBD-GST-sepharose for 1h. a) RhoB western blot. The smaller band at 25kDa shows RhoB while the thicker band at approximately 28kDa most probably arises from unspecific binding of the anti-RhoB antibody to

another Rho protein. b) Quantitative analysis of RhoB bands as determined by ImageJ gel analyzer. c) Pulldown controls: First two probes after pulldown. Probes three and four were incuabated with GST-sepharose without coupled RBD domain. Unincubated RBD-GST-sepharose shows a band at approx. 35kDa which is probably the dettached RBD domain.

Simvastatin induces morphological changes in dhSKM

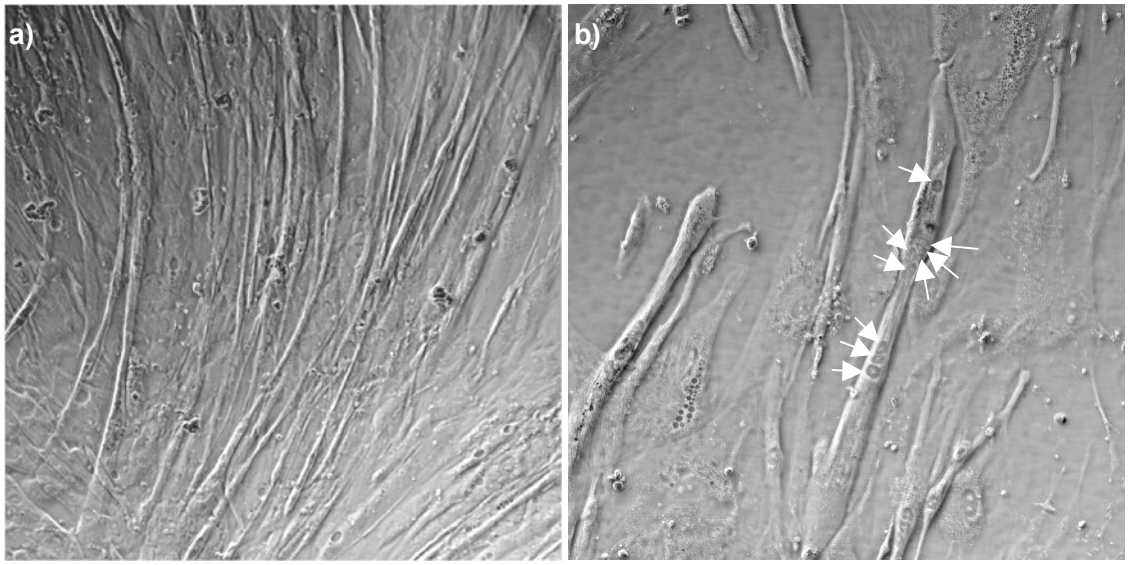


Figure 20. Untreated human differentiated skeletal muscle cells. a) Myotubes are neatly aligned in the more dense regions of the dish. 20x objective / 1x zoom. **b)** Newly formed, multi nuclei containing (arrows) myotubes can be better observed in less dense regions with a higher zoom factor (20x objective / 3x zoom). Pictures were taken with a confocal laser microscope in phase contrast mode 2 with a 20x objective.

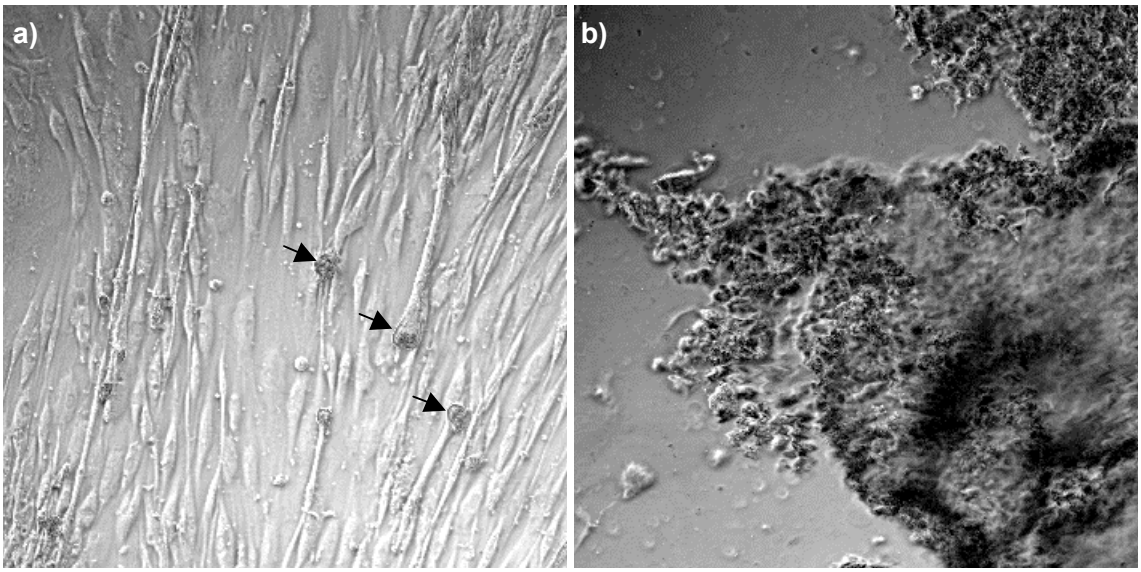


Figure 21. Simvastatin treated dhSKM cells after 15 hours. Controls for this experiment are shown in Figure 20 a) and b). **a)** Most cells treated with 2μM Simvastatin are already shortened and have lost their fibrous phenotype. A few rounded, black cells (arrows) show already an

apoptotic morphology. **b)** Treatment with 20μM Simvastatin results in a total loss of the original phenotype. All cells have dettached from the surface and are visible as dense, swimming clusters. Pictures were taken with a confocal laser microscope in phase contrast mode 2 with a 20x objective.

Human differentiated skeletal muscle cells were treated with different concentrations of Simvastatin for different times. After treatment with 10μM Simvastatin for 24h strong morphological changes could be observed. The fibrous phenotype of myotubes is lost, cells dettach from the surface and show typical rounded apoptotic structures. Higher doses lead to rounding quickly, but with enough time even low concentrations of 0,1μM Simvastatin lead to dettachment of the cells. ***Figure 21*** shows the changing morphology of a dhSKM cell layer after 2μM and 20μM Simvastatin treatment for 15 hours.

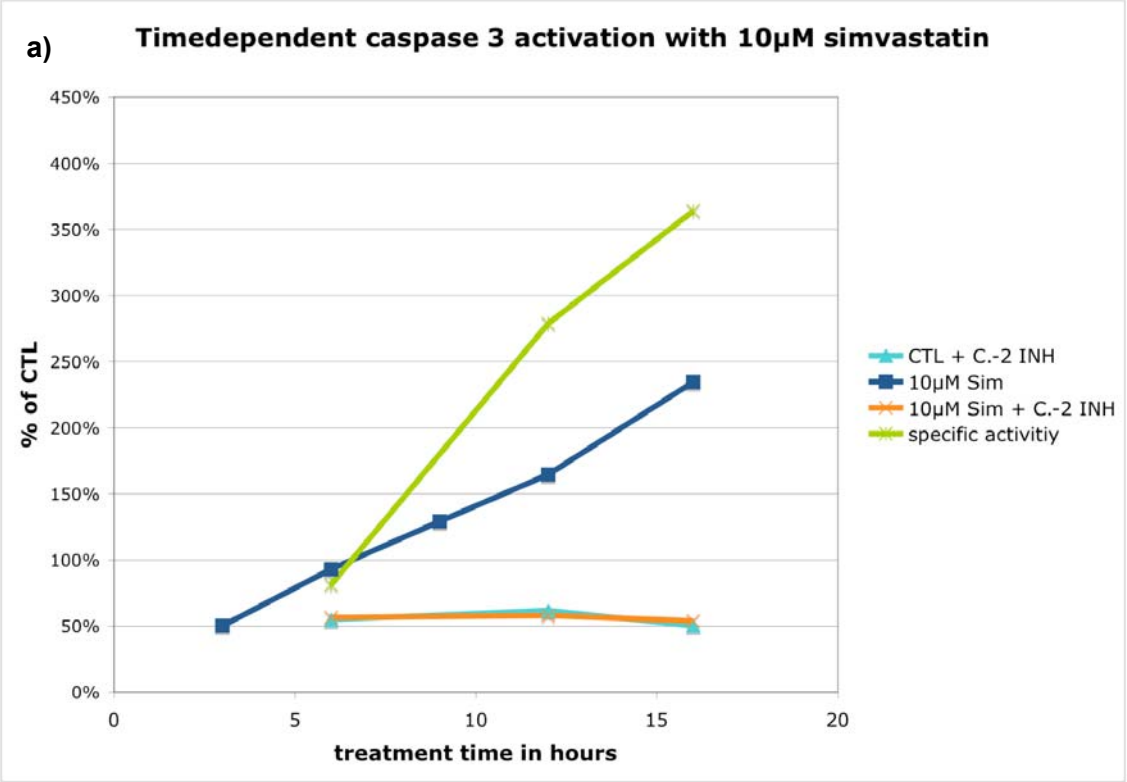
Caspase-3 and 2 are activated in parallel to RhoB

The morphological changes suggest involvement of an apoptotic process. This has been confirmed by caspase-3 activation in dhSKM cells after treatment with Simvastatin ***(Figure 22 a))*** as it has been shown before.[119] Furthermore we show that caspase-2 is activated by Simvastatin in a time dependent manner, too ***(Figure 22 b))***.
Caspase-3 is a central effector caspase. Thus, its activation shows clear ongoing apoptosis . After 16h of treatment with 10μM Simvastatin we measured an activity of about 230% in relation to an untreated control. With the same concentration of Simvastatin, no caspase-3 activation was detectable after 6h. Surprisingly after 3h of treatment caspase-3 was even only 50% as active as in control cells. Early RhoB activation could play an important role in this phenomenon, since RhoB is known for its role in positive and negative regulation of apoptosis.
Caspase-2 shows a very similar activation pattern with 10μM Simvastatin, although upregulation of total activity reaches only 175% after 16h. We used a caspase-2 inhibitor, which we added directly to the cell dishes in a final concentration of 2μM. With this inhibitor it was possible to determine not only the total caspase-substrate cleaving activity of the lysates, but in fact the specific activity of caspase-2. Therefore we substracted the remaining unspecific activity of inhibitor treated control and Simvastatin treated cells from the total activity values and calculated the activity in relation to control cells again.

