Identification and Functional Characterisation of Genes regulated by Monomeric Actin

Arnaud Descot

München 2009

Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.) der Fakultät für Biologie der Ludwig-Maximilians-Universität München

Identification and Functional Characterisation of Genes regulated by Monomeric Actin

Arnaud Descot

Aus La Teste de Buch, Frankreich

December 2009

Diese Arbeit wurde am Max-Planck Institut für Biochemie, Martinsried angefertigt.

Eingereicht am 3. December 2009

Mündliche Prüfung am 10.März 2010

Prof. Dr. Stefan Jentsch	(Erstgutachten)
Prof. Dr. Angelika Böttger	(Zweitgutachten)
Prof. Dr. Dirk Eick	(Rigorosum)
Prof. Dr. med. Thomas Cremer	(Rigorosum)
Prof. Boshart	(Umlauf)
PD Dr. Guido Posern	(Umlauf)

Ehrenwörtliche Erklärung und Erklärung über frühere Promotionsversuche

Hiermit erkläre ich, dass ich, Arnaud Descot, die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt habe. Sämtliche Experimente sind von mir selbst durchgeführt, ausser wenn explizit auf Dritte verwiesen wird. Ich habe weder anderweitig versucht, eine Dissertation einzureichen oder eine Doktorprüfung durchzuführen, noch habe ich diese Dissertation oder Teile derselben einer anderen Prüfungskommission vorgelegt.

München, den …… ……..

(Arnaud Descot)

1 Summary

Monomeric actin controls the activity of the transcription factor Serum Response Factor (SRF) via its coactivator MAL/MRTF-A. Upon signal induction, MAL is released from actin, binds SRF and activates target gene expression. In order to characterise the physiological role of this signalling pathway, I screened on a genome wide basis for target genes by transcriptome analysis.

A combination of actin binding drugs (Cytochalasin D and Latrunculin B), targeting monomeric actin, was used to specifically and differentially interfere with the complex between MAL and actin. 210 genes primarily controlled by monomeric actin were identified in mouse fibroblasts. Among them more than 30% have been already found in screens for SRF target genes, supporting the validity of the screening approach. As expected, a lot of genes were involved in cytoskeleton organization. However, genes having anti-proliferative or pro-apoptotic features were identified surprisingly to the same extent. Consistently, I could demonstrate an antiproliferative function of MAL. More specifically, several genes interfering with the MAPK pathway were identified.

One of them was Mig6/Errfi1, a negative regulator of EGF receptors. Mig6 induction by LPA or FCS revealed to be dependent on MAL, monomeric actin and the small GTPases Rho. Activated forms of MAL or SRF were sufficient to induce Mig6 expression. Subsequently, a Mig6 promoter element was found to be necessary to mediate MAL/SRF induction. Moreover, induction of Mig6 through the Actin-MAL pathway led to the downregulation of the mitogenic EGFR-MAP kinase cascade. For the first time a transcriptional link between G-actin levels sensed by MAL and the regulation of EGFR signalling was established.

Furthermore, after having demonstrated that MAL induces apoptosis, I focused on the characterisation of two proapototic targets identified in the screen: Bok and Noxa. Bok and Noxa were induced by activators of the Rho-Actin-Mal-Srf pathway on a MAL dependent manner. The study of the Bok promoter revealed the existence of a response element that was necessary for the induction by MAL-SRF. Interestingly, apoptotic inducers like staurosporine, TNFα, or the DNA damaging agent Doxorubicin triggered MAL-SRF mediated transcription. As SRF controls the expression of the anti-apoptotic genes Bcl2 and Mcl1, the results from this work places thus SRF as a key transcription factor controlling the balance between pro and anti apoptotic genes in response to external cues.

2 Introduction

2.1 The Actin-Mal-SRF signalling pathway

2.1.1 SRF mediated transcription

Transcription factors mediate the execution of genetic programs in response to extra- or intracellular signals. They play major roles to adapt the cells and tissues to changed demands, to control cell differentiation or the cell cycle. One of them, the Serum Response Factor (SRF) is essential for development as knock out of SRF leads to an early embryonic death in mice (Arsenian et al., 1998). SRF controls many genes including “immediate early” genes, cytoskeletal genes and muscle specific genes (Miano, 2003; Pipes et al., 2006; Posern and Treisman, 2006). However, on his own, SRF is a weak transcription factor. SRF is considered as a transcriptional platform controlled by its combinatorial association with different co-activators or repressors.

The first described signalling pathway leading to the activation of SRF relies on the activation of a MAP kinase cascade by the Ras GTPase. Upon stimulation with serum or growth factors, the Ternary Complex Factors (TCFs) are phosphorylated by the MAP kinase/Erk cascade and bind SRF to trigger the expression of the target genes like the immediate early gene c-FOS (Figure 1).

The serum response element of the cFos promoter is composed of one TCF binding site adjacent to a SRF binding site also called a CARG box that has for sequence $CC(A/T)_6GG$ (Figure 1). Interestingly, when the TCFs binding site of the cFos promoter was mutated, the cFos promoter was still partly responsive to serum and to Lysophosphatidic Acid (LPA) in a SRF dependent manner (Hill and Treisman, 1995). These results lead to the hypothesis of a new signalling pathway towards SRF.

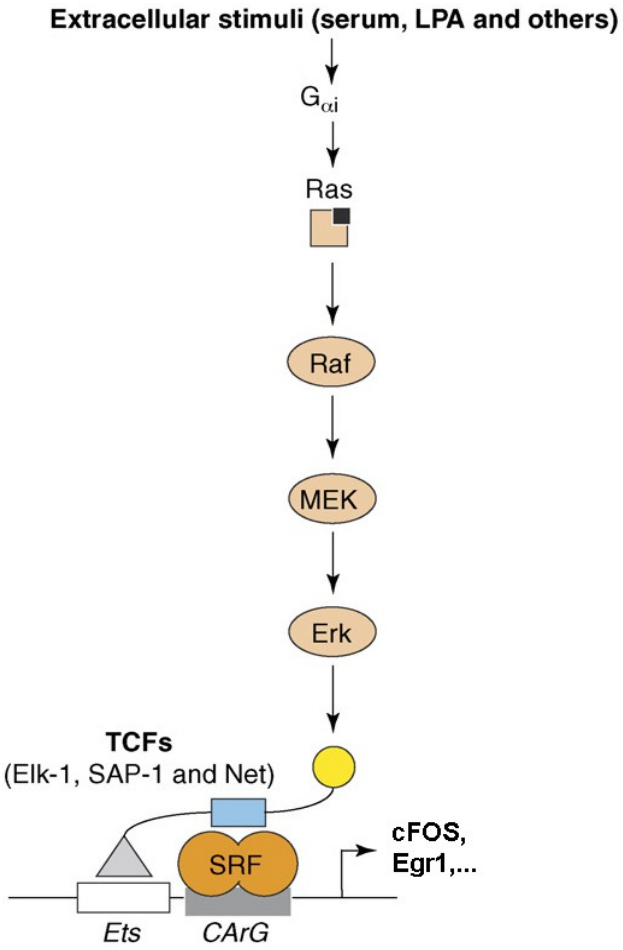


Figure 1. Activation of SRF by the Classical MAP Kinase Pathway

From (Posern and Treisman, 2006).

2.1.2 Rho A controls TCFs independent SRF mediated transcription through actin

The TCF independent activation of SRF is blocked by inhibition of Rho proteins by C3 transferase and induced by the microinjection of activated RhoA (Hill et al., 1995). Moreover, activated forms of the Rho family GTPases Rac1 and CDC42 also activate transcription via SRF but they function independently of RhoA. Hence, functional Rho is required for TCF-independent SRF activation by serum but not by activated CDC42 or Rac1.

In a screen for SRF activators, LIM kinase-1 (LIMK1) was identified as a potent activator of SRF mediated transcription (Sotiropoulos et al., 1999). LIMK1 activation of SRF was dependent upstream on RhoA and downstream on its ability to regulate actin treadmilling by stabilising F-actin. Yet, the overexpression of a dominant negative LIMK1 mutant had no effect on SRF activation by LPA or serum showing that LIMK1 activity is not essential for SRF

activation by these stimuli. Finally, LIM-kinase was shown to cooperate with Diaphanous to regulate SRF (Geneste et al., 2002). Thus, signals leading to the stabilisation of F-actin mediated via LIMK or Diaphanous lead to the activation of SRF (Figure 2). But it was not clear if SRF activity was sensing the F actin level, the ratio F/G actin or the G actin level.

Further experiments with actin binding drugs like Cytochalasin D or Swinholide A and overexpression of wild type actin which does not modify the ratio F/G actin suggested that SRF activity responds in fact to G-actin levels (Sotiropoulos et al., 1999). The confirmation came by overexpressing the non polymerisable actin mutants G13R and R62D which inhibited SRF activity (Posern et al., 2002) (Figure 2).

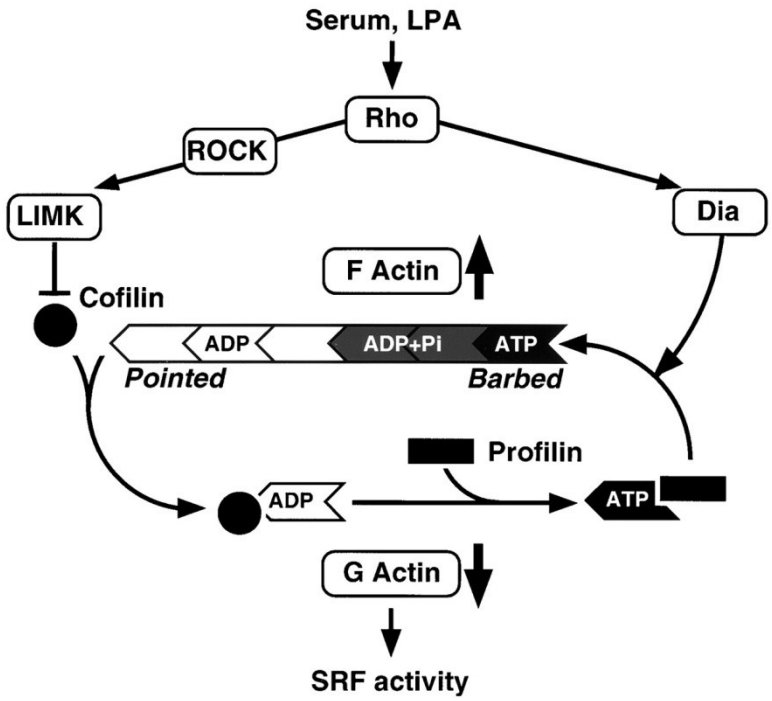


Figure 2. Control of SRF Activity by the Action of Rho Effectors on the Actin Cytoskeleton

From (Geneste et al., 2002).

Altogether, G-actin or a sub-population of it controls SRF activity. The problem was that no direct physical interaction between G-actin and SRF could be found by mammalian or yeast two hybrid assay.

2.1.3 The discovery of MAL as a SRF coactivator regulated by actin

In 2001, a new family of SRF coactivator was discovered (Wang et al., 2001). The founding member of this family, Myocardin, is specifically expressed in cardiac and smooth muscles.

Looking in silico for homologs of Myocardin ubiquitously expressed, Wang et al. discovered MAL (also known as MKL1, MRTFA and BSAC) and MAL-16 (also known as MKL2 or MRTFB) (Wang et al., 2002)(Figure 3). Independently, two other groups identified MAL. First, MAL was shown to be part of a fusion protein linked to a Megakaryoblastic Acute Leukaemia (Ma et al., 2001; Mercher et al., 2001). The fusion protein consists of nearly the whole sequences of MAL and OTT (also known as RBM15), a gene inhibiting myeloid differentiation in hematopoietic cells (Ma et al., 2007)(Figure 3). We could show that the resulting fusion protein OTTMAL is constitutively activating SRF mediated transcription because of a loss of regulation by upstream signalling (Descot et al., 2008). Second, a shorter isoform of MAL (BSAC) was identified in a screen for cDNA able to reverse the TNFα induced apoptosis in the highly apoptosis susceptible TRAF2/TRAF5 double knock out MEFs (Sasazuki et al., 2002).

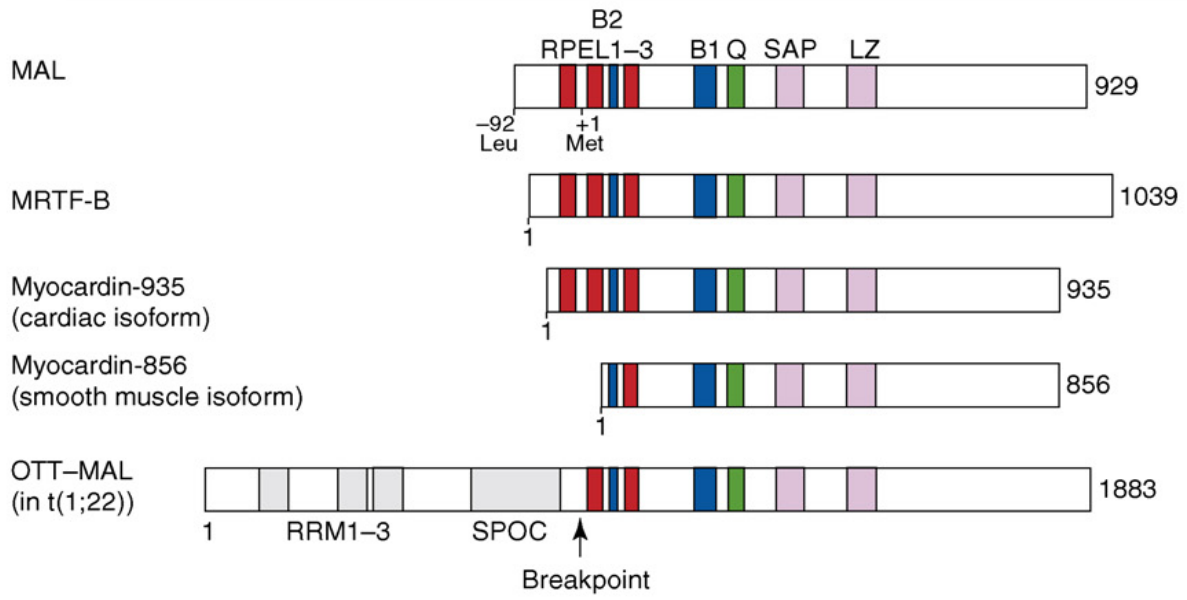


Figure 3. Schematic Representation of the Myocardin-Related Transcription Factor (MRTF) Family and the OTT–MAL Fusion Protein.

From (Posern and Treisman, 2006).

MAL was shown to accumulate in the nucleus upon serum stimulation (Miralles et al., 2003). Nuclear accumulation of MAL was also observed following expression of actin mutants S14C, V159N or G15S which lead to F-actin stabilisation and strongly activated SRF. The serum-mediated nuclear accumulation of MAL was inhibited by blockade of RhoA by C3 transferase

or toxin B. The overexpression of wild type actin or the non polymerisable mutant R62D which inhibit SRF activity was also sufficient to inhibit MAL nuclear accumulation (Miralles et al., 2003). In conclusion MAL activity is regulated via the control of its localisation by the Rho actin pathway.

2.1.4 Regulation of the Actin-MAL complex by actin binding drugs

MAL localisation could be as well modulated by the actin binding drugs Cytochalasin D and Latrunculin B (Miralles et al., 2003).

Cytochalasin D (from Zygusporium mansonii) represses actin polymerisation and induces depolymerisation by capping F-actin barbed ends and stimulates G-actin ATP hydrolysis (Sampath and Pollard, 1991). Latrunculin B (from Latrunculia magnificans) binds G-actin monomers and blocks polymerization into filaments (Spector et al., 1983). The two drugs impair F-actin formation and increase thereby G-actin levels but have different impact on MAL localisation and activity.

Treatment of starved fibroblasts with Cytochalasin D induced the nuclear localisation of MAL while treatment with Latrunculin B could block the serum induced MAL nuclear localisation. The nuclear relocalisation of MAL by Cytochalasin D correlated with an activation of SRF mediated transcription (Figure 4). Latrunculin B didn't activate SRF mediated transcription, but was able to block the induction serum.

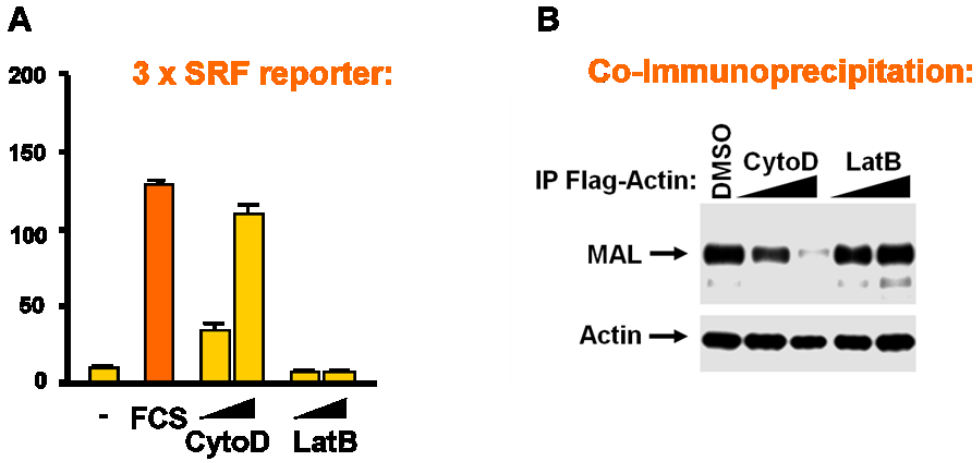


Figure 4. Regulation of MAL by Actin binding drugs

(A) Induction of MAL-SRF transcription by Cytochalasin D compared to serum and repression by Latrunculin B. (B) Dissociation of the inhibitory complex Actin-MAL by Cytochalasin D and stabilisation by Latrunculin B evaluated by co-immunoprecipitation. Adapted from (Miralles et al., 2003).

The explanation of the differences between Cytochalasin D and Latrunculin B lies in the differential impact of the two drugs on an Actin-MAL complex. Cytochalasin D induced the dissociation of MAL from Actin while Latrunculin didn't or even stabilized the complex (Figure 4).

The modulation of this direct interaction between actin and MAL is the mechanism of regulation of MAL mediated transcription by endogenous stimuli like FCS or LPA.

2.1.5 MAL binds actin via a new actin binding motif

The affinity of MAL to actin is similar to Gelsolin, another actin binding protein (Posern et al., 2004). The domain of MAL responsible for the interaction with actin is situated at the N-terminus and is composed of a repetition of 3 RPEL motifs (Miralles et al., 2003)(Figure 5).

Crystal structure of one RPEL motif with actin show that the helix α1 of RPEL motifs and WH2 (WASP homology domain-2) domains of actin binding proteins share the same 3D structure and the same interaction surface with actin (Mouilleron et al., 2008) (Figure 5).

Each RPEL motif can bind actin. Moreover the Actin-MAL complex has a ratio of 3 to 1 (Vartiainen et al., 2007). Therefore it is likely that one MAL molecule binds 3 actin molecules.

However, not all RPEL motifs have the same importance for MAL regulation (Guettler et al., 2008). Mutation of RPEL 1 or 2 doesn't influence MAL localisation while mutation of RPEL 3 is sufficient to partially relocalize MAL to the nucleus. Combined mutation of RPEL 1 and 2 lead to the same partial nuclear localisation of MAL but mutation of RPEL 2 and 3 resulted in a complete nuclear localisation. Thus, RPEL motifs collaborate to regulate MAL localisation with a primordial role for RPEL 3. The importance of the RPEL 3 motif can be linked to its lower affinity for actin compared to RPEL 1 or 2 (Guettler et al., 2008). When the G actin level decreases, the RPEL domain 3 is the first to be free from actin leading immediately to a partial relocalisation of MAL to the nucleus. Hence, RPEL 3 depletion of actin is a critical regulatory step for sensing G-actin levels.

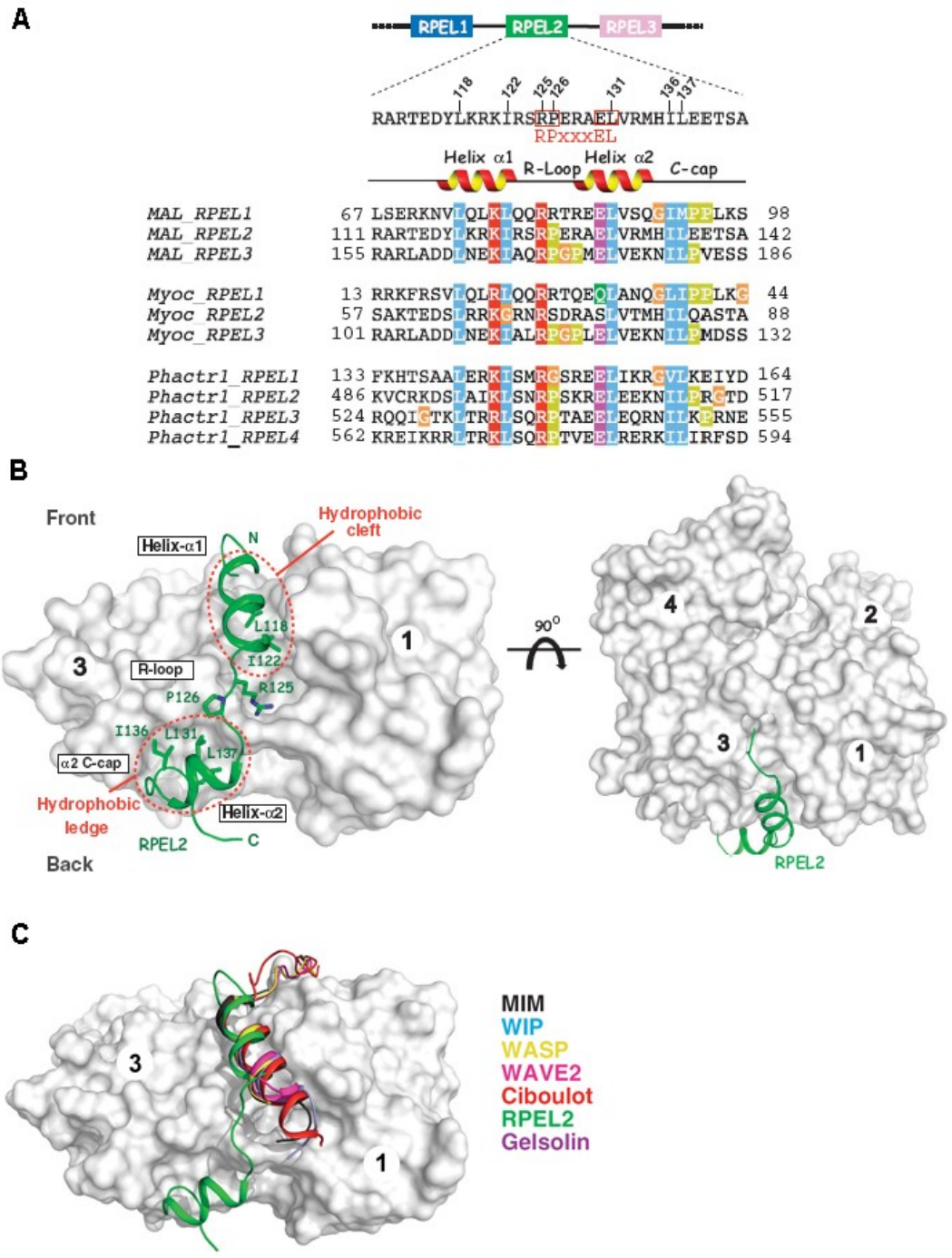


Figure 5. Structure of one RPEL Motif of MAL in Complex with G-Actin

(A) Sequence alignment of individual RPEL motifs from murine MAL, myocardin (transcript variant A) and Phactr1. RPEL2MAL secondary structure and are shown above the sequence. Selected conserved residues are highlighted. (B) Two views of the RPEL2MAL:G-actin complex, related by a 901 rotation around the horizontal axis. Right-hand panel is the classical view of the 'front' surface of actin (white with subdomains labelled 1–4). RPEL2MAL is drawn in green (cartoon) with highly conserved RPEL residues that interact with actin shown as sticks. The hydrophobic cleft and the subdomain 3 ledge of actin are indicated by red dashed circles. (C) Structural Comparison with Known G-Actin-Binding Proteins Bottom view of superposed WH2 motif containing proteins together with RPEL2 motif of MAL bound to G-actin. From (Mouilleron et al., 2008).

2.1.6 Nuclear actin regulates the nuclear localisation and activity of MAL

Monitoring of MAL shuttling between the cytoplasm and the nucleus by FRAP (Fluorescence Recovery After Photobleaching) revealed that upon stimulation the decrease of the nuclear export rate of MAL is the main contributor to MAL relocalisation in the nucleus (Vartiainen et al., 2007). The increase in import rate contributes only marginally. Moreover, a mutant MAL defective for actin binding harbours a strongly reduced nuclear export. Thus, nuclear actin regulates nuclear localisation of MAL by controlling its nuclear export (Figure 6).

Leptomycin B, which blocks Crm1 dependent export, induced the relocalisation of MAL to the nucleus with the same kinetics as activating actin binding drugs (Vartiainen et al., 2007). However, Leptomycin B treatment is not sufficient to induce a strong MAL mediated transcription, even though MAL was enriched on the promoter of target genes in Leptomycin B treated cells. The transcription is triggered only when interaction of MAL with actin is abolished (Figure 6).

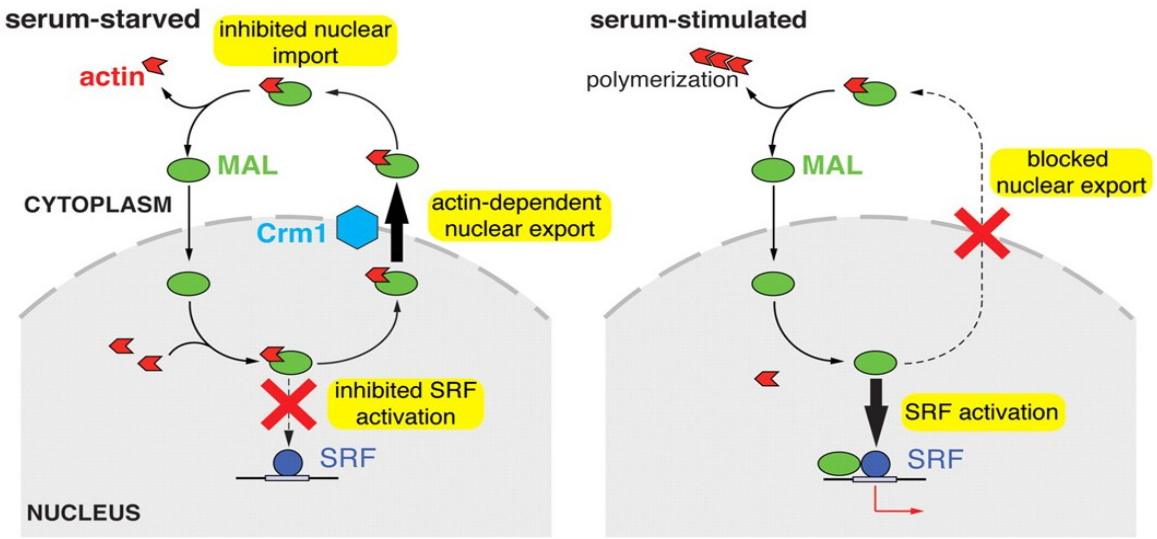


Figure 6 Multiple Roles for Actin in MAL Regulation

In unstimulated cells, high export rates ensure MAL is mainly cytoplasmic, whereas nuclear actin prevents SRF activation. Upon stimulation, decreased export induces nuclear MAL accumulation, and diminished interaction with actin allows SRF activation. Proteins are shown as monomers for simplicity. From (Vartiainen et al., 2007).

It is not known so far if the role of nuclear actin concerning MAL regulation is independent or not from the functions of actin in chromatin remodelling or transcription described so far

(Miralles and Visa, 2006). For the RNA polymerase II, actin has to be present in the complex in an early step of transcription (Hofmann et al., 2004) contradicting the requirement of loss of actin binding for MAL mediated transcription. Therefore, the regulation of MAL by nuclear actin is likely to be independent from the role of nuclear actin in RNA polymerase II mediated transcription.

2.1.7 Competition between TCFs and MAL family members for SRF binding

Both the MAP kinase cascade and the Rho-Actin-MAL pathway lead to activation of SRF mediated transcription (q.v. 2.1.1). The two families of transcription factors TCFs and MRTFs contact the same surface on SRF in a mutually exclusive manner (Wang et al., 2004). Growth promoting signals inducing the MAP kinase cascade activate TCFs which are then able to displace MRTFs from SRF. In the case of smooth muscle genes, the replacement of Myocardin by Elk1 results in an overall repression of transcription as Myocardin is a much more potent transcription factor than Elk1. The competition between Elk1 and Myocardin has been characterised in smooth muscle cells which switch between proliferation and differentiation upon extra cellular signals. When the smooth muscle cells proliferate, the MAP kinase cascade is active and the differentiation program controlled by Myocardin is off. Yet, when the smooth muscle cells enter differentiation, the MAP kinase cascade is switched off, Myocardin can then bind SRF and trigger the expression of smooth muscle specific genes (Figure 7).