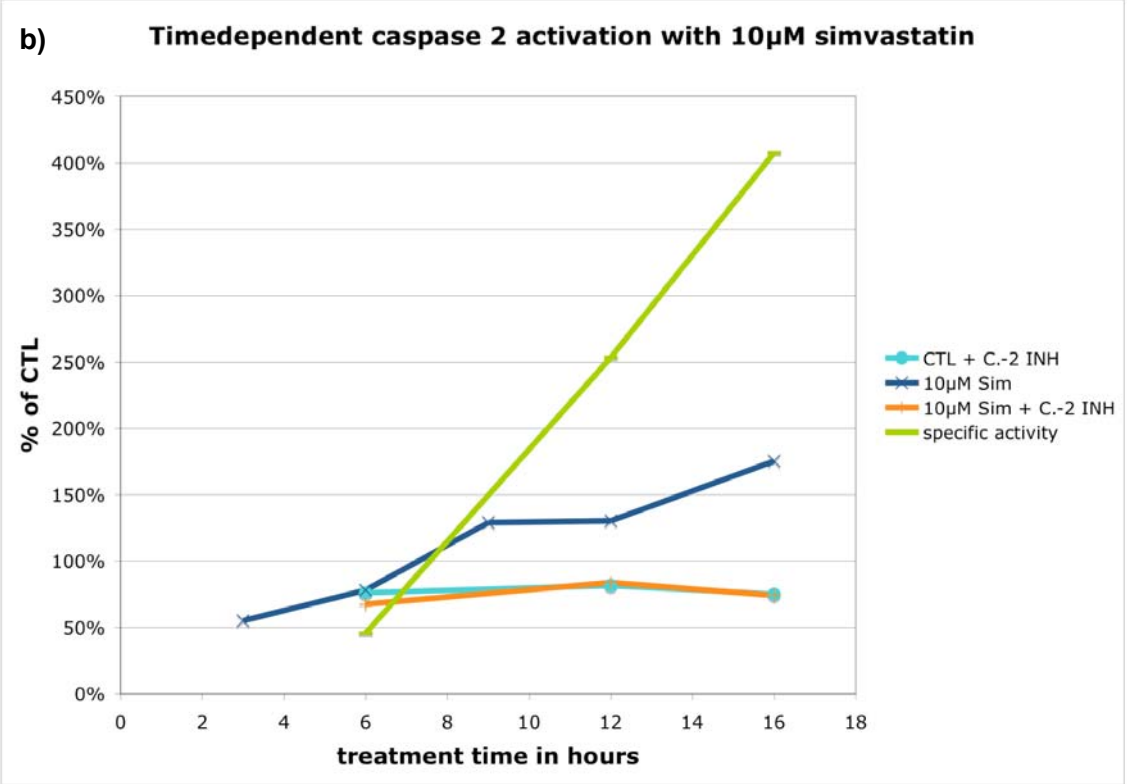
As a result we found that only 20-25% of caspase-2-substrate cleavage was specifically cleaved by caspase-2. This resulted in a specific activation of around 400% for caspase-2 after 16h with 10μM Simvastatin.
When measuring caspase-3 after treatment with the same caspase-2 specific inhibitor, we observed an activity inhibition of 50-60%. To determine if this caspase-3 inhibition was an indirect effect of caspase-2 inhibition or just unspecifity of the caspase-2 inhibitor, we added the inhibitor to lysates before measurement instead of adding it to the growing cells. The same effects could be observed, which leads to the conclusion that the inhibitor has some specificity for caspase-3, too (Data not shown: The probes used for ***Figure 23*** were treated with caspase-2 inhibitor only shortly before measurement. A second measurement showed that caspase-3 activity was also inhibited).
Thus it should be legitimate to calculate specificity values for caspase-3 in the same way as it was done for caspase-2. After 16h the specific caspase-3 activity induced by 10μM Simvastatin was 360%.

Complete reversibility of caspase-2 activation (values even lower than in the control cells) can be achieved by adding 1mM MA to dhSKM cells in parallel to Simvastatin. We also tried to reverse the effect by adding 10μM Farnesyl pyrophosphate (FPP) and 10μM Geranylgeranyl pyrophosphate (GGPP), respectively. GGPP had no effect but FPP could rescue around 50% of 10μM Simvastatin induced apoptotic activity.
Interestingly, addition of 1mM MA or 10μM GGPP alone induced specific caspase-2 activity to a similar level of more than 150%, while 10μM FPP did not have an effect.
These results underline the importance of membrane anchors for RhoB localization and thus its proper action. GGPP is responsible for RhoB's endosomal localization and FPP promotes a shift to the plasma membrane. A sensitive equilibrium between both lipid anchors is important to distribute RhoB correctly in the cell.

a)


Timedependent caspase 3 activation with 10µM simvastatin
450%
400%
350%
300%
250%
200%
150%
100%
50%
0%
% of CTL
0
5
10
15
20
treatment time in hours
CTL + C.-2 INH
10µM Sim
10µM Sim + C.-2 INH
specific activitiy

b)


Timedependent caspase 2 activation with 10µM simvastatin
450%
400%
350%
300%
250%
200%
150%
100%
50%
0%
% of CTL
0
2
4
6
8
10
12
14
16
18
treatment time in hours
CTL + C.-2 INH
10µM Sim
10µM Sim + C.-2 INH
specific activity

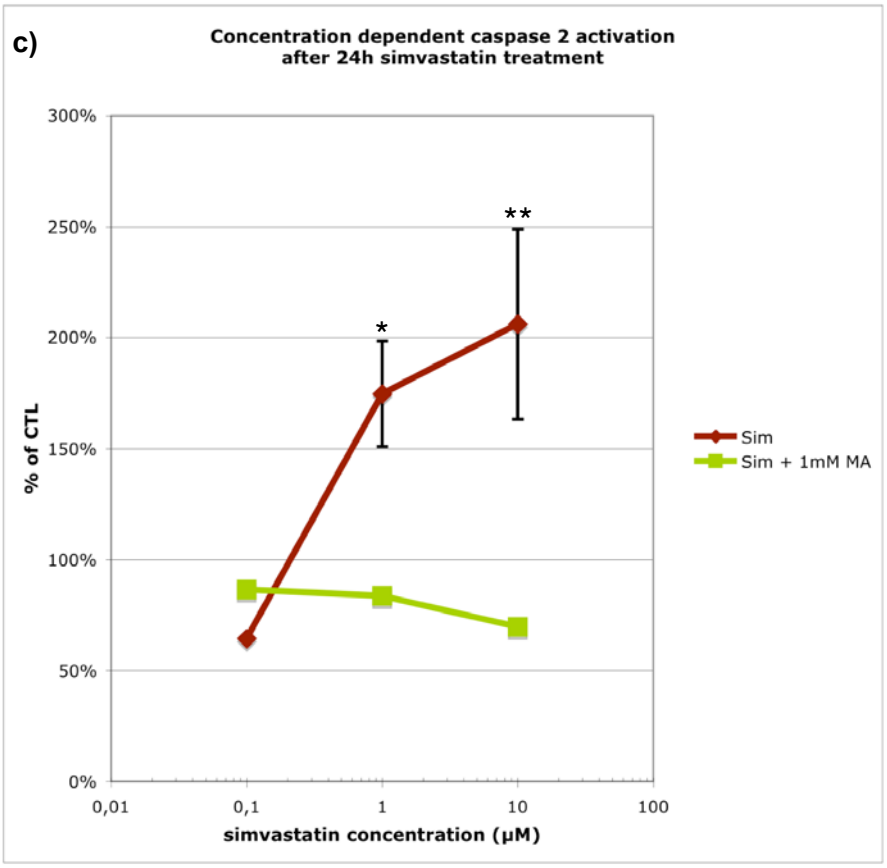


Figure 22. Caspase-3 and caspase-2 are activated by the addition of Simvastatin (Sim) in dhSKM cells in a time- and concentration dependent manner. The activation of both caspases can be abrogated by the addition of 2μM caspase-2 specific inhibitor (INH) to living cells. Specific activities are then obtained by substracting inhibited values from total values and calculating the percentage in relation to the control. **a)** Caspase-3 shows an absolute activity of up to 230% and a specific activity of 360% after 16 hours of treatment, compared to untreated cells (CTL). Caspase-2 inhibitor also inhibits caspase-3 activity very effectively. Controls and treated cells both show caspase-3 levels of around 50% of CTL at all measured timepoints. Two independet experiments were combined into one figure. **b)** Caspase-2 could be activated up to 175% of CTL after 16 hours. Moreover a downregulation by 50% could be observed after 6 hours. Caspase-2 inhibitor reduced caspase-2 substrate cleavage in control cells by ca. 20-25% at all timepoints. **c)** Caspase-2 is being activated with rising Simvastatin concentrations.