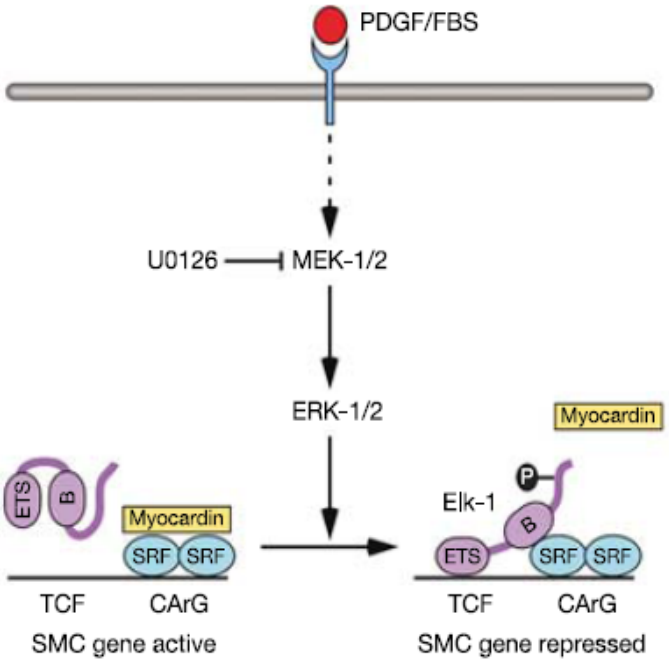


Figure 7. A Model to Account for the Modulation of Smooth Muscle Genes By Competition between Myocardin and Elk-1 for SRF in Response to Growth Signals

2.1.8 Additional ways to inhibit MRTFs-SRF mediated transcription

2.1.8.1 Posttranslational modifications of MRTFs and SRF

2.1.8.1.1 Phosphorylation of SRF

Another possible way to switch between proliferation and myogenic programs controlled by SRF is to phosphorylate SRF in the DNA binding domain at S162 (Iyer et al., 2006). This phosphorylation event suppresses MRTFs-SRF mediated transcription of muscle specific genes by preventing DNA binding. On the opposite, the pro-proliferative immediate early genes controlled by Elk1-SRF like Egr1 or cFos were unaffected, likely due to the stabilisation of the complex on the DNA by the DNA binding domain of Elk1.

Yet, it is worth to note that SRF phosphorylation in the DNA binding domain doesn't lead always to repression of myogenic targets. The Myotonic Dystrophy Protein kinase (DMPK) and PKCα phosphorylate SRF at T159 to activate expression of cardiac α actin (Iyer et al., 2003).

2.1.8.1.2 Phosphorylation of MRTFs

Serum stimulation activates MAL by inducing its relocalisation to the nucleus (q.v. 2.1.3). However, serum stimulation promotes as well the opposite (Muehlich et al., 2008). Serum or phorbol ester 12-*O*-tetradecanoyl-13-acetate (TPA) renders MAL phosphorylated by Erk 1/2 at S454. This phosphorylation increases actin binding and therefore nuclear export. Additionally, a non phosphorylable MAL mutant showed a constant nuclear localisation and activation. This phosphorylation is likely happening after the start of MAL mediated transcription to terminate the induction of the target genes. Hence, modulation of MAL binding to nuclear actin by the MAPK regulates MAL mediated transcription (Figure 8).

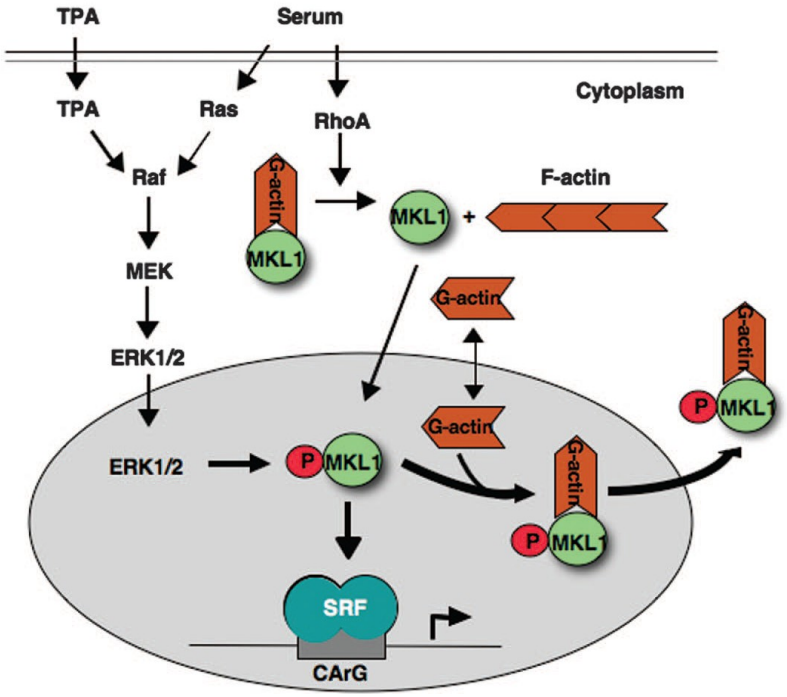


Figure 8. The Model for Serum Regulation of MKL1

Serum induction results in the activation of the RhoA- and Ras/MEK/ERK pathways. RhoA activation stimulates MKL1 nuclear localization due to the formation of actin stress fibers and a decrease in G-actin levels, while nuclear export is stimulated due to ERK1/2 phosphorylation of MKL1 and increased G-actin binding. Export of actin-bound MKL1 reduces the amount of MKL1 available to bind to SRF and activate transcription. From (Muehlich et al., 2008)

Erk 1/2 phosphorylate as well Myocardin impairing the expression of smooth muscle specific genes. The phosphorylation occurs on four residues (S812, S859, S866 and T893), in the transactivation domain of Myocardin inhibiting its interaction with acetyl transferase or

CREB binding proteins (Taurin et al., 2009) which are known to promote Myocardin activity (Cao et al., 2005).

Moreover, Myocardin was also shown to be inhibited by GSK3 beta mediated phosphorylation at serines 455 to 467 and 624 to 636 to reduce cardiac hypertrophy (Badorff et al., 2005). The phosphorylation of Myocardin by GSK3 beta potentiates its degradation by the proteasome (q.v. 2.1.8.1.3; (Xie et al., 2009)).

Other phosphorylation sites for MRTFs and SRF are described in large scale phosphoproteomic experiments but await functional validations e.g. (Olsen et al., 2006)(http://www.phosida.com/).

2.1.8.1.3 MRTFs sumoylation or ubiquitinylation

Myocardin is ubiquitinylated by the E3 ligase C terminus of Hsc70-interacting protein (CHIP) and sent for degradation by the proteasome (Xie et al., 2009). The outcome is a repression of smooth muscle genes due to a decrease in Myocardin transcription activity.

Transcriptional activity of transcription factors like p53 (Gostissa et al., 1999; Rodriguez et al., 1999), c-Jun (Muller et al., 2000) or LEF1/TCF (Sachdev et al., 2001) is modulated by sumoylation. MAL undergoes as well sumoylation at K499, 576, and 624 by interacting with the E2 conjugating enzyme UBC9 (Nakagawa and Kuzumaki, 2005). The sumoylation of MAL results in an impairment of MAL-SRF mediated transcription. Interestingly, sumoylation of MAL is triggered by serum stimulation or active RhoA which could provide a negative feedback. Conversely, Myocardin activity is enhanced after sumoylation by the E3 ligase PIAS1 at K445 in pluripotent 10T1/2 fibroblasts (Wang et al., 2007). This discrepancy could be explained by differences between Myocardin and MRTFs or by the different reporters used. More work is needed to delineate clearly the outcome of MRTFs sumoylation.

2.1.8.2 Mutual inhibition of NFκB and Myocardin

On top of the inhibition mechanisms presented above, Myocardin can be inhibited by interaction with NFκB (Tang et al., 2008). Binding to NFκB inhibits the formation of the complex between Myocardin SRF and DNA. The inhibition of Myocardin by NFκB is reciprocal as overexpression of Myocardin inhibits binding of p65 to DNA and abrogates LPS induced TNFα expression. As the domains required for Myocardin to interact with NFκB are

conserved in MAL, MAL should be reciprocally inhibited by NFκB. Repression of NFκB signalling is proposed to explain antiproliferative properties of Myocardin (Milyavsky et al., 2007) (Figure 9).

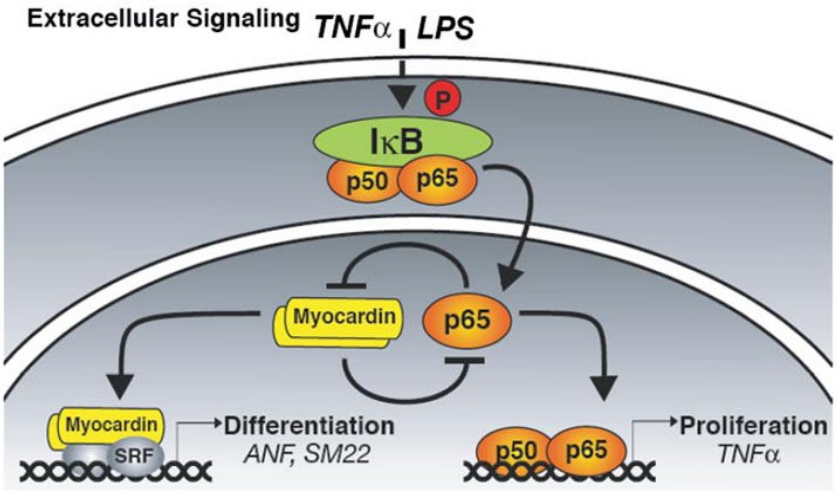


Figure 9. Model for the Mutual Inhibition of Myocardin and P65

From (Tang et al., 2008).

2.1.8.3 Competitive inhibitory binding of SRF by FHL2

Inhibitors of MRTF-SRF mediated transcription can compete for binding to SRF like Elk1 which displaces Myocardin from SRF (q.v. 2.1.7). The four and a half Lim domain protein 2 (FHL2) has been identified as a transcriptional target of Rho-Actin-MAL-SRF signalling able to abolish MAL binding to SRF (Philippar et al., 2004). Hence, FHL2 is part of a transcriptional negative regulatory loop to control MAL-SRF mediated transcription.

2.1.8.4 Inhibition of the MRTF-SRF complex by SCAI or Foxo4

Another protein, the Suppressor of Cancer cell Invasion (SCAI) inhibits as well the complex MAL-SRF but on a different way than FHL2 or ELK1 (Brandt et al., 2009b). In presence of SCAI, the binding of MAL to SRF is preserved. SCAI binds the MAL-SRF complex sitting on the promoter of the target genes like Integrin β1 preventing MAL-SRF mediated transcription (Brandt et al., 2009a). SCAI is even able to repress the constitutive active MAL fusion protein OTTMAL involved in leukaemia.

Supposed to work on the same way as SCAI to inhibit MRTF-SRF mediated transcription, Foxo4 has been shown to mediate PI3K/Akt signals controlling smooth muscle cell differentiation by inhibiting Myocardin (Liu et al., 2005). Upon treatment of smooth muscle cells with the differentiation inducer insulin like growth factor 1, Akt phosphorylates Foxo4 which is then relocalized to the cytoplasm. The Myocardin-SRF complex is then free from Foxo4 inhibition and can trigger the expression of smooth muscle genes.

2.1.8.5 Inhibition by HOP

The Homeodomain Only Protein (HOP) inhibits MRTF mediated transcription to modulate cardiac development likely by two mechanisms. First, HOP inhibits the binding of SRF to DNA (Chen et al., 2002a; Shin et al., 2002). Second, HOP recruits deacetylases to the targeted promoters (Kook et al., 2003).

2.1.9 Cooperation of the MRTF-SRF complex with other transcription factors

Depending on the tissue or the external cues, the MRTF-SRF transcription factor complex cooperates with other transcription factors to increase the expression of target genes.

For example, in smooth muscle cells, Myocardin controls the expression of the smooth muscle gamma actin gene (ATG2) by cooperating with the transcription factor Nkx3.1 (Sun et al., 2009). The ATG2 promoter has several CARG boxes which could be bound by Myocardin/SRF complexes. Myocardin binds preferentially the CARG box which has in close vicinity a binding site for Nkx3.1. Binding of Nkx3.1 to Myocardin enhances the transcription of ATG2 on a specific manner as Nkx2.5 binds as well Myocardin but without increasing transcription.

Transcription factors of the GATA family can promote Myocardin-SRF mediated transcription of smooth muscle genes like smooth muscle myosin heavy chain and smooth muscle alpha-actin. However, the coactivation mediated by GATA 4 or 6 is not valid for all target genes. In some cases, like telokin, the interaction of GATA transcription factors with Myocardin results in repression of transcription (Oh et al., 2004; Yin and Herring, 2005).

2.1.10 MRTFs controls chromatin remodelling

By searching how MAL-SRF discriminates between smooth muscle targets and genes controlled by Rho-Actin, MAL was shown to interact with Brahma-related gene 1 (Brg1) part

of the ATP dependent SWI/SNF chromatin remodelling complex (Zhang et al., 2007a). Dominant negative Brg1 could repress MAL dependent expression of smooth muscle genes without affecting Rho-actin targets. In SW13 cells which lack Brg1, ectopic expression of Brg1 restored the ability of MAL to control the expression of smooth muscle genes. The same result could be shown for Myocardin (Zhou et al., 2009).

MRTF may play as well a direct role in chromatin remodelling in a context dependent manner through their SAP domain, a putative DNA-binding motif involved in chromosomal organization (Aravind and Koonin, 2000). For instance, deletion of the SAP domain of Myocardin completely abolished ANF induction mediated by Myocardin but only impaired the induction of SM22 (Wang et al., 2001).

2.1.11 MRTFs mediated transcription independent of SRF

MRTFs in complex with SRF contact DNA (Zaromytidou et al., 2006) but the interaction is too limited to provide MRTFs the ability to bind CARG boxes without SRF. However, MRTFs can bind promoters on a SRF independent manner in complex with other transcription factors.

2.1.11.1 Interaction of MRTFs with SMAD

TGF beta 1 signalling plays an important role in the development of smooth muscle cells from embryonic stem cells by activating Smad mediated transcription (Sinha et al., 2004). One of the targets is SM22, an early marker of smooth muscle cell development. The promoter of SM22 is known to be responsive to Myocardin-SRF transactivation (Du et al., 2003). However, Myocardin is able to cooperate synergistically with Smad3 to activate a CARG mutated SM22 promoter (Qiu et al., 2005). The Smad 3-Myocardin complex is dependent on the Smad binding element to bind the SM22 promoter and on the transactivation domain of Myocardin to activate transcription.

The interaction with Smad 3 is also valid for MAL as demonstrated for the control of the Slug promoter (Morita et al., 2007). During TGFβ induced Epithelial-Mesenchymal Transition (EMT), MAL binds Smad3 which recognizes a cis-element in the Slug promoter. Induction of Slug induces cell-cell contact dissociation by repressing E-cadherin expression (Bolos et al., 2003; Hajra et al., 2002).