Errorbars show standard error of three independent experiments which were carried out in duplicates. Statistical significance for multiple comparison was calculated with ANOVA and post hoc Scheffé's test (*, $p < 0,1$; **, $p < 0,05$).

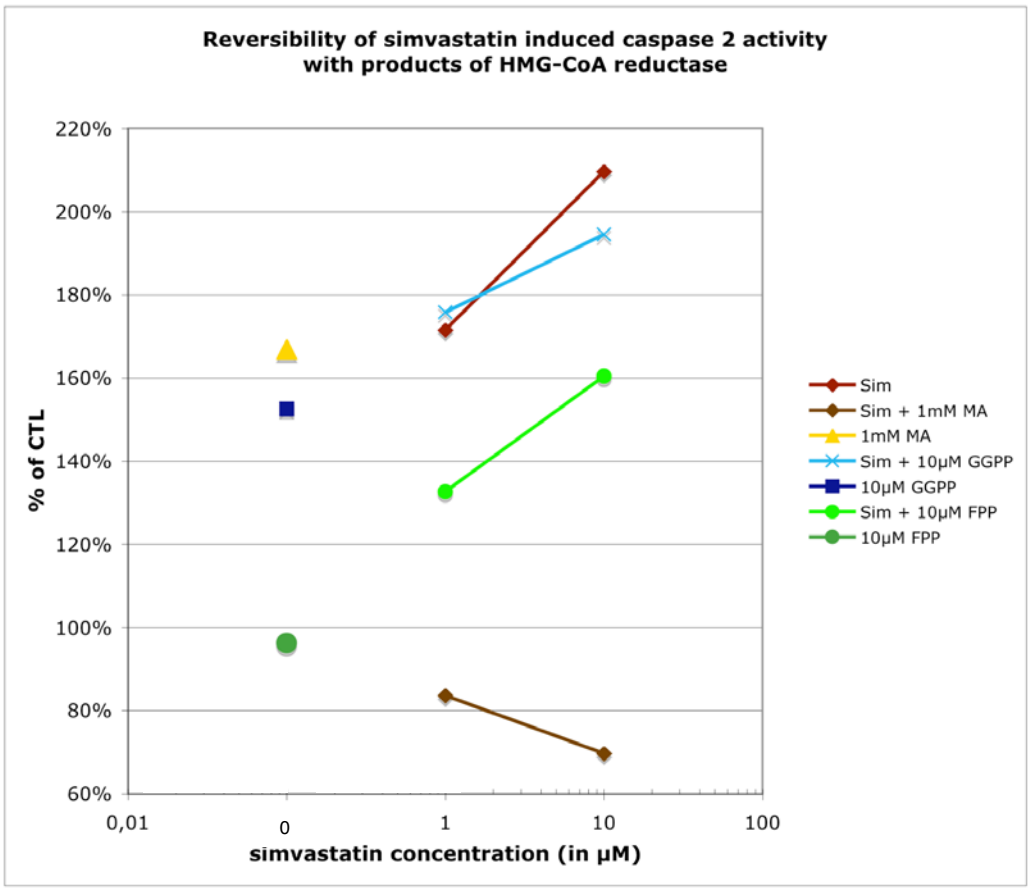


Figure 23. Reversibility of Simvastatin (Sim) induced caspase-2 activity with products of the HMG-CoA reductase pathway in dhSKM cells treated for 24 hours. Mevalonic acid (MA, 1mM), geranylgeranyl pyrophosphate (GGPP, 10μM) and farnesyl pyrophosphate (FPP, 10μM). Specific activity values were obtained by adding caspase-2 inhibitor to lysates shortly before use. While higher concentrations of Simvastatin induce caspase-2, 0,1μM to reduce its activity. MA reduces Sim induced activity to levels lower than control. MA alone strongly activates caspase-2. GGPP and FPP could only reduce Sim induced effects by a small extent. GPP alone induces caspase-2 activity while FPP acts neutrally. *Dublicates. Error bars show standard deviation. Caspase-2 inhibitor reduced measured activities in all probes to a level of 40-60% of control cells.*

Simvastatin treatment leads to a loss of RhoB's vesicular localization pattern

Immunohistochemistry of dhSKM cells grown on special cover slip dishes shows the intra cellular localization of RhoB. The same monoclonal antibody was used as in the western blots.

RhoB is is only abundant in a very small amount in control cells. A vesicular localization can nevertheless be detected. 10μM Simvastatin are brighter, but RhoB seems to be totally mislocalized. No more vesicular structures are detectable. A combination of

10μM Simvastatin and 1mM MA leads to the interesting observation that RhoB is still abundant but a vesicular-like localization pattern becomes visible again.
Lysosome staining with lysotracker to check for a possible colocalization with the observed vesicular structures was unfortunately unsuccesful.

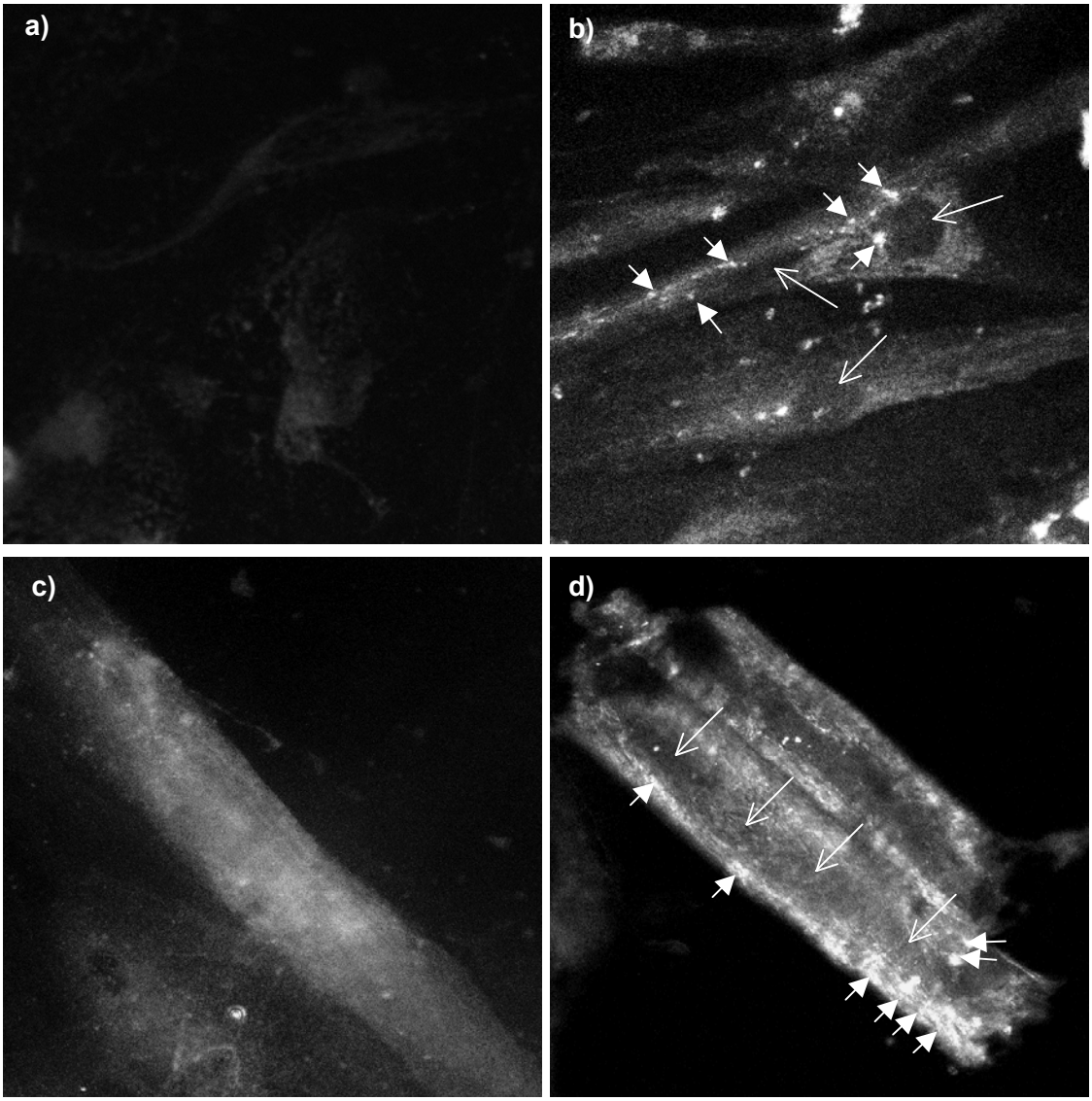


Figure 24. Immunofluorescene (monoclonal RhoB antibody) after 16h treatment of dhSKM cells. a) Secondary antibody negative control. **b)** Untreated cells. Fluorescence is clearly stronger then the negative control. The cells show accumulations of RhoB (filled arrows) which could be due to a vesicular localization. Several nuclei are visible (stroke arrows). **c)** Cells treated with 10μM Simvastatin appear much brighter but RhoB appears to be localized into the cytosol rather than to vesicles in a large extend. **d)** 10μM Simvastatin and 1mM mevalonic acid show a very bright staining close to the plasma membrane. Vesicular-like structures (filled arrows) can also be identified outside of the nuclei (stroked arrows) again. Pictures were taken with a confocal laser microscope with an 40x objective and 2x zoom.

Lethal toxin upregulates RhoB

lethal toxin (LT) from *Clostridium sordellii* deactivates Ras proteins which leads to a subsequent upregulation of Rho proteins. HIV patients who are infected with this pathogen show similar skeletal muscle cell damage as patients under Statin treatment do. Moreover an upregulation of RhoB in fibroblasts as a result of LT treatment has already been published. We used this system as a comparison to Simvastatin and we could show an upregulation of RhoB in LT treated dhSKM cells in a time dependent manner ***(Figure 25).*** An increase in RhoB levels is already visible after 4 hours incubation with 1*µ*g/ml LT. Amounts increase only slightly until 12h, but no more RhoB is detectable after 24h. However the according loading control is also very low. In another experiment which is shown in ***Figure 27***, small amounts of RhoB are still detectable after 16h.

When treated for 12h with Simvastatin concentrations from 0,3*µ*g/ml up to 10*µ*g/ml LT, RhoB upregulation is visible at all concentrations, but the intensity is not increasing with rising concentrations. It is possible that the concentrations chosen were already too high. This becomes more evident when looking at LT induced caspase activation, which is stronger in a concentration of 1*µ*g/ml than 4 *µ*g/ml after 24h ***(Figure 32).***

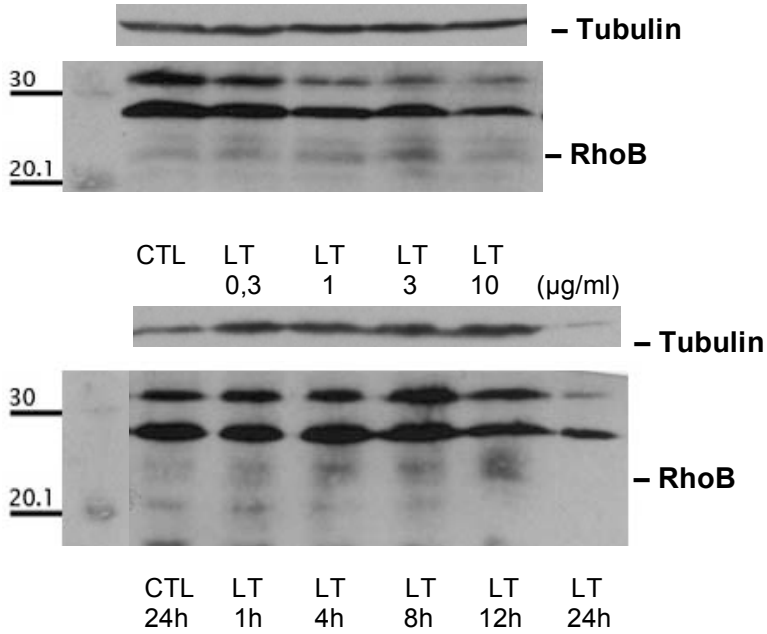


Figure 25. Concentration and time dependency of RhoB upregulation in dhSKM cells under lethal toxin treatment after 16 hours. a) Cells were treated with different concentrations of LT for 12 hours. Strongest RhoB upregulation could be detected with a concentration of 3*µ*g/ml. **b)** Time series with 1*µ*g/ml lethal toxin (LT). RhoB is upregulated in a time dependent manner with the strongest upregulation after 12 hours. After 24 hours no RhoB but also almost no tubulin is present.

C3 inactivates RhoB

C3 is a bacterial toxin from *Clostridium botulinum* which deactivates Rho proteins by ribosylating their GTP binding site. Inactivation of Simvastatin induced RhoB through C3 could lead to new insights into the role of RhoB in caspase activation.

Cells treated with 1 or 2 μg/ml C3 for 12 hours show similarly to control cells almost no detectable RhoB. When cell lysates of LT treated cells are loaded on the same gel as C3 + LT treated cell lysates, a band shift due to ADP-ribosylation is obvious. This ensures that C3's is enzymatically active. Moreover the experiement shows that C3 seems not to have an intense influence in regulation of total RhoB amounts.

A simultaneous treatment with C3 and Simvastatin has a similar effect as the combination of C3 and LT, although an even higher RhoB amount can be observed.

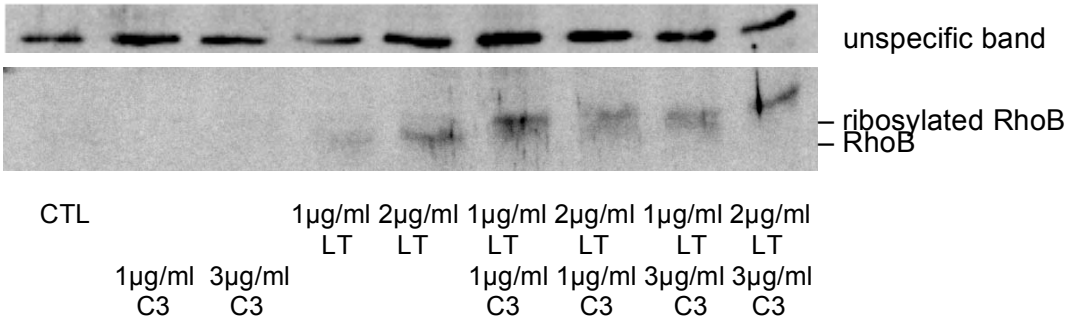


Figure 26. RhoB regulation by C3 and lethal toxin after 12 hours. RhoB inactivation by C3 is not compensated by upregulation of the general RhoB amount. Lethal toxin induces RhoB in a concentration dependent manner. This inducing effect is not reversible but induced even more by simultaneous treatment with C3. However, a band shift can easily be seen in cells treated with C3, which is due to ADP-ribosylation of RhoB by C3 and ensures C3's enzymatic activity.

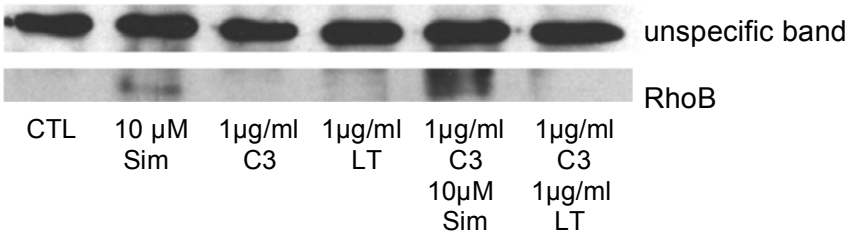


Figure 27. Influence of Simvastatin, C3, LT and combinations of the latter on total RhoB amounts after 16 hours. This blot confirms RhoB upregulation upon 10μM Simvastatin treatment. C3 does not alter total RhoB levels. 1μg/ml lethal toxin induces RhoB slightly. Interestingly, Simvastatin induced RhoB upregulation is even enhanced by the addition of C3. The effect of C3 on LT induced RhoB is not completely clear from this blot, which might be the result of a wetting problem on parts of the last lane.