The Smad 1/4 have been shown as well to interact with MRTFs. MAL/Smad complexes are formed in response to RHO/ROCK signalling in proliferating myoblasts to trigger the expression of the myogenic differentiation inhibitor Id3 (Iwasaki et al., 2008). Interestingly, this complex can be inhibited by a forkhead transcription factor. During myoblast differentiation, FKHR translocates to the nucleus to prevent the association of the MAL/Smad complex with the Id3 promoter.

2.1.11.2 Myocardin binds MEF2 to control the expression of smooth muscle genes

A cardiac enriched isoform of Myocardin has been shown to interact with MEF2 through an additional C terminus domain which is not present in other MRTFs (Creemers et al., 2006). However, overexpression of MRTFA and B didn't lead to activation of MEF2 mediated transcription. Then, this SRF independent mediated transcription seems to be limited to Myocardin.

2.1.12 Stimuli inducing Actin-MAL-mediated transcription

LPA signals through the GPCRs LPAR 1 to 3 to trigger MAL mediated transcription. Another phospholipid, sphingosine-1-phosphate (S1P) is also able to activate MAL by binding and stimulating the GPCRs S1PR 1 to 5. Once induced the GPCRs leads to the activation by the Gα subunits 12 or 13 of Guanine nucleotide exchange factors (GEFs) which then activate the Rho GTPAse.

However, new ways have been described recently to activate the Actin-MAL pathway. We could show that dissociation of epithelial cell-cell junctions activates RhoA and Rac1 and triggers MAL-SRF mediated transcription on a Rac1 dependent manner (Busche et al., 2008). Others could demonstrate as well the activation of RhoA upon loss of epithelial cell-cell contacts but gave a central to the Rho-ROCK-actomyosin instead of Rac1 activation to induce SRF (Fan et al., 2007). E-cadherins is the sensor of cell-cell contacts controlling SRF transcription as cells that do not express E-cadherin cannot activate SRF mediated transcription upon epithelial cell-cell junction dissociation (Busche et al., 2008) while reexpression of E-cadherin restored SRF activation (Busche and Posern, Submitted).

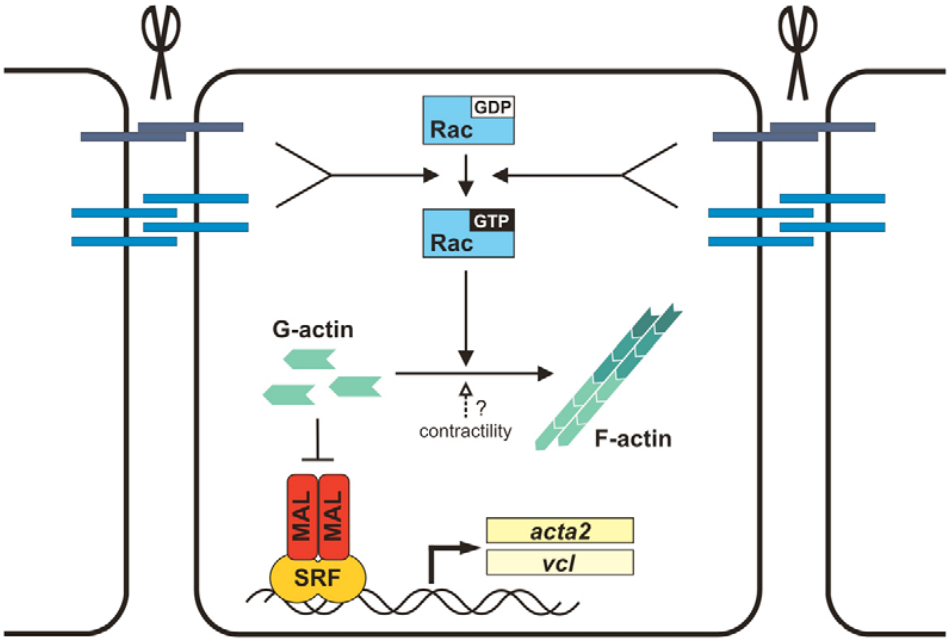


Figure 10. Model of the Regulation of MAL/SRF-Dependent Transcription by Epithelial Junctions.

Disassembly of E-cadherin-mediated cell-cell contacts leads to transient activation of Rac and alterations in actin treadmilling. This releases the G-actin-mediated inhibition of MAL. GTP-loaded Rac is both required and sufficient for MAL and SRF activation upon epithelial disintegration, and subsequent transcription of endogenous target genes such as vinculin (vcl) and smooth muscle α-actin (acta2). From (Busche et al., 2008).

Accordingly, in epithelial cells loss of cell-cell contacts during Epithelial-Mesenchymal Transition (EMT) correlates with an induction of MAL-SRF activity. Importantly, TGFβ induced EMT is dependent on MAL as dominant negative MAL or knockdown of MAL prevents TGFβ mediated EMT. It seems to be a feed forward mechanism as overexpression of a dominant active MAL construct is sufficient to trigger EMT (Morita et al., 2007).

On top of the dissociation of cell-cell contacts, shear forces sensed by integrins can activate as well MAL-SRF mediated transcription on a ROCK dependent manner (Zhao et al., 2007). This result could explain why in pressure or volume overload, the myocardium myofibroblasts differentiate as marked by the increased expression of the MAL-SRF target smooth muscle alpha actin 2 (Acta2).

Finally, in neurons, increased synaptic activity or stimulation with the Brain Derived Neurotrophic Factor (BDNF) triggers as well MAL-SRF mediated transcription. Intriguingly,

these two stimuli were dependent on the MAP kinase pathway as treatment with the MEK inhibitor UO126 impaired the induction of MAL-SRF driven transcription (Kalita et al., 2006). This result contrasts with the Erk mediated inhibiting phosphorylation described for MAL (Muehlich et al., 2008)(q.v. 2.1.8.1.2).

2.1.13 Known functions of MRTF mediated transcription

2.1.13.1 Phenotypes of the MRTFs knockouts

In mice, loss of MAL has a limited phenotype. Only the development of mammary myoepithelial cells is affected (Li et al., 2006; Sun et al., 2006b). These cells share common traits with smooth muscle cells even if they originate from a different lineage. Their altered development in mutant mice leads to their apoptosis and the inability of the mothers to nurse their offspring.

In mice, loss of MRTFB induces a defect in smooth muscle cell differentiation and cardiovascular development leading to death between embryonic day 17.5 and postnatal day 1 from cardiac outflow tract defects (Li et al., 2005; Oh et al., 2005). Remarkably, MRTFB restoration only in cardiac neural crest was sufficient to rescue the pathology induced by complete loss of MRTFB.

Finally, loss of function of all three members of the MRTF family by expression of a dominant negative construct in the skeletal muscle lineage leads to a severe skeletal muscle hypoplasia (Li et al., 2005). The muscle fibers formed but failed to undergo hypertrophic growth like in the mutant mice where SRF is as well specifically deleted in the skeletal lineage.

In conclusion, the straight single MRTFs knockouts have a mild phenotype likely due to the compensation mechanisms between the different MRTFs.

2.1.13.2 Control of actin homeostasis and cytoskeleton genes

The β-actin gene itself is a target of the actin-MAL-SRF pathway. Increased expression of β-actin inhibits MAL-SRF signalling and therefore β-actin transcription. This regulatory loop is a critical element of the actin homeostasis.

Other genes involved in cytoskeleton dynamics like Vinculin, Zyxin, Integrin β1 or Gelsolin have been characterised as MAL SRF targets (Miralles et al., 2003; Philippar et al., 2004; Schratt et al., 2002). Vinculin is an adapter protein that links the actin cytoskeleton to the

plasma membrane. The N terminal part of Vinculin binds Talin which interacts with β integrins while the C terminal part of Vinculin binds F actin. Integrin β1 associates with Integrin α chains to bind elements of the Extra Cellular Matrix (ECM) like fibronectin. Interestingly, loss of Vinculin or Integrin β1 leads to the impairment of cell adhesion and spreading resembling the loss of SRF (Fassler et al., 1995; Schratt et al., 2002; Xu et al., 1998). Gelsolin is a remodelling agent of actin cytoskeleton. Gelsolin severs actin filaments and cap the resulting fragments to prevent regrowth. Uncapping of Gelsolin provides new growth competent actin filament end to adapt the actin cytoskeleton to a new situation (Sun et al., 1999). Accordingly, Gelsolin knockout MEFs show an increase in stress fibers (Witke et al., 1995).

2.1.13.3 Role in adhesion and migration

SRF-/- MEFs have a defect in adhesion, migration and cell spreading (Schratt et al., 2002). The restricted panel of known specific MAL targets lead to the assumption that the phenotype of MEFs SRF-/- was in part due to the loss of the expression of MRTF dependent SRF targets. Knockdown of MRTF A and B confirmed this hypothesis (Medjkane et al., 2009). Adhesion, spreading, motility and invasion were impaired in the MBA-MB231 breast carcinoma cell line and in the B16F2 melanoma cell line. In a colonisation model of distant organs by metastatic cells, cancer cells depleted for MRTFs fails to colonize lungs. This is likely due to the adhesion defects as the cells were able to reach the lungs but were cleared away by the blood flow. Two target genes of MRTF-SRF, MYH9 and MYL9 were proposed to mediate this phenotype (Medjkane et al., 2009).

Moreover, inhibition by SCAI of MAL-SRF mediated signalling in MDA-MB435 inhibits invasion (Brandt et al., 2009a). The MAL-SRF target Integrin β1 is proposed to mediate this effect as knockdown of Integrin β1 or an Integrin β1 a blocking antibody rescue the gain of invasion mediated by knockdown of SCAI.

2.1.13.4 Cell differentiation

MRTFs play a major role in the switch between proliferation and differentiations of smooth muscle cells or myoblasts notably by competing with TCFs controlled by the MAP kinase pathway (Wang et al., 2004).

Moreover, MAL has been shown as well to control the differentiation of megakaryocytes (Cheng et al., 2009). MAL level is increased during megakaryocytes differentiation in mice. The platelet counts in the peripheral blood of mice MAL-/- are reduced as well as the ploidy of megakaryocytes in the bone marrow which is a megakaryocyte differentiation marker. Forced expression of MAL induced the megakaryocytic differentiation of primary human CD34(+) cells cultured in the presence of thrombopoietin.

Finally, MKL1 plays a major role in the differentiation of two nervous system cell types.

A first trait that is affected by MAL and SRF is the neurite outgrowth. Depletion of SRF or overexpression of a dominant negative MAL construct leads to an impairment of neurite outgrowth while overexpression of a constitutive active SRF construct promoted neurite elongation (Knoll et al., 2006; Shiota et al., 2006). Inhibition of MAL-SRF mediated transcription by the inhibiting actin mutant R62D inhibited neurite outgrowth and neuronal motility while activation by the mutant actin G15S activated neurite outgrowth and filopodia formation (Stern et al., 2009). Impairment of MAL-SRF transcription perturbs axon guidance and brain circuit formation (Knoll et al., 2006).

A second trait affected by MAL-SRF is the differentiation of oligodendrocytes (Stritt et al., 2009). SRF depletion in the forebrain of mice inhibits terminal differentiation of oligodendrocytes. Consistently, SRF mutants harbour a myelination defect and axon degeneration. Yet, the differentiation defect is non cell autonomous as an expression of the activated SRF construct SRFVP16 limited to neurons is sufficient to restore the differentiation of oligodendrocytes. CTGF was proposed to mediate the inhibition of oligodendrocytes maturation observed in SRF mutants. Interestingly, CTGF is a validated target of MAL-SRF upon serum stimulation in fibroblast but its expression is increased in the corpus callosum of SRF knockout mice, meaning that SRF negatively regulates CTGF expression in neurons.

2.1.13.5 Control of proliferation and apoptosis

MAL was discovered as an antiapoptotic gene involved in TNFα signalling (Sasazuki et al., 2002). However, no further result so far supported that MRTF could promote cell survival. In latter reports, Myocardin has been shown to reduce cell proliferation (Chen et al., 2002b; Milyavsky et al., 2007; Shats et al., 2007). Myocardin inactivation by a dominant negative

construct or by SiRNA increased fibroblast proliferation (Milyavsky et al., 2007). Interestingly, the repression of proliferation mediated by Myocardin is cell density dependent: Myocardin is able to repress the proliferation of WI-38 human fibroblasts only at low cell density. Finally, Myocardin expression has been shown to be impaired in colon and prostate cancer. One possible mechanism for Myocardin to impair cell proliferation is to inhibit NFκB dependent cell cycle progression (Tang et al., 2008). Yet, it is worth to note that in the MDA-MB231 breast carcinoma cell line and in the B16F2 melanoma cell line, knock down of MRTF A and B didn't affect either proliferation or apoptosis (Medjkane et al., 2009).

2.1.13.6 Knowledge out of genome wide screen for SRF targets

Until recently (Medjkane et al., 2009), the majority of the screens to identify SRF target genes were carried out without being able to specify if the target genes were controlled by the Actin-MAL pathway, by the MAPK-TCFs pathway or by other coactivators of SRF (Cooper et al., 2007; Philippar et al., 2004; Sun et al., 2006a). Yet, one screen did try to identify genes specifically controlled by MAL, but the use of the dominant negative MALΔC weakens the proposed classification of the targets between MAL dependent and MAL independent (Selvaraj and Prywes, 2004). MALΔC works as dominant negative construct because of the loss of the transactivation domain but it retains the ability to interact with SRF. Therefore MALΔC may displace TCFs from SRF and consequently block indiscriminately the two pathways leading to SRF activation.

2.2 Aim of the project: Identification of G-actin regulated genes

The primary goal of the project was to identify on a genome wide basis the genes specifically controlled by the Actin-MAL-SRF pathway in order to better characterise its physiological role and importance for the cell (Figure 11).

The signalling pathway was targeted at the actin step by the actin binding drugs Cytochalasin D and Latrunculin B which respectively activates or represses MAL-SRF mediated transcription by destabilizing or stabilizing the G-actin-MAL complex (q.v. 2.1.4; Figure 11). This strategy allows screening for genes controlled by Actin-MAL-SRF but also for genes controlled by potentially new sets of transcription factors dependent on monomeric actin.