Treatment with Simvastatin, lethal toxin and C3

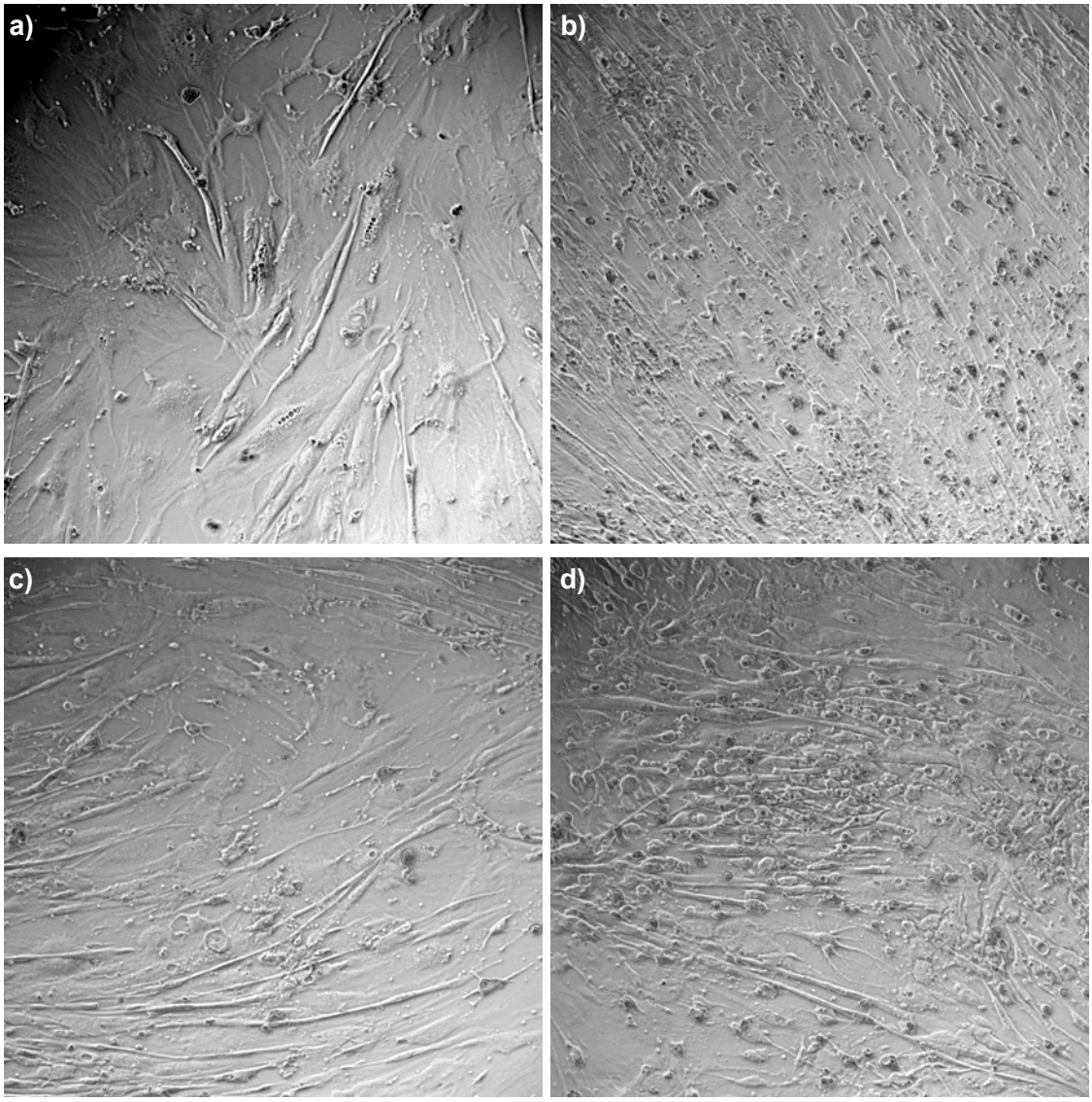


Figure 28. C3 + Simvastatin treated dhSKM cells after 16 hours. Controls for this experiment are shown in Figure 20 a) and b). **a)** Cells treated with 120μg/ml C3 show already severe morphological changes. Many cells have a rounded shape and retraction of the cellular fibers can be observed. **b)** 240μg/ml C3 treatment results in severe rounding and shortening of myotubes. Many retracted cells (black spots) are visible. We could not observe a rescuing effect of C3 on Simvastatin treated cells after treating cells with both substances for 16 hours: **c)** 10μM Simvastain + 120ng/ml C3 **d)** 10μM Simvastain + 240ng/ml C3. Pictures were taken with a confocal laser microscope with a 20x objective.

Intruigingly, morphological changes do not always correlate with caspase activation. When we performed caspase assays on C3 treated cells, an increase of 5 to 6 times compared to control cell actvitiy was visible after 24h ***(Figure 32)***, all cells have rounded up and detached from the dish ***(Figure 29)***. LT treated cells changed their morphology only by rounding and shortening to a little extend but showed elevated

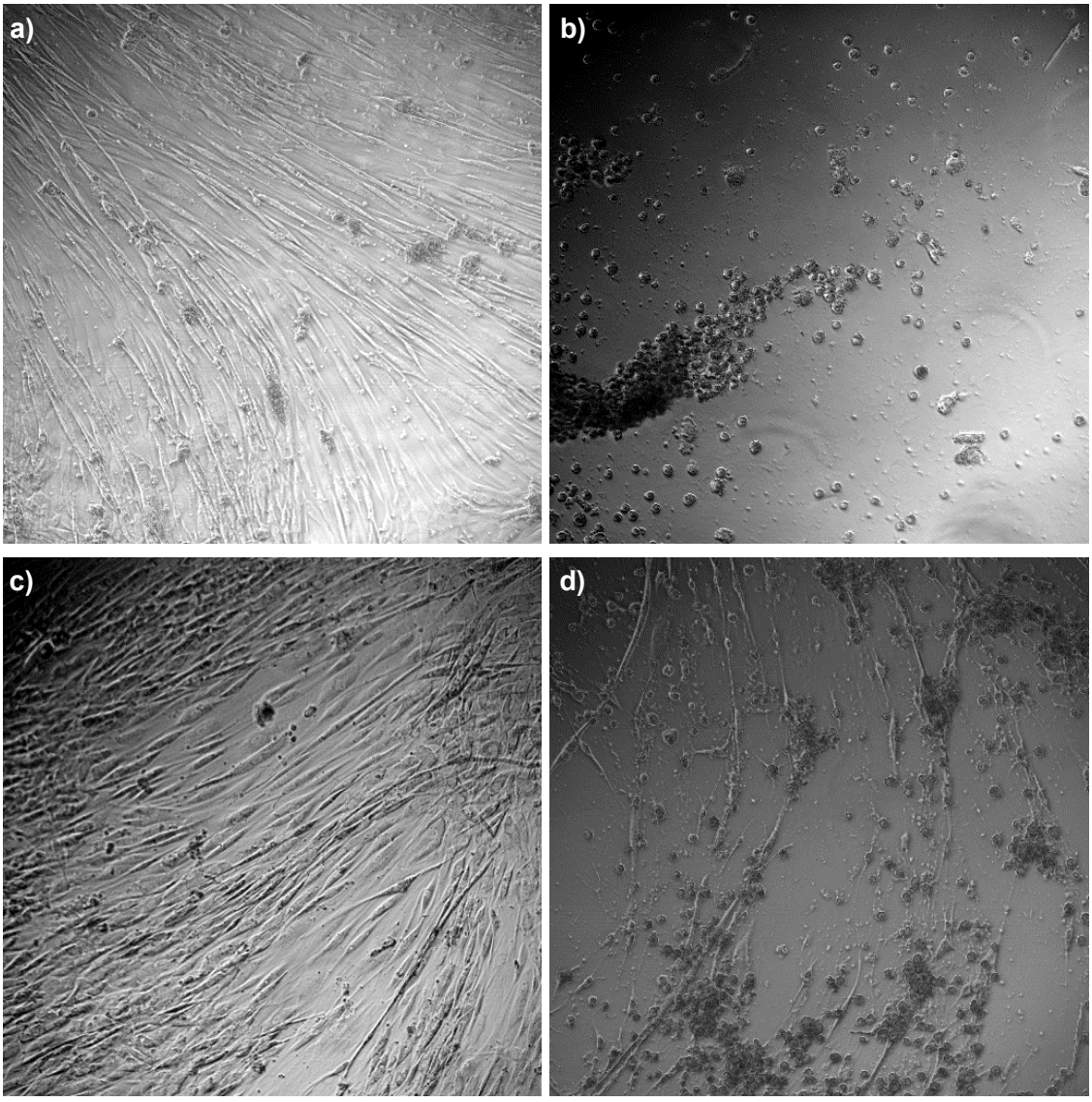


Figure 29. C3 + lethal toxin treated dhSKM cells after 15 hours. a) Control cells **b)** 1μg/ml C3 treatment results in complete rounding and detachment of the underground of all cells. **c)** Cells treated with 1μg/ml lethal toxin show morphological changes only in a relatively small extent. Alignment of cells is still visible, although they are a bit shortend. **d)** It seems that simultaneous treatment with 1μg/ml C3 and 1μg/ml lethal toxin saves a few cellular structures from detaching. Nevertheless most cells show the same changes as with C3 treatment alone. Pictures were taken with a confocal laser microscope with a 20x objective.

caspase activities of up to 14 fold of control. Simultaneous treatment with LT and C3 led to elevated caspase-3 activity that was twice as high as the one induced by LT alone after 16 and 24 hours. Simvastatin and C3 add up to a higher activity as well. Interestingly results differed, when dhSKM cells were treated with C3 for only 12 hours. Caspase-3 activity levels were reduced by C3 after 12h to a small extent. Experiments for even shorter treatment periods would probably give a better insight in the supposably early caspase-activity inhibiting effects of C3.

C3 mediated inactivation of RhoB in LT induced apoptosis

Caspase-2 was activated in dhSKM cells after 12h and 16h when incubated with lethal toxin. Thereby caspase activity rises over time and concentration ***(Figure 30, 31)***. Caspase-2 was induced 5 fold in relation to untreated control cells after 16h by 1μg/ml LT, but simultaneous inactivation of RhoB by C3 doubled this activity to 10 fold instead of reducing it. When the same concentrations were used to treat cells for only 12h, the results were different: LT induced activation was only 130% but it could be reduced to 110% by C3. Other LT and C3 concentrations were in line with this finding, only one value fell out of the pattern (1μg/ml LT, 1μg/ml C3). The caspase-2 deactivating effect could eventually be stronger after even shorter incubation times. The main issue is to find the time where caspase activation with LT alone is already significant but yet not toxic enough to trigger apoptosis via different pathways.

After 24h 1μg/ml LT and C3 induce caspase-3 to levels of 14 fold and 6 fold ***(Figure 32)***. It is interesting that C3 induces caspase activity after 16h and 24h but decreases it by 15-20% after 12h. It seems that apoptosis is downregulated in a first step and than activated due to the strong toxicity of this compound which ribosylates other Rho proteins, too.

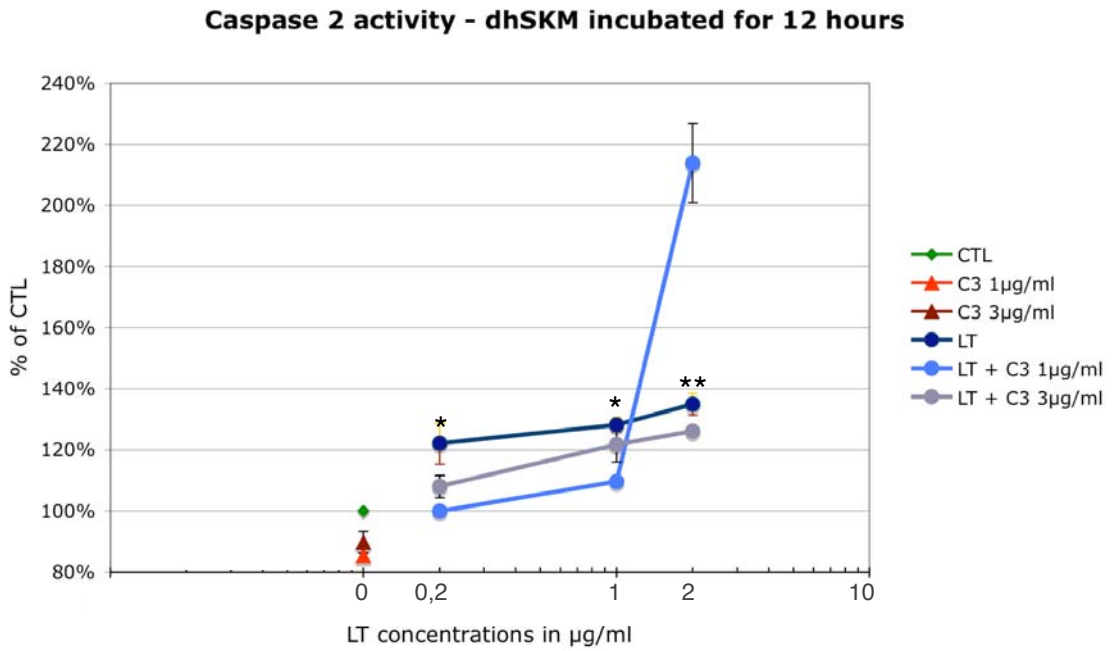


Figure 30. Caspase-2 activation in dhSKM cells treated for 12 hours with different concentrations of C3 and lethal toxin. LT induced caspase-2 activity is reversible by 1μg/ml C3 to a level near the control. However this is only true for 0,2 and 1 μg/ml LT; 2μg/ml show a huge induction of activity. C3 in a very high concentration of 3μg/ml also reduced the induced activity but only to a smaller extent. Errorbars show standard deviation of dublicates. A Caspase-2 inhibitor reduced the measured activities in all probes to a level of 40-60% of control (data not shown). Errorbars show standard deviation of duplicates. Statistical significance for multiple comparison was calculated with ANOVA and post hoc Scheffé's test (*, $p < 0,1$; **, $p < 0,05$).

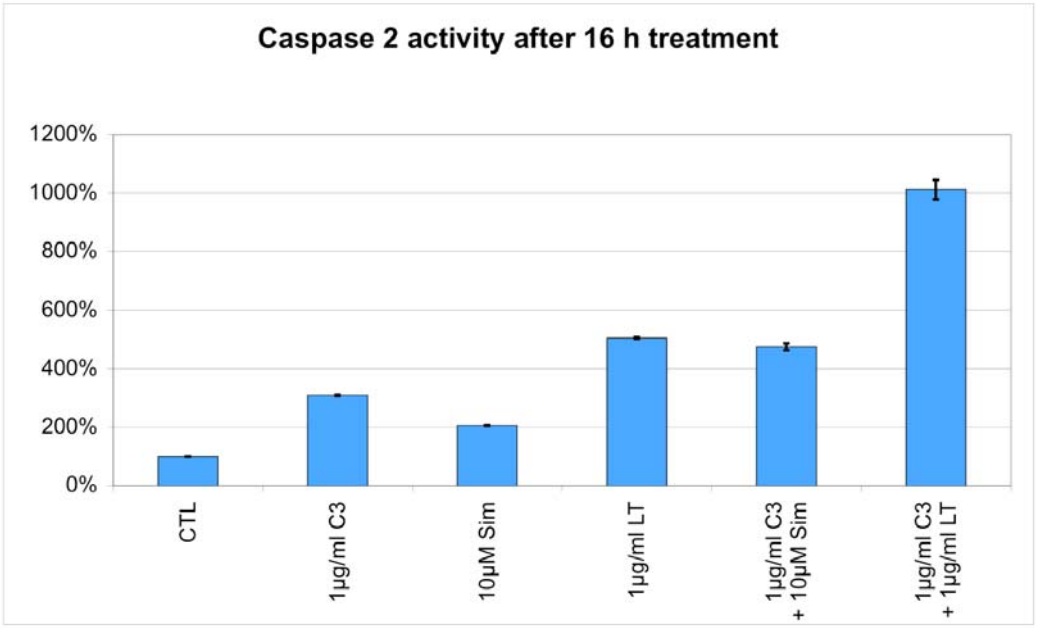


Figure 31. Caspase-2 activation in dhSKM cells treated for 16 hours with different concentrations of C3 and lethal toxin. All agents induced caspase-2 activity. C3 was not able to reduce Simvastatin or LT induced activity after 16 hours. Instead it induced caspase-2 activity even more. Errorbars show standard deviation of dublicates.

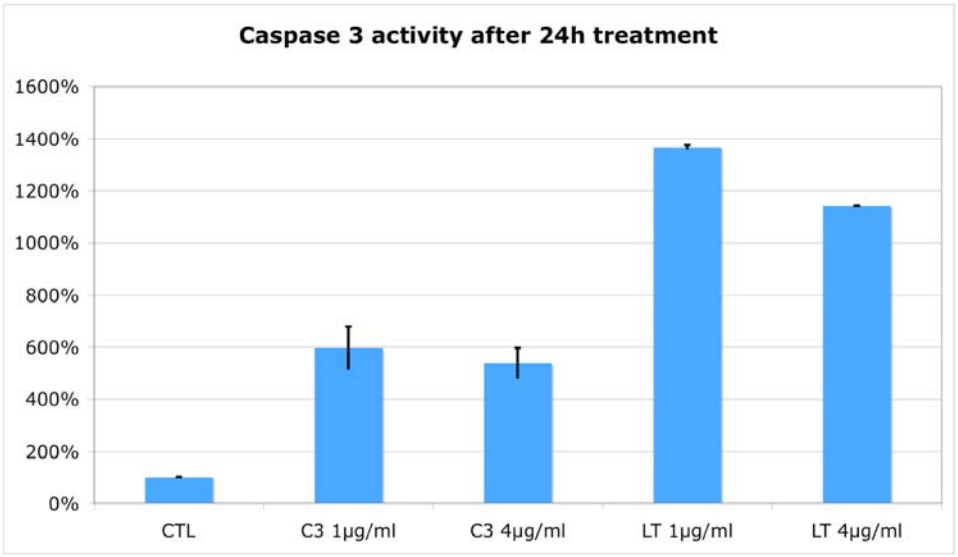


Figure 32. Caspase-3 activation in dhSKM cells treated for 24 hours with different concentrations of C3 and lethal toxin. C3 and LT both strongly induced caspase-3. C3 toxin activates caspase-3 only half as strong as LT. Higher concentrations of the toxins did not result in higher caspase activity. Errorbars show standard deviation of dublicates.

RhoB cleavage by caspase-2?

A western blot against caspase-2 shows a clear caspase-2 upregulation and procaspase-2 cleavage in 10μM Simvastatin treated dhSKM cells. The activation was abrogated with mevalonic acid. These results are in line with the findings we obtained with caspase activity assays. Additionally we treated cells with caspase-2 inhibitor. This inhibitor does neither cause an upregulation of the caspase-2 protein nor does it induce procaspase cleavage. But when the inhibitor is added to cells simultaneously with Simvastatin, it prevents Simvastatin mediated caspase-2 upregulation ***(Figure 33)***.