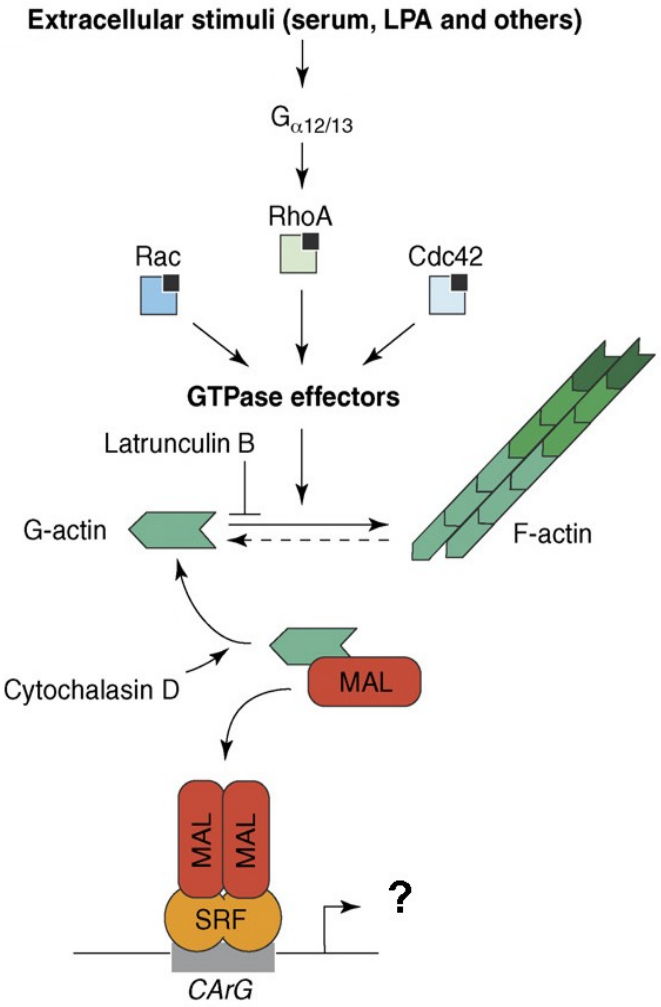


Figure 11. Control of SRF Activity by the Rho-Actin-MAL Pathway and Actin Binding Drug

From the screen, only the genes which were differentially regulated by Cytochalasin D and Latrunculin B were considered for further characterisation. As the two drugs depolymerise the F-actin cytoskeleton, genes dependent on F-actin could not be differentially modulated and hence were excluded.

To identify without bias on a genome wide basis the genes controlled by G-actin, the transcriptome of NIH3T3 fibroblast after actin binding drug treatment was monitored with microarrays. By this approach, the hope was to identify and characterize new target genes controlled by monomeric actin mediating unexpected functions sharing G-actin as a sensor.

3 Results part I

3.1 Establishment of the conditions used for the identification of G-actin regulated genes

The screen for G-actin regulated genes was realised in the mouse fibroblast NIH3T3 cell line (Todaro and Green, 1963; Todaro et al., 1963) where the Actin-MAL-SRF pathway is best characterised. As mentioned before, in order to identify G-actin regulated genes, the expression of the target genes was triggered with Cytochalasin D and repressed with the inhibiting drug Latrunculin B.

To determine the proper concentration of the two drugs the 3DA luciferase reporter (Geneste et al., 2002) which monitors the SRF activity controlled by the Actin-MAL pathway was utilised (Figure 12 A). The best ratio between the induction mediated by Cytochalasin D and the repression by Latrunculin B was obtained for a concentration of 2µM Cytochalasin D and 5µM Latrunculin B.

Then, the induction of the known MAL-SRF target genes Ctgf and Srf, which controls its own expression, was monitored over the time after actin binding drug treatment to determine the best time point for the screen (Figure 12 B). After 90 min of Cytochalasin D treatment, while Srf expression was increasing further, Ctgf expression attained a maximum and the repression of SRF and Ctgf mediated by Latrunculin B reached a plateau. At that time point, the induction by Cytochalasin D of the two target was more than 2,5 times as well as the repression by Latrunculin B of the Cytochalasin D induction. Therefore, the 90 minutes time point was chosen. This time point has the advantage as well to be short limiting the possibility to detect secondary regulated targets.

In order to restrict even more the screen to primary targets, the cells were pretreated with the translation inhibitor cycloheximide (CHX). The final conditions are summarized on (Figure 13).

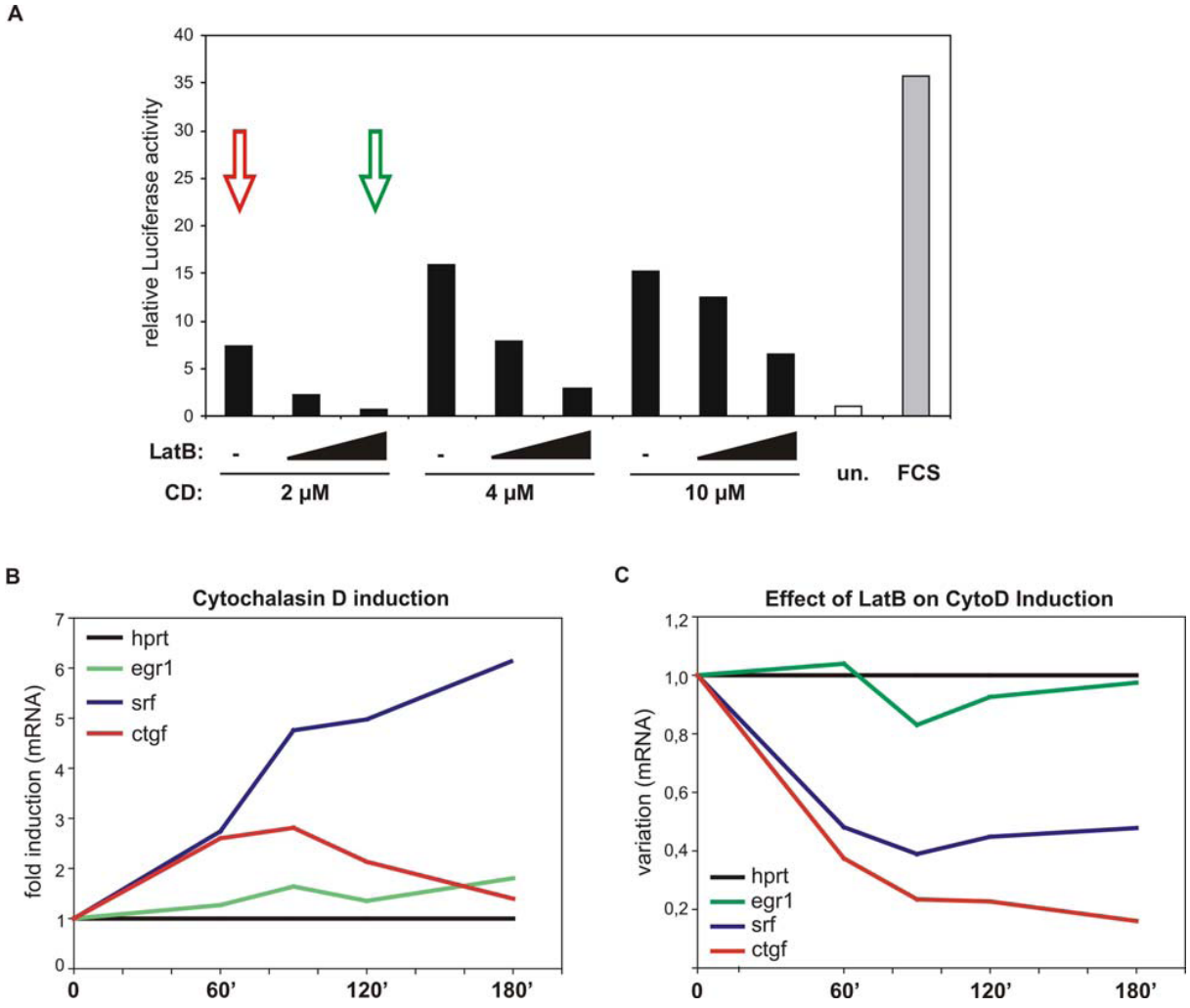


Figure 12. Establishment of the Conditions Used for Gene Expression Analysis

(A) NIH 3T3 cells were transiently transfected with a 3xSRF luciferase reporter and serum starved overnight. Following pretreatment with 0 µM, 1 µM, or 5 µM latrunculin B, cells were treated for 7 hr with 2 µM, 4 µM, or 10 µM of cytochalasin D, as indicated. Unstimulated and serum-induced reporter activity is shown as a control. Arrows indicate the conditions used for subsequent microarray analysis.

(B and C) Time course analysis of endogenous gene expression, which was analyzed following mRNA isolation by quantitative RT-PCR using primers specific for *egr1, srf, ctgf*, *and hprt*. The relative mRNA amount was calculated by normalisation to *hprt*. Cells were treated with 2 µM cytochalasin D for 0, 60, 90, 120, and 180 min (B). Cells were preincubated with 5 µM latrunculin B and subsequently treated with 2 µM cytochalasin D for 60, 90, 120 and 180 min (C). Shown is the ratio of the relative mRNA amounts of cells treated with cytochalasin and cytochalasin D + Latrunculin B.

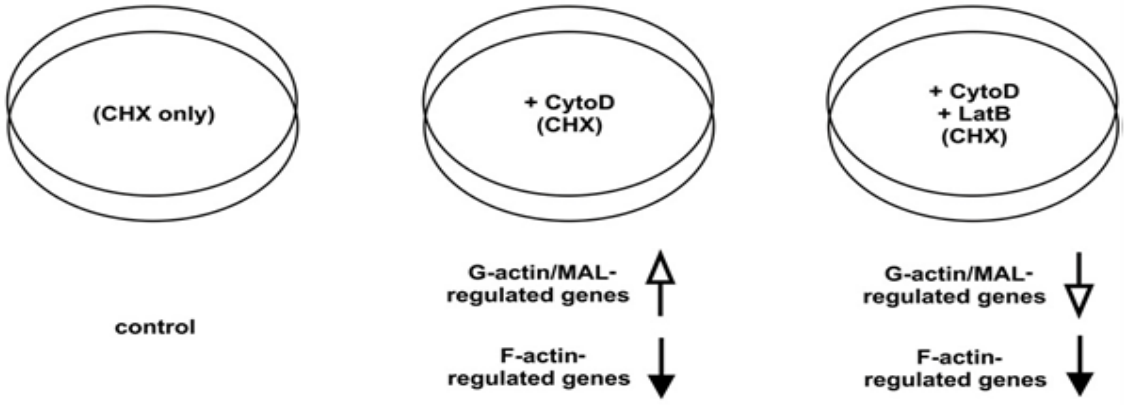


Figure 13. Schematic Description of the Three Conditions Used for Screening in NIH 3T3 Fibroblasts.

Control cells were treated with cycloheximide only (CHX, 3 mg/ml, 2 hr). G-actin-regulated genes were induced by treatment with cytochalasin D (CytoD, 2 mM, 90 min) and inhibited by latrunculin B (LatB, 5 mM).

A first concern about the screening approach was that actin binding drugs could activate stress response kinases like p38 and JNK. Actually, JNK activation was not detected in the conditions used for the screnn while p38 was activated by the two drugs Cytochalasin D and Latrunculin B (Figure 14).Therefore, genes controlled by p38 phosphorylation will not score as differentially regulated and then then will not be considered as potential G-actin targets.

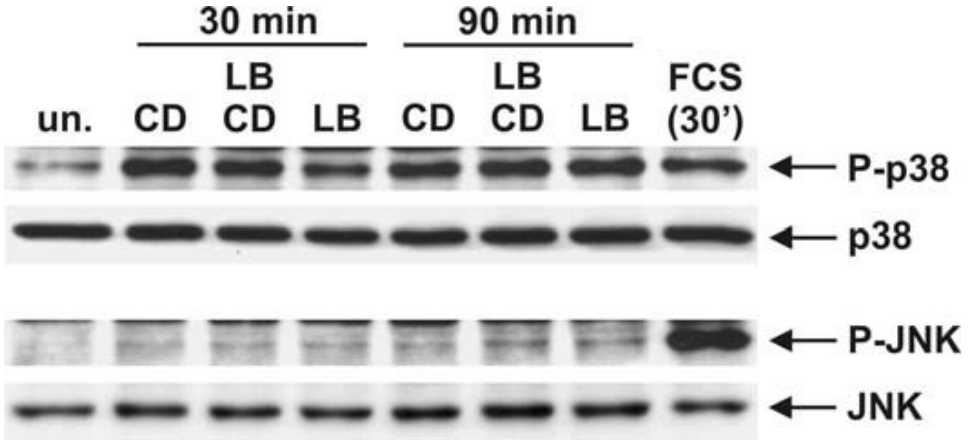


Figure 14. Drug Treatment Used for Identification and Characterization of Targets Does Not Differentially Regulate Stress Kinases

Cells were serum starved for 24 hr and then stimulated with cytochalasin D (CD, 5 µM), latrunculin B (LB, 5 µM) alone or in combination for 30 min and 90 min. Total protein lysates were immunoblotted using antibodies specific for anti-phospho-p38 and anti-phospho-JNK. Reblots showing equal loading were done with anti-p38 and anti-JNK1. Serum stimulation for 30 min is shown as a positive control.

A second concern was that mRNAs could be degraded after actin binding drug treatment. The RNA samples of the 3 independent experiment used for the screen were run on agarose gels. No sign of degradation of the 28S or 18S ribosomal DNA could be observed (Figure 15).

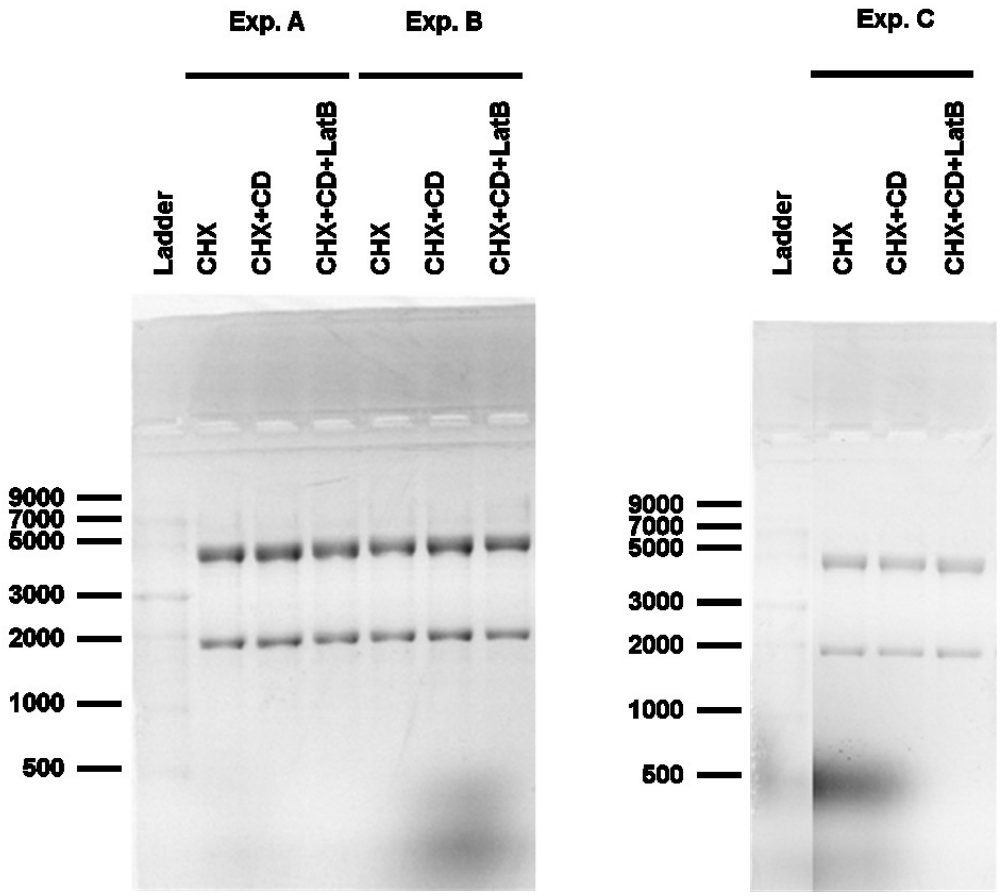


Figure 15. RNA Gels of Samples Treated with Actin Binding Drugs Used for the Microarrays

NIH 3T3 fibroblasts were treated with cycloheximide (CHX, 3 mg/ml, 2 hr), cytochalasin D (CytoD, 2 mM, 90 min) and latrunculin B (LatB, 5 mM, 90 min) as indicated.