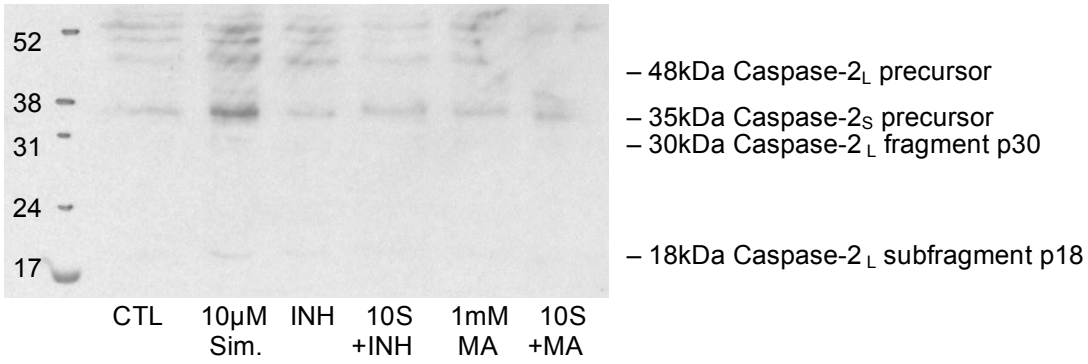


Figure 33. Caspase-2 western blot of dhSKM cell lysates treated for 16h. The blot shows several caspase-2 precursors and fragments which are produced by caspase mediated cleavage. 10μM Simvastatin were used to induce caspase-2. The caspase-2 inhibitor (INH) was added in a concentration of 2μM. The concentration of mevalonic acid (MA) was 1mM.

In order to see if the caspase-2 inhibitor is able to prevent Simvastatin mediated upregulation of RhoB, a westernblot against RhoB was carried out ***(Figure 34)***. Interestingly the inhibitor itself induced RhoB to some amount. An explanation for this effect could be that RhoB is a target of caspase-2, which can no longer cleave RhoB when it is inhibited. RhoB's short half life of 2 hours supports this idea. Cells that were simultaneously treated with Simvastatin and the inhibitor showed lower RhoB amounts than Simvastatin treated cells. Eventually this could mean that caspase-2 is needed to induce RhoB.

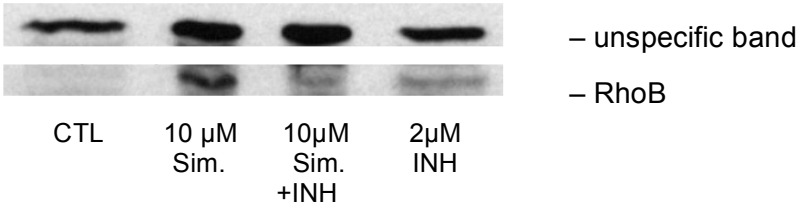


Figure 34. RhoB western blots of dhSKM cell lysates treated for 16h. 10μM Simvastatin strongly induced RhoB while 2μM caspase-2 inhibitor (INH) had a weak upregulating effect RhoB. The inhibitor abrogated RhoB upregulation to some amount.

IV. Discussion

Statins are well tolerated inhibitors of the HMG-CoA reductase which reduce the risk of cardio vascular and cardiac events by about 30%. Thus they are prescribed to millions of patients worldwide and belong to the group of blockbuster drugs with revenues of more than one billion US dollar per year.

Despite their effectiveness in reducing blood cholesterol levels, statins often elicit side effects which especially affect skeletal muscle. While most statin induced skeletal muscle related side effects result in muscle pain or sometimes myositis, an estimated 50 patients have died of rhabdomyolysis caused by Bayer's drug Lipobay® (Cerivastatin). The drug was therefore voluntarily withdrawn from the market by Bayer in August 2001 and the molecular backgrounds of these side effects have since been subject of intensive research.

Principal mechanisms of statin action on cholesterol synthesis are well understood, but the molecular cause of most side effects still remains unclear. We performed several whole human genome Affymetrix gene chip analyses with mRNA obtained from differentiated human skeletal muscle (dhSKM) cells treated with Simvastatin. The results approved our textbook knowledge of Statin action, since almost all of the enzymes in the cholesterol synthesis pathway were compensatorily upregulated and also among the strongest regulated genes.
Among the top ten significantly regulated genes we identified RhoB as one of the most interesting candidates. Beneath RhoB we found members 2, 4 and 6 of the Kruppel-Like-Factor (KLF) family, which are similarily strong upregulated and show a regulation pattern analog to RhoB. These KLFs act downstream of Rho proteins and influence the activity of transcriptions factors NFkB and JUN, which are known to affect cellular functions like proliferation, inflammation and phagocytosis.

Since RhoB acts upstream of KLFs, we decided to focus on this small G-protein. Its mRNA levels are highly upregulated upon Simvastatin treatment in a time and concentration dependent manner ***(Figure 16)***. RhoB belongs to the group of Rho proteins, whose most obvious roles are in modifying the cytoskeleton. RhoB has not yet been intensively investigated but it was claimed to be an important regulator of apoptosis.[99] RhoB is the only small G-protein that uses three different anchors for its intracellular compartimentalisation, two of which are intermediate products of the cholesterol synthesis pathway: farnesyl- and geranylgeranylpyrophosphate. Thus these anchors are of high relevance for RhoB's intracellular localization and function.

We could show a Simvastatin induced upregulation of RhoB on protein level in dhSKM cells ***(Figure 17)***. This is important since elevated mRNA levels do not necessarily result in higher protein levels. Using a Rhotekin binding domain (RBD) pulldown assay, we also showed that upregulated RhoB is in an active GTP bound state ***(Figure 19)***. All Simvastatin induced RhoB levels (mRNA as well as protein) and RhoB activation could be abrogated by simultaneous treatment with mevalonic acid, which is the product of the statins's target HMG-CoA reductase ***(Figure 18)***.

Moreover, RhoB upregulation and activation are paralleled by an activation of caspase-2 and caspase-3 which are important effector molecules of apoptosis ***(Figure 22)***. Strong activation of the main effector caspase caspase-3 shows that Simvastatin induced apoptosis has been triggered. The meaning of caspase-2 could be especially interesting since it is one of the most conserved caspases in different animal species.

We tried to revert caspase activation by the addition of mevalonic acid as well as by the addition of farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate, which are the key metabolites for lipid-anchor synthesis. It has been proposed that farnesyl anchors facilitate RhoB localization to the plasma membrane and geranygeranyl anchors promote endosomal localization. While the addition of mevalonic acid to Simvastatin treated cells was able to abrogate caspase activation completely, the reactivation of geranylgeranyl anchor synthesis showed absolutely no effectiveness in this regard and farnesyl pyrophosphate was able to reduce half of the Simvastatin induced caspase activation ***(Figure 22 and 23)***.
These results show a clear connection between missing lipid anchors and caspase activation in dhSKM cells. Since RhoB is being upregulated upon Simvastatin treatment, upregulation could be a compensatory mechanism to reestablish proper RhoB localization to endosomes and the plasma membrane.

The importance of properly localized RhoB was supported by immunostaining of RhoB. While RhoB was localized to vesicles in control cells and cells treated with Simvastatin and mevalonic acid, Simvastatin treated cells showed a homogenic abundance of RhoB without identifiable vesicular structures ***(Figure 24).***

An infection with *Clostridium sordellii*, which was observed in HIV positive drug abusers and pregnant women, shows similar symptomes on human skeletal muscle cells as statin treatment. The active toxic compound produced by *C. sordellii* is lethal toxin (LT), which specifically inactivates Ras proteins and thereby leads to an upregulation of Rho

proteins. According to this, rising RhoB levels have been shown in fibroblasts treated with LT.[108]

We could show that LT increases RhoB levels in dhSKM cells with a peak after 12 hours ***(Figure 25)*** and that it also activates caspases 2 and 3 ***(Figure 29, 30 and 31)***. Although LT activates caspases even stronger than Simvastatin, dhSKM cells treated with LT do not undergo the same drastic morphological changes as Simvastatin treated cells. A comparison of these two compounds in regard of caspase activation and RhoB upregulation shows an obvious similarity and underlines the possibility of RhoB as an activator of apoptosis.

C3 toxin, a bacterial toxin from *Clostridium botulinum*, was previously used to experimentally inactivate RhoB. It inactivates Rho proteins by covalently adding an ADP-ribosyl residue to their GTP binding site and thus disables activation of Rho proteins. We used this system to check for a possible reversibility of RhoB induced apoptotic effects. Although we could confirm successful C3 mediated RhoB inactivation in LT and Simvastatin treated cells via a band shift on a western blot ***(Figure 26)***, caspase activation was accumulating with C3 treatment after 16 and 24 hours. Only treatment of 12 hours showed a reduction of LT induced apoptosis by C3 ***(Figure 30)***. This phenomenon most probably results from activation of different apoptotic pathways in response to the toxin. It is likely that RhoB plays a role only in early events. To answer this question more clearly, experiments with shorter treatment times should be done. Another approach could be the use of siRNA lower total RhoB levels. A comparison of RhoB inactivation in Simvastatin and LT treated dhSKM cells could also shed some light on the importance of other lipid anchor using proteins than RhoB, since LT does not affect these anchors.

We observed a strong caspase-2 activation, which is particularly interesting in the context of increased RhoB levels, since Rabkin and Kong described direct interaction and complex formation of RhoB and caspase-2 in lovastatin treated mouse cardiomyocytes via caspase-2 activity assay and a caspase-2 western blot.[123] However we could not verify this complex after a Rhotekin binding domain (RBD) pulldown of probes from cells treated with Simvastatin for 12 and 16 hours (data not shown). Possibly the complex was dissolved during the procedure. Westernblotting for caspase-2 after a RBD pulldown would be a better approach to verify a RhoB / caspase-2 complex. Moreover a westernblot would detect procaspase-2 as well as the cleaved and active caspase-2. Interestingly the caspase-2 inhibitor led to enriched total RhoB levels in two experiments ***(Figure 34)***. This could mean that RhoB might constantly be cleaved by caspase-2. RhoB's short half life time of 2 hours supports this

idea. Simultaneous treatment with Simvastatin abrogated upregulation to some amount, but more experiments would have to be performed to allow a clear statement.

Altogether these findings suggest a key regulatory function of the small G-protein RhoB in activation of apoptosis in Simvastatin and lethal toxin treated differentiated human skeletal muscle cells. Simvastatin leads to mislocalization of RhoB by prohibiting the synthesis of lipid anchors, which is most probably the reason for a compensatory upregulation of RhoB. Correct localization is necessary for the proper functioning of RhoB. RhoB upregulation as seen under LT treatment is sufficient to activate the caspases-2 and -3. Inactivation of elevated RhoB by ADP-ribosylation is not effective in avoiding apoptosis in cells treated with LT for periods longer than 12 hours.

siRNA should be used in order to inactivate RhoB to confirm a proapoptotic function of this yet quite unexplored small G-protein in Simvastatin treated cells. If it could be verified that RhoB siRNA prevents Simvastatin mediated apoptosis in dhSKM cells it would not only explain why statins act toxically on skeletal muscles but it could also shed light on RhoB as regulator of apoptosis, possibly in connection with caspase-2.

V. A) Abstract

Statins are well tolerated inhibitors of HMG-CoA reductase which are used to effectively lower blood cholesterol levels. However in some cases statins elicit myotoxic effects. The goal of this diploma thesis was to identify candidate genes which are responsible for Simvastatin induced myotoxicity in differentiated human skeletal muscle (dhSKM) cells. A whole human genome gene chip analysis was performed. After an extensive evaluation via in silico methods, the small G-protein RhoB was chosen for furter investigation. This choice was based on the following arguments: RhoB is among the top ten regulated genes, its upregulation is causally linked to statin action and it was previously ascribed a role in regulation of apoptosis.

Here we show for the first time an upregulation of RhoB on mRNA and on protein level in dhSKM cells in response to Simvastatin treatment. RhoB activation was observed by a Rhotekin binding domain (RBD) pulldown assay. RhoB upregulation upon Simvastatin treatment was paralleled by a strong activation of the apoptosis effector molecules caspase-2 and caspase-3. RhoB and caspase activation could be completely prevented by mevalonic acid (MA), the product of HMG-CoA reductase, which demonstrates the causal relation to Simvastatin action. MA downstream products farnesyl- and geranygeranyl pyrophosphate showed each less ability to reduce caspase activity.

Furthermore bacterial toxins were used to investigate the role of RhoB. *C. sodellii* lethal toxin (LT) was known to upregulate RhoB levels by inactivating Ras in murine fibroblasts. *C. botulinum* C3 toxin specifically inactivates Rho proteins by ADP-ribosylation. We could show that LT induces RhoB, caspase-2 and caspase-3 activity in dhSKM cells. When Simvastatin or LT induced RhoB was deactivated by C3, reduced caspase-2 activitiy after 12 hours. Contrary to previous results the existence of a complex between caspase-2 and RhoB could not be confirmed.

V. B) Zusammenfassung

Statine sind gut vertragene und effektive Inhibitoren der HMG-CoA-Reduktase, die hauptsächlich als Senker des Blutcholesteringehalts eingesetzt werden. In einigen Fällen lösen Statine allerdings myotoxische Effekte aus. Das Ziel dieser Diplomarbeit war es, Kandidaten-Gene zu identifizieren, die für die Simvastatin-induzierte Myotoxizität in differenzierten menschlichen Skelettmuskelzellen (dhSKM) verantwortlich sind. Dazu wurde eine Gen-Chip-Analyse für das komplette menschliche Genom durchgeführt. Nach intensiver Auswertung mittels in silico Methoden wurde das kleine G-Protein RhoB für eine weitere Analyse ausgewählt. Diese Wahl wird von folgenden Argumenten gestützt: RhoB gehört zu den zehn am stärksten regulierten Genen, seine Hochregulation hängt kausal mit der Funktion der Statine zusammen und in der Literatur wurde bereits eine Rolle RhoB's in der Regulation von Apoptose erwähnt.

Hier zeigen wir zum ersten mal eine Hochregulation von RhoB sowohl auf mRNA- als auch auf Protein-Ebene in dhSKM-Zellen als Folge einer Simvastatin-Behandlung. Eine Aktivierung von RhoB konnte in einem Rhotekin-Bindedomäne (RBD)-Pulldown-Experiment nachgewiesen werden. Die RhoB Hochregulation nach Simvastatin-Behandlung wurde von einer starken Aktivierung der Apoptose-Effektor-Moleküle Caspase-2 und Caspase-3 begleitet. RhoB- und Caspase-Aktivierung konnten durch Mevalonsäure (MA), dem Produkt der HMG-CoA-Reduktase, komplett verhindert werden. Das beweist den kausalen Zusammenhang der RhoB-Regulation durch Simvastatin-Aktivität. Die nachgeordneten Metaboliten der MA, Farnesylpyrophosphat und Geranylgeranylpyrophosphat zeigten jeweils eine niedrigere Fähigkeit die Caspase-Aktivität zu reduzieren.

Desweiteren wurden bakterielle Toxine genutzt um die Rolle von RhoB zu untersuchen. Von *C. sodellii* lethal toxin (LT) ist bekannt, dass es RhoB in Maus-Fibroblasten induziert indem es Ras inaktiviert. *C. botulinum* C3 toxin deaktiviert spezifisch Rho-Proteine durch ADP-Ribosylierung. Wir konnten zeigen, dass LT RhoB, caspase-2 und caspase-3 in dhSKM-Zellen induziert. Wenn Simvastatin oder LT induziertes RhoB durch C3 deaktiviert wurde, konnte die Caspase-2-Aktivität nach 12 Stunden reduziert werden. Im Gegensatz zu publizierten Daten konnte die Existenz eines Caspase-2-RhoB-Komplexes nicht bestätigt werden.

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Curriculum Vitae
Timm Linus Zörgiebel

Personal information

Date of birth:	April 28th, 1982
Place of birth:	Darmstadt, Germany
Adress:	Rosenstr. 15, 01445 Radebeul, Germany
Mail:	timmworldwide@gmail.com

Education

1992 – 1993	High School [Gymnasium] Oberhaching, Germany
1993	St.-Benno High School [Gymnasium] Dresden, Germany
2001	High School Degree [Abitur] at St.-Benno High School
Since Sept. 2002	Course of study „molecular biology“ [Molekulare Biologie] at Vienna University, Austria

Academic publications

Timm Zörgiebel, Lukas Weigl, Harald Genth and Martin Hohenegger
"Activation of RhoB in simvastatin-induced apoptosis of differentiated human skeletal muscle cells."
BMC Pharmacology 2007, 7 (Suppl 2): A16 (Meeting Abstract)

Internships

Summer 2000	Advanced Micro Devices (AMD), Dresden
April – Sept. 2002	Biotype AG, Dresden
Summer 2005	Max Planck Institute for Cell Biology and Genetics (MPI-CBG), Dresden, group Eckhard Lammert
December 2005	Institute of Molecular Biotechnology Austria (IMBA), Vienna, group Vic Small
December 2006	Max F. Perutz Laboratories (MFPL), Vienna, group Andrea Barta
Jan. – Feb. 2007	Max F. Perutz laboratories (MFPL), Vienna, group Roland Foisner

Additional activities

2001 – 2002	9 months of Military Service, Kassel (German Bundeswehr)
Jan. '01 – Jan. '08	Cofounder of the webdesign company „Zörgiebel & Gähler GbR"(civil law association), Dresden
July – Aug. 2006	Scientific input/output analysis for Max F. Perutz laboratories (MFPL), Vienna
Sept. '06 – Aug. '07	Scientific output analysis for Vienna science, research and technology fund (WWTF)

Honorary activities

Since Dec. 2002	Member of the Austrian students association „AktionsGemeinschaft"
2003 – 2007	Member of the Executive Committee of „AktionsGemeinschaft at the faculty of sciences at Vienna University" as chairman's substitute / treasurer
May 03 – July 07	Elected students representative for the course of study "molecular biology" [Molekulare Biologie] at Vienna University - Reelected until 2007 in 2005
Since Dec. 2005	Member of the "Molecular Biological Society – Wissenschaftliches Netzwerk", Vienna