These 9 samples were then processed and hybridised to 9 Affymetrix GeneChip Mouse Genome 430 2.0 arrays allowing the analysis of the expression of over 39000 transcripts. The processing of the arrays as well their analysis was done externally by a collaboration partner (Reinhard Hoffmann, Institute of Medical Microbiology and Immunology, Technical University of Munich).

3.2 Analysis and quality control of the microarray experiment

One of of the quality method used is the RNA digestion plot. On the GeneChip 230, a transcript can be monitored by several probe sets. Each probe set is composed of 11 probes with a perfect match plus 11 probes with a single mismatch. The in vitro reverse transcription proceeds from the 3' end of the transcript towards the 5' end. If the reverse transcription stops, a signal increase of probe sets present at the 3' end of the transcripts is expected. This artefact from the reverse transcription is then used as a first quality control. The hybridisation of the samples gave rise to the expected pattern on the 9 arrays (Figure 16). The probes located at the extreme 5' end position (probe number=0) gave a weaker signal than the probes located at the extreme 3' end position (probe number=10). The difference in signal intensity between the probes according to their position on the transcript is in the acceptable range: the samples were not degraded.

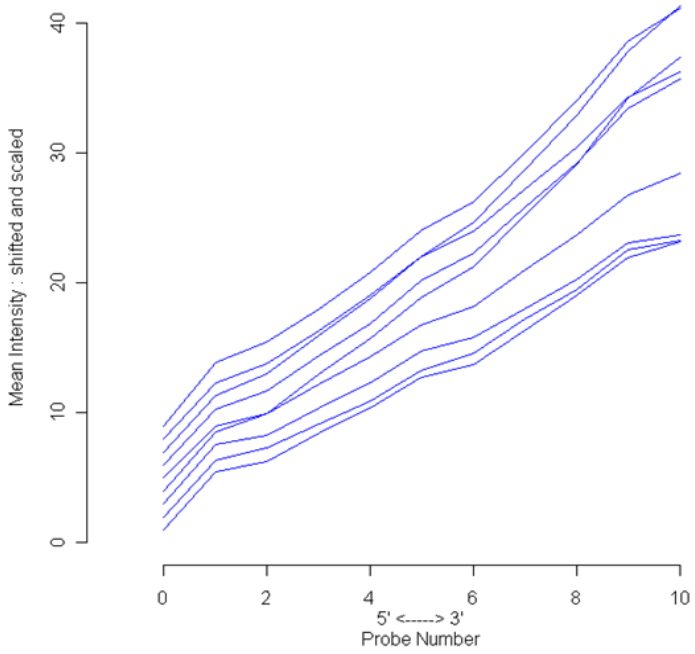


Figure 16. RNA Digestion Plot

Each curve represents the overall average expression intensity by probe position per array. The probes #0 are located close to the 5' end while the probe #10 is located close to the 3' end of the transcripts.

Another quality control is the comparison of the probe signals of each array to the probe signals of a virtual mean array. The virtual mean array is the average of the signal of the 9 arrays of the experiment. This comparison is done thanks to a MA plot (Dudoit et al., 2002).

A problem concerning the normalisation process would lead to a deviation of the probe signal scatter away from the horizontal M=0 line which is not the case (Figure 17). Only a small fraction of the transcriptome has been affected by the experiment like expected in any microarray experiment.

The two quality controls presented support the idea that the actin binding drug treatment didn't perturb massively RNA stability or expression and is therefore a compatible stimulus with a transcriptomic approach.

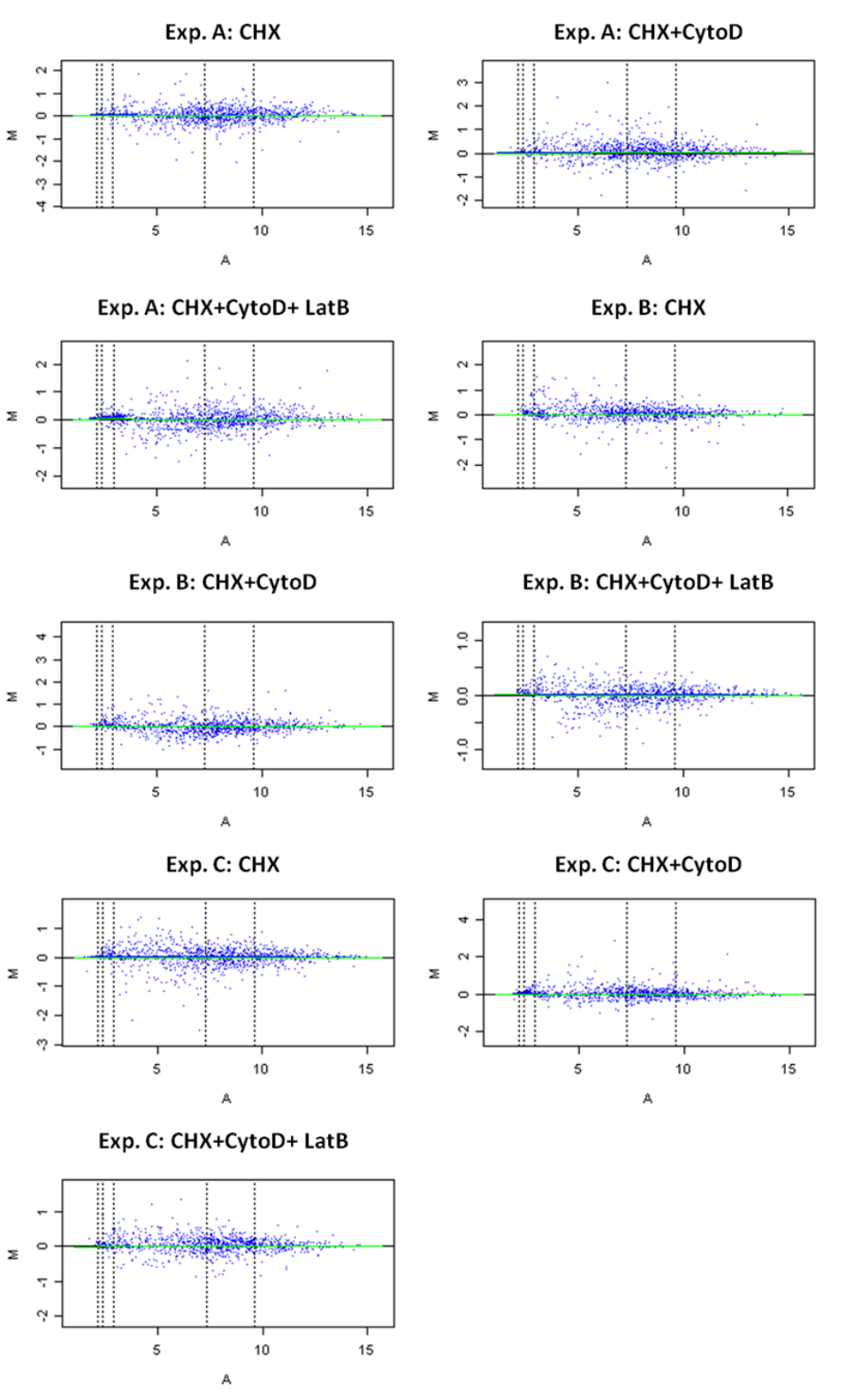
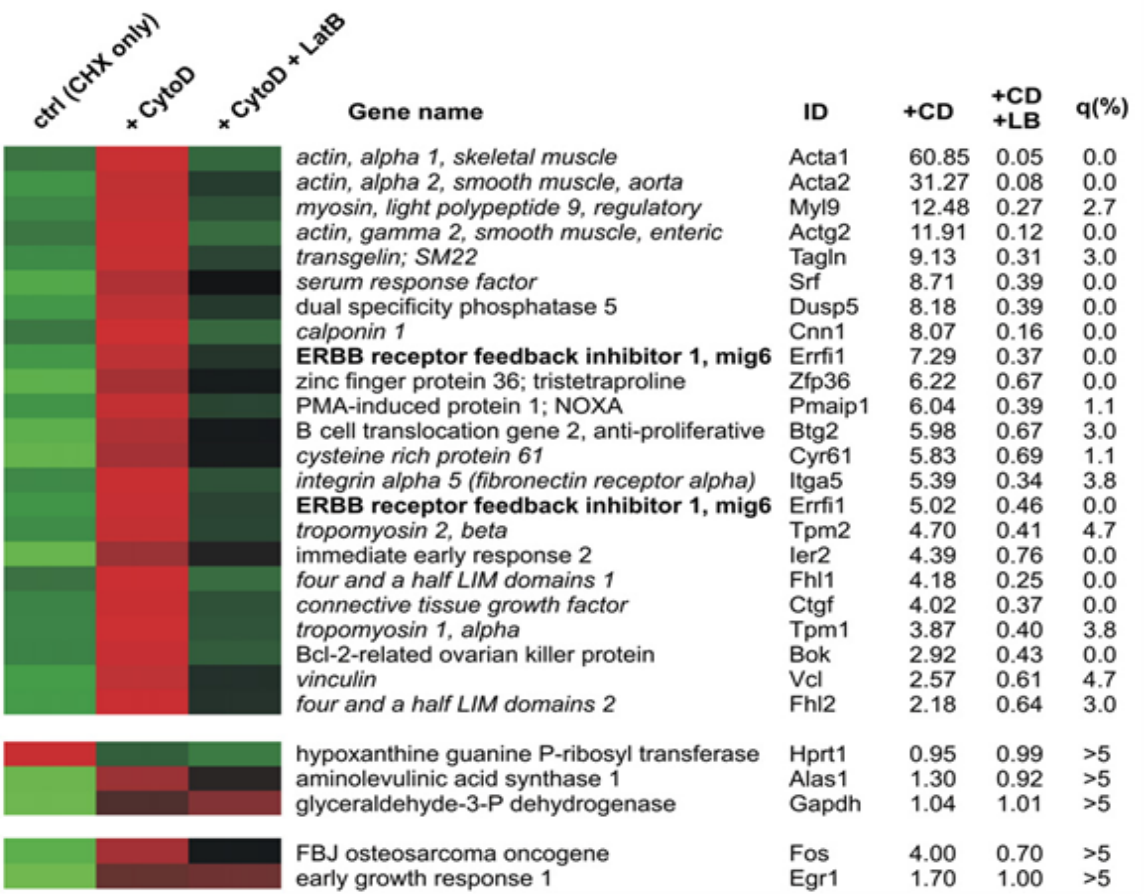


Figure 17. Control of the Normalisation of the Microarrays by M/A Plot

M is the intensity log ratio ($M= \log_2 I_{probe\ x,\ array\ y} - \log_2 I_{probex,\ mean\ array}$). A is the average intensity ($A= - (\log_2 I_{probex,\ array\ y} + \log_2 I_{probex,\ mean\ array})$) where I is the probe intensity. See text and (Dudoit et al., 2002).

3.3 Putative G-actin regulated genes identified by microarray

The arrays were analysed with the RMA (Irizarry et al., 2003) algorithm and its improved version GC-RMA (Wu et al., 2004) which takes into account the GC content of the probes during background normalisation. By setting a False discovery rate of 5.43 for GCRMA and 5,17 for the RMA analysis a combined list of probe sets differentially regulated of 255 corresponding to 210 genes was obtained. More than 30% of the genes identified were already present in previous screens for SRF targets supporting the screening approach. For example, several actin genes, the early marker of smooth muscle cell development SM22, the Connective Tissue Growth factor (CTGF) or Srf itself were found (Figure 18).



Gene name	ID	+CD	+CD +LB	q(%)
actin, alpha 1, skeletal muscle	Acta1	60.85	0.05	0.0
actin, alpha 2, smooth muscle, aorta	Acta2	31.27	0.08	0.0
myosin, light polypeptide 9, regulatory	Myl9	12.48	0.27	2.7
actin, gamma 2, smooth muscle, enteric	Actg2	11.91	0.12	0.0
transgelin; SM22	Tagln	9.13	0.31	3.0
serum response factor	Srf	8.71	0.39	0.0
dual specificity phosphatase 5	Dusp5	8.18	0.39	0.0
calponin 1	Cnn1	8.07	0.16	0.0
ERBB receptor feedback inhibitor 1, mig6	Errfi1	7.29	0.37	0.0
zinc finger protein 36; tristetraproline	Zfp36	6.22	0.67	0.0
PMA-induced protein 1; NOXA	Pmaip1	6.04	0.39	1.1
B cell translocation gene 2, anti-proliferative	Btg2	5.98	0.67	3.0
cysteine rich protein 61	Cyr61	5.83	0.69	1.1
integrin alpha 5 (fibronectin receptor alpha)	Itga5	5.39	0.34	3.8
ERBB receptor feedback inhibitor 1, mig6	Errfi1	5.02	0.46	0.0
tropomyosin 2, beta	Tpm2	4.70	0.41	4.7
immediate early response 2	Ier2	4.39	0.76	0.0
four and a half LIM domains 1	Fhl1	4.18	0.25	0.0
connective tissue growth factor	Ctgf	4.02	0.37	0.0
tropomyosin 1, alpha	Tpm1	3.87	0.40	3.8
Bcl-2-related ovarian killer protein	Bok	2.92	0.43	0.0
vinculin	Vcl	2.57	0.61	4.7
four and a half LIM domains 2	Fhl2	2.18	0.64	3.0
hypoxanthine guanine P-ribosyl transferase	Hprt1	0.95	0.99	>5
aminolevulinic acid synthase 1	Alas1	1.30	0.92	>5
glyceraldehyde-3-P dehydrogenase	Gapdh	1.04	1.01	>5
FBJ osteosarcoma oncogene	Fos	4.00	0.70	>5
early growth response 1	Egr1	1.70	1.00	>5

Figure 18. Transcriptome Analysis of Genes Regulated by G-Actin-MAL Signaling

List of some differentially regulated genes and controls. Shown is the induction (red) or repression (green) as a heat map. Genes are sorted according to the average fold induction by cytochalasin D (n = 3), with known MAL/SRF targets in italics. Average repression by latrunculin B is in comparison to the CytoD induction. The q value represents a measure of statistical significance. As controls, three housekeeping genes as well as two known MAL-independent SRF targets are depicted (lower panels). See annexe 1 for a complete list.

Unsupervised clustering of the probe sets, lead to the identification of 5 major groups of genes sharing the same pattern of induction/repression by the actin binding drugs (Annexe 1). The groups 3, 4 and 5 correspond to the genes which are induced by Cytochalasin D and are repressed by Latrunculin B. With a total of 163 genes, they form the majority of the genes with significant variations. Several known MRTF-SRF or SRF dependent genes are part of these groups.The group 2 genes are induced by Cytochalasin D but not repressed by Latrunculin B and may represent genes dependent on F-actin status. Finally the group1 genes are inversely regulated. Instead of being induced by Cytochalasin D, the expression of group 1 genes is repressed. These genes could be the best candidate genes controlled by monomeric actin but by another set of transcription factor than MAL-SRF. However, the relatively low induction by Latrunculin B and repression by Cytochalasin D limited the interest to consider at first this group.

To validate the results monitored on the microarrays, 20 out of 210 genes were selected. The induction by Cytochalasin D and the repression by LatrunculinB could be confirmed by Real Time Quantitative PCR (RT-QPCR) for 19 genes. Only, collagen type I alpha 2 (Col1a2) showed a different tendency by RT-QPCR than on the arrays (Tableau 1).

Tableau 1. Verification by Quantitative RT-PCR of the Gene Expressions Monitored on the Microarrays

Gene	Microarray (n=3)		qRT-PCR (n=1)	
Arc	17,39	0,14	9,04	0,31
Pkp2	12,85	0,16	4,43	0,34
Srf	7,90	0,56	5,59	0,63
Pall	7,37	0,34	5,62	0,37
Errfi1	7,29	0,37	5,59	0,46
Rassf6	7,01	0,20	4,55	0,34
Zfp36	6,22	0,67	5,99	0,81
Ankrd1	6,12	0,25	4,08	0,35
Itga5	5,39	0,34	3,13	0,55
Lima1	5,21	0,47	3,18	0,63
Lims2	4,53	0,27	3,32	0,45
Rassf3	4,38	0,45	2,44	0,57

Gene	Microarray (n=3)		qRT-PCR (n=1)	
Gpr23	4,28	0,23	3,06	0,31
Rgs16	4,25	0,31	3,61	0,49
Ctgf	4,02	0,37	5,20	0,26
Syde2	3,31	0,40	2,87	0,49
Ankrd15	3,08	0,36	3,52	0,33
Inversely regulated:				
Gadd45B	0,38	1,46	0,48	1,41
Ccl2	0,29	1,69	0,30	1,64
Col1a2	0,22	1,22	1,14	1,07
Arrdc3	0,16	2,57	0,30	2,30

3.4 Mig6/Errfi1 is a Target of Actin Signalling

The first fonctional studies were focused on Mig6 which is a negative regulator of the EGFR MAP kinase axis (Hackel et al., 2001) because of the importance of the interaction between the MAP kinase cascade and MRTFs mediated transcription. Mig6 binds preferentially to the activated EGF receptors (Anastasi et al., 2003; Fiorentino et al., 2000; Hackel et al., 2001) by an EGFR Binding Domain (EBD) shared with the Ack1 tyrosine kinase. Mig6 inhibits EGFRs by blocking the formation of the dimer interface between EGFRs necessary for the activation of downstream effectors (Zhang et al., 2007b).

Mig6 expression was monitored on the microarrays by two different sets of pobes with high level of confidence (q value=0 for both probe sets). Mig6 was induced by Cytochalasin D and repressed by Latrunculin B like a MAL-SRF target gene. The variations monitored for Mig6 on the microarrays were confirmed in triplicates by qRT-PCR and compared with the variations observed for the known MAL-SRF target gene Ctgf (Figure 19 A).

The induction of Mig6 was confirmed at the protein level in several cell lines by Cytochalasin D but also by Swinholide A and Jasplakinolide two other actin binding drugs which are known to activate MAL-SRF mediated transcription (Figure 19 B). Swinholide A is supposed to work like Cytochalasin D. On the opposite, Jasplakinolide, which stabilizes F-actin filaments (Bubb et al., 1994; Bubb et al., 2000), is supposed to activate MAL-SRF mediated transcription by depleting G-actin. Repression of Cytochalasin D induction by Latrunculin B could be also observed at the protein level (Figure 19 B).

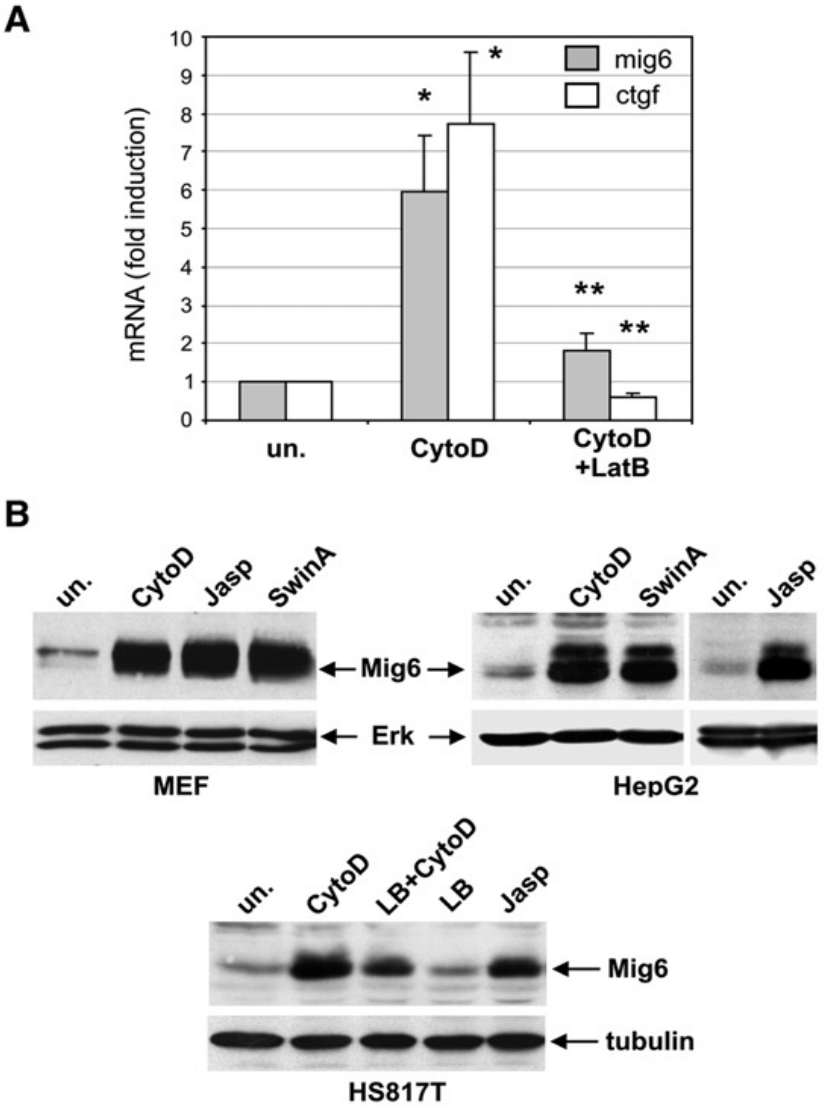


Figure 19. Validation of Mig6 Regulation by Actin Signaling

(A) Induction of mig6 mRNA by cytochalasin D and latrunculin B. NIH 3T3 cells were treated with vehicle (un.) or cytochalasin D (2 mM) for 120 min or with Cytochalasin following 30 min pretreatment with latrunculin B (5 mM). The mRNA was isolated and subjected to quantitative RT-PCR. Shown is the average induction of mig6 after normalization to hprt, and the known actin-MAL-regulated gene ctgf for comparison. Error bars, SEM (n = 3). *, significant activation; **, significant repression ($p < 0.05$, unpaired Student's t test). (B) Mig6 protein induction following treatment with various actin drugs in MEFs and liver cell lines HepG2 or HS817T. Cells were treated with vehicle, CytoD (2 mM), jasplakinolide (Jasp, 0.5 mM), swinholide A (SwinA, 0.3 mM), or LatB (5 mM) for 7 hr and analyzed by immunoblotting as indicated.

To characterise more precisely, the induction of Mig6 by Cytochalasin D, a dose response and a time course were realised. A concentration of 2µM and an induction time of 7 hours were sufficient to trigger a full induction of Mig6 (Figure 20 A). At any time, no significant increase of Erk phosphorylation was detected excluding an induction of Mig6 by the MAP

kinase cascade. Jasplakinolide didn't induce either Erk phosphorylation and required as well around 7 hours to fully induce Mig6 expression at the protein level (Figure 20 B).

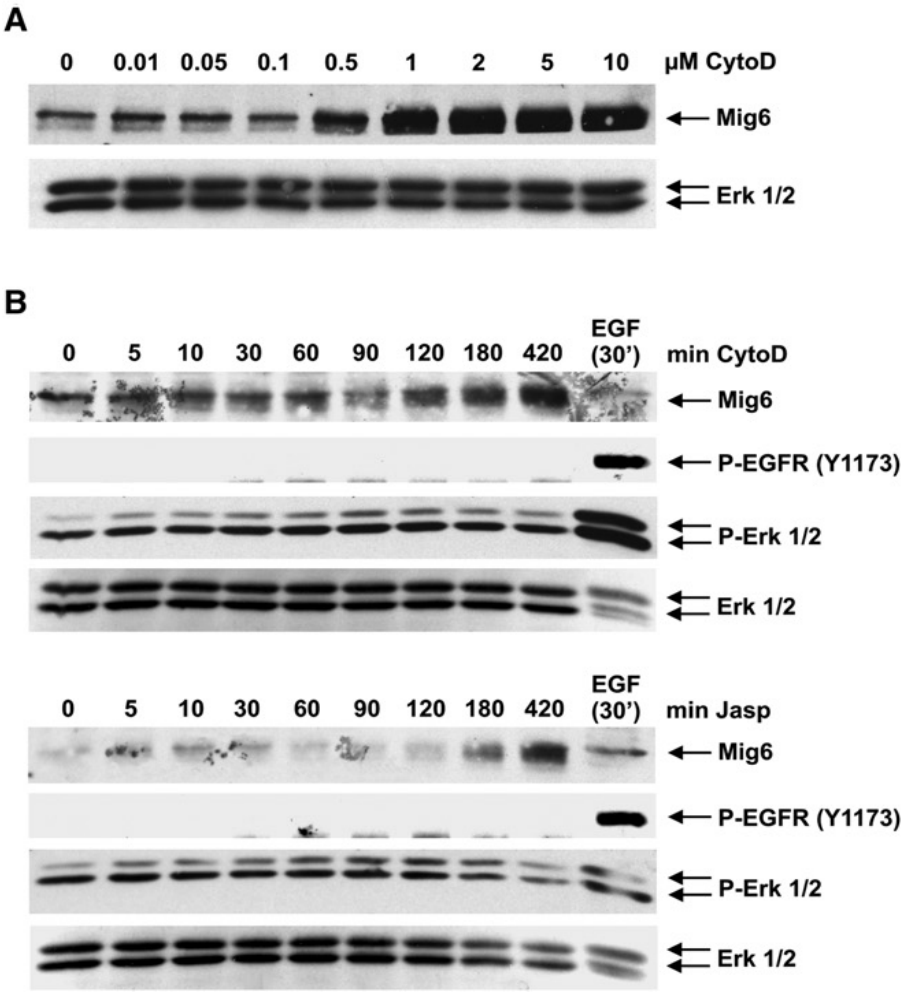


Figure 20. Time Course and Dose Response of the Induction of Mig6 Mediated by Actin Binding Drugs

(A) Dose response of Mig6 protein induction by CytoD in MEF. (B) Time course of Mig6 protein induction by CytoD (upper panels) or Jasp (lower panels) in MEFs.

3.5 Mig6 induction by members of the Rho-Actin-Mal-Srf pathway

More specific than actin binding drugs, over expression of activated MAL, SRF constructs or Myocardin was sufficient to induce the expression of Mig6 in MEFs (Figure 21 A). Over expression of activated MAL, SRF or Rho were sufficient as well to induce Mig6 expression in NIH3T3 in the same range as the other known MRTF-SRF targets Integrin alpha 5 (Itga5) and

Ctgf. Overexpression of MAL full length didn't induce Mig6 expression in accordance with its much lower transcriptional activity than the activated form MALΔN (Figure 21 A and B).

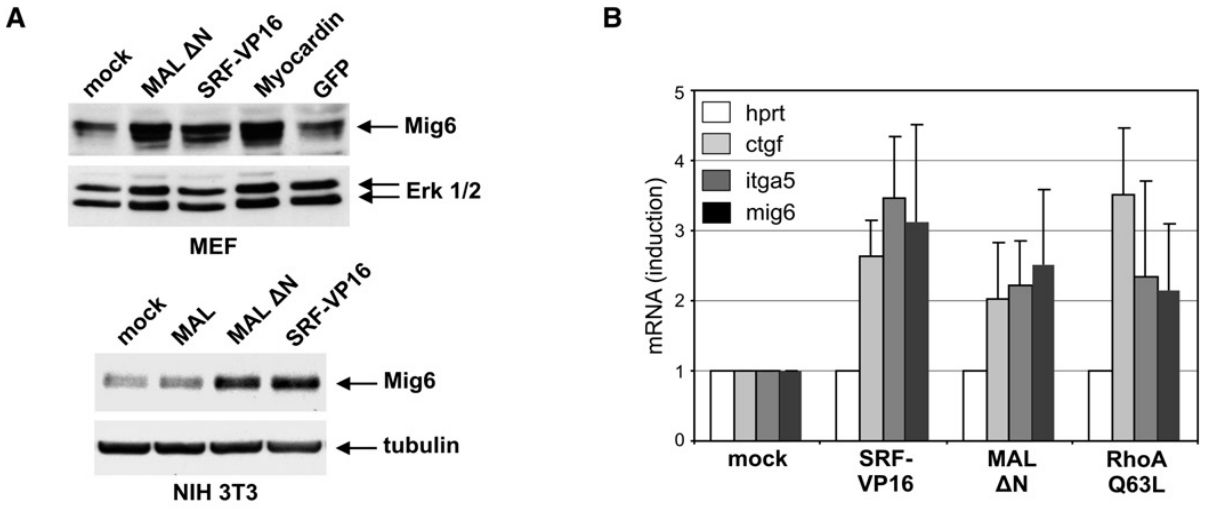


Figure 21. Induction of Mig6 by Expression of Overexpression of Members of the Rho-Actin-Mal-Srf Pathway

(A) Induction of Mig6 protein by transient transfection with activated MAL DN, SRF-VP16, or Myocardin. Total lysates from electroporated MEFs (top panels) or lipofected NIH 3T3 (bottom panels) were immunoblotted for Mig6 and Erk or tubulin.(B) Regulation of mig6, itga5, and ctgf mRNA by MAL, SRF, and RhoA. NIH 3T3 cells were transiently transfected with the indicated constructs, and endogenous gene expression was analyzed by quantitative RT-PCR and normalized to hprt (error bars indicate half-range).

3.6 Identification of MAL/SRF response element in the Mig6 promoter

In order to identify in the Mig6 promoter a MAL-SRF response element, different versions in length of the consensus Mig6 promoter were cloned in a luciferase reporter. Promoters starting at -1635bp to -330bp from the transcription start site were responsive to MALΔN and SRFVP16 while a promoter construct starting at -147bp was not inducible anymore. Between the base pairs -330 and -147, a degenerated possible SRF binding site (CCTTCTAAGG) could be identified at position -260. Deletion of this site in the -726ΔCARG construct lead to a complete loss of inducibility by MAL and a decrease by SRF (Figure 22 A).

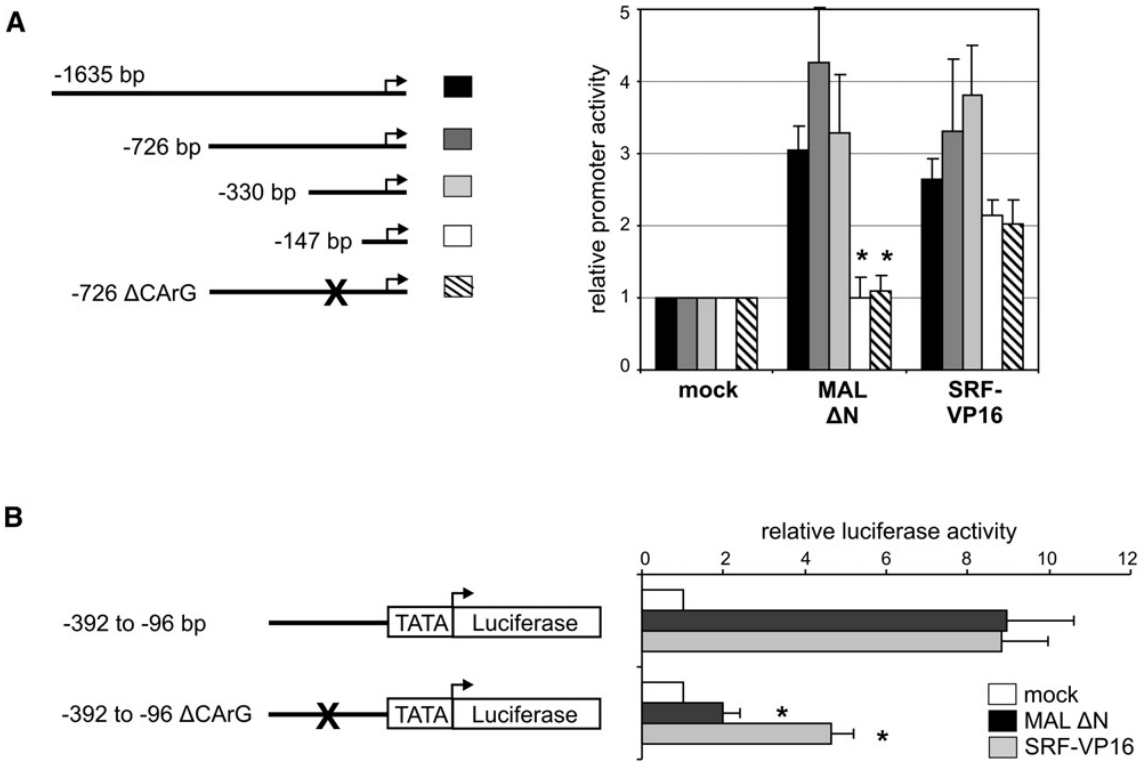


Figure 22 Identification of a MAL/SRF Response Element in the Mig6 Promoter

(A) Deletion analysis of the mig6 promoter. The indicated promoter fragments, ranging from -1635 to +1, were cloned in front of a luciferase reporter and cotransfected with activated ΔNMAL and SRF-VP16. The mutated promoter -726 ΔCArG contains a 10 bp deletion of a putative CArG box at -260, indicated by X. Shown is the mean relative luciferase activity, normalized to Renilla luciferase. Error bars, SEM (n = 3). (B) Responsiveness of a heterologous promoter containing a -392 to -96 fragment in front of a TATA box. Error bars, SEM (n = 4). *, significant repression compared to the WT promoter ($p < 0.05$).

As the Mig6 promoter is strongly active, induction by MAL or SRF of the cloned Mig6 promoter could be impaired by this strong background activity. Therefore, a new luciferase reporter composed of the bases ranging from -392 to -96 upstream of a TATA box controlling the expression of a luciferase gene was created. This new reporter showed a better inducibility by MALΔN or by SRFVP16. Mutation of the degenerated CARG box induced the loss of inducibility by MALΔN like for the original Mig6 promoter. SRFVP16 induction was impaired by around 50% meaning that SRF may bind somewhere else in the promoter (Figure 22 B).

Intriguingly, it was not possible to induce the Mig6 promoter construct with stimuli like LPA. The only way a Mig6 promoter could be induced by a physiological stimulus like LPA was to

overexpress MAL full length. LPA and MAL full length synergised to induce the activity of the Mig6 promoter (Figure 23).

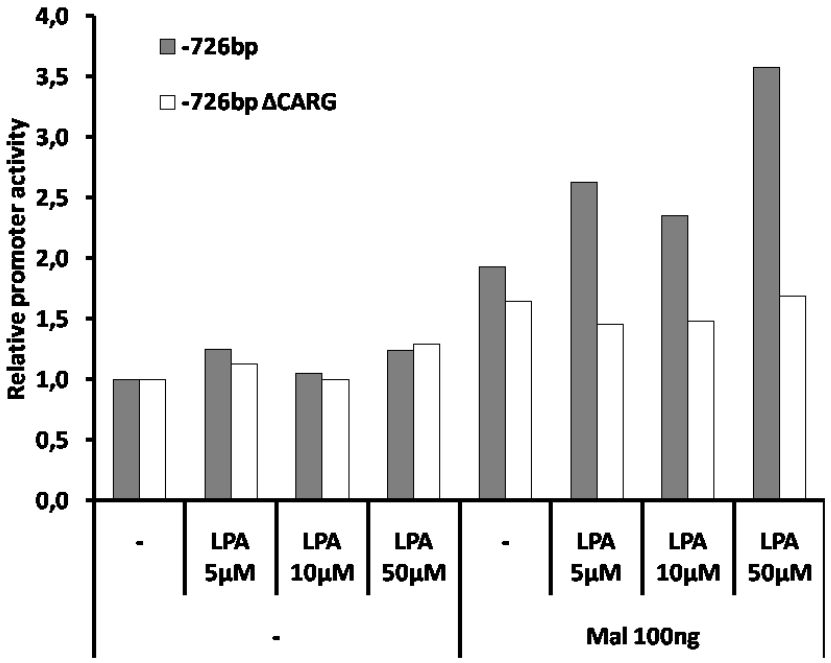


Figure 23 : Induction of the Mig6 Promoter by LPA when MAL is Overexpressed.

Responsiveness to LPA of the Mig6 promoter was evaluated with or without MAL overexpression and with or without the putative CARG box at -260bp.

3.7 Role of NFκB signalling in Mig6 expression

A MAL-SRF response element could be identified in the mouse Mig6 promoter. However, the response element spotted in the mouse Mig6 promoter aligned with a sequence having 2 further mismatches in the human Mig6 promoter compared to a consensus SRF binding site. Analysis of the Mig6 promoter with rVista (Loots and Ovcharenko, 2004), revealed that the MAL response element resembles a NFκB response element (Figure 24 A and B).

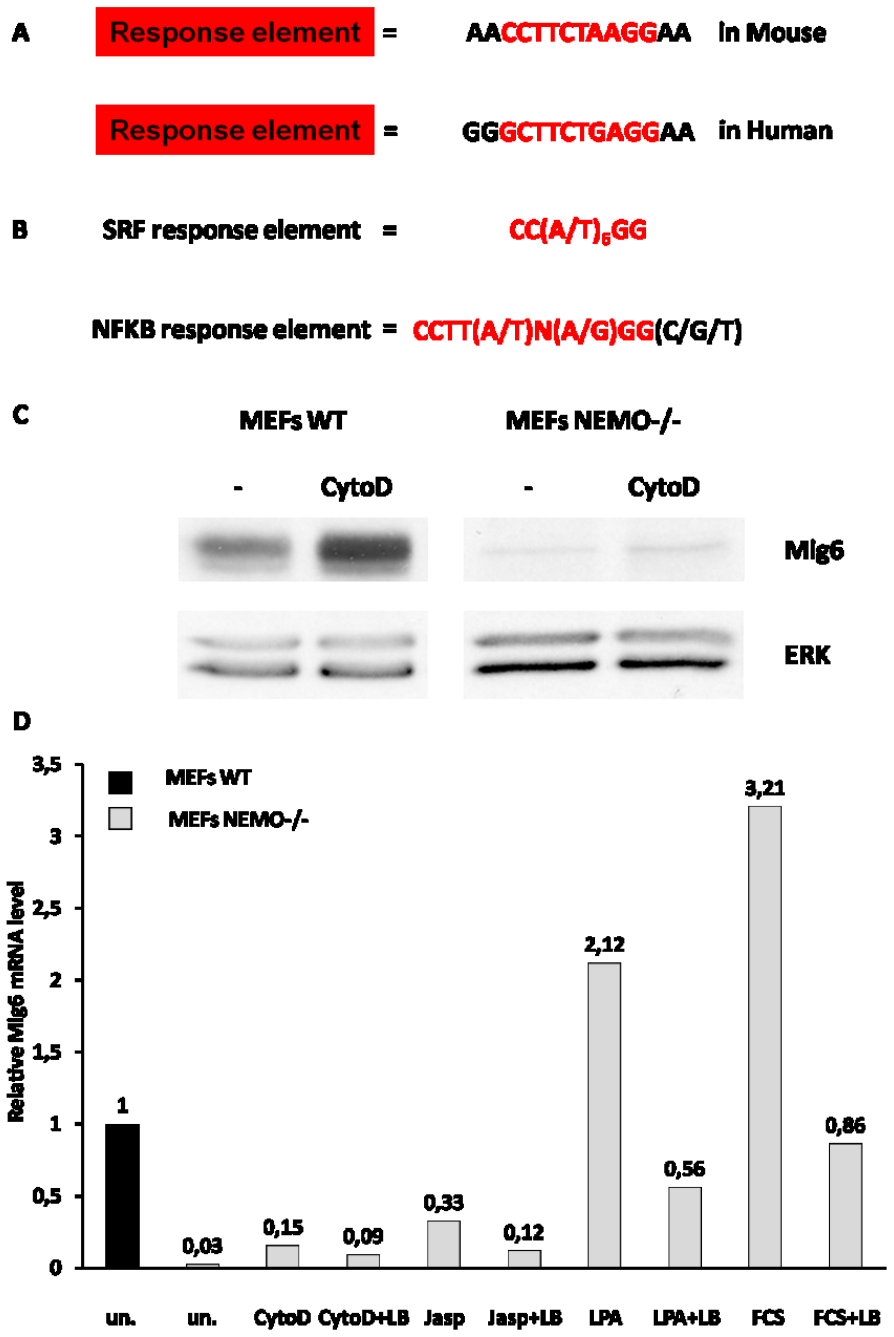


Figure 24. Importance of the NFκB Signalling for the Expression of Mig6

(A) Sequence of the putative Actin-Mal-Srf response element in the murine Mig6 promoter aligned with the corresponding sequence in the human Mig6 promoter. (B) Consensus sequences for a SRF or a NFκB response element. (C) Induction of Mig6 in the NEMO-/- versus WT MEFs at the protein level or (D) at the mRNA level.

Therefore, the induction of Mig6 in MEFs NEMO-/- where NFκB signalling is strongly impaired (Schmidt-Supprian et al., 2000) was monitored. In these cells, the background expression of Mig6 was almost completely lost. Yet, Cytochalasin D treatment could induce Mig6 expression by approximately 5 fold meaning that Actin-MAL signalling is able to induce

Mig6 expression in the absence of NFκB in the nucleus. Therefore, MAL is likely to control Mig6 expression independently of NFκB (Figure 24).

Moreover, MAL and SRF actually bind the Mig6 promoter element identified after serum stimulation as shown by chromatin immunoprecipitation (Descot et al., 2009). Therefore, it is likely that the MAL-SRF complex controls Mig6 expression through the identified element even if the binding site is degenerated.

3.8 The actin cytoskeleton controls EGFR-MAPK signalling by regulating Mig6 expression

The physiological role of the transcriptional control of Mig6 by the Rho-Actin-MAL-SRF signalling was then investigated.

Mig6 inhibits the phosphorylation of several EGFR residues including EGFRY1173 (Anastasi et al., 2007). Then, EGFRY1173 was used as a sensor to monitor EGFR phosphorylation after actin binding drug treatment. At first, HepG2 cells were chosen as a cell model because they express high levels of EGFR allowing biochemical analysis compared to NIH which have a very low density of EGFR on their plasma membrane (Velu et al., 1987). Cytochalasin D or Swinholide A mediated induction of Mig6 correlated with the decrease of EGF induced phosphorylation of EGFRY1173 (Figure 25 A). On the same way, amphiregulin induced phosphorylation of Erk was decreased in cells where Mig6 expression had been triggered by Cytochalasine D (Figure 25 B).

In fact, Mig6 is necessary for the Cytochalasin D or Swinholide A regulation of phospho-EGFR as knockdown of Mig6 by ShRNA resulted in the loss of the dephosphorylation of EGFRY1173 induced by the two actin binding drugs (Figure 25 C).

This result could be confirmed in MEFs knockout for Mig6 where Cytochalasin D or Jasplakinolide can not impair EGFRY1173 phosphorylation induced by a short treatment with EGF like in MEFs wildtype (Figure 25 D).

Thus, the Rho-Actin-MAL-SRF pathway controls the EGFR-MAP kinase axis by the transcriptional control of Mig6.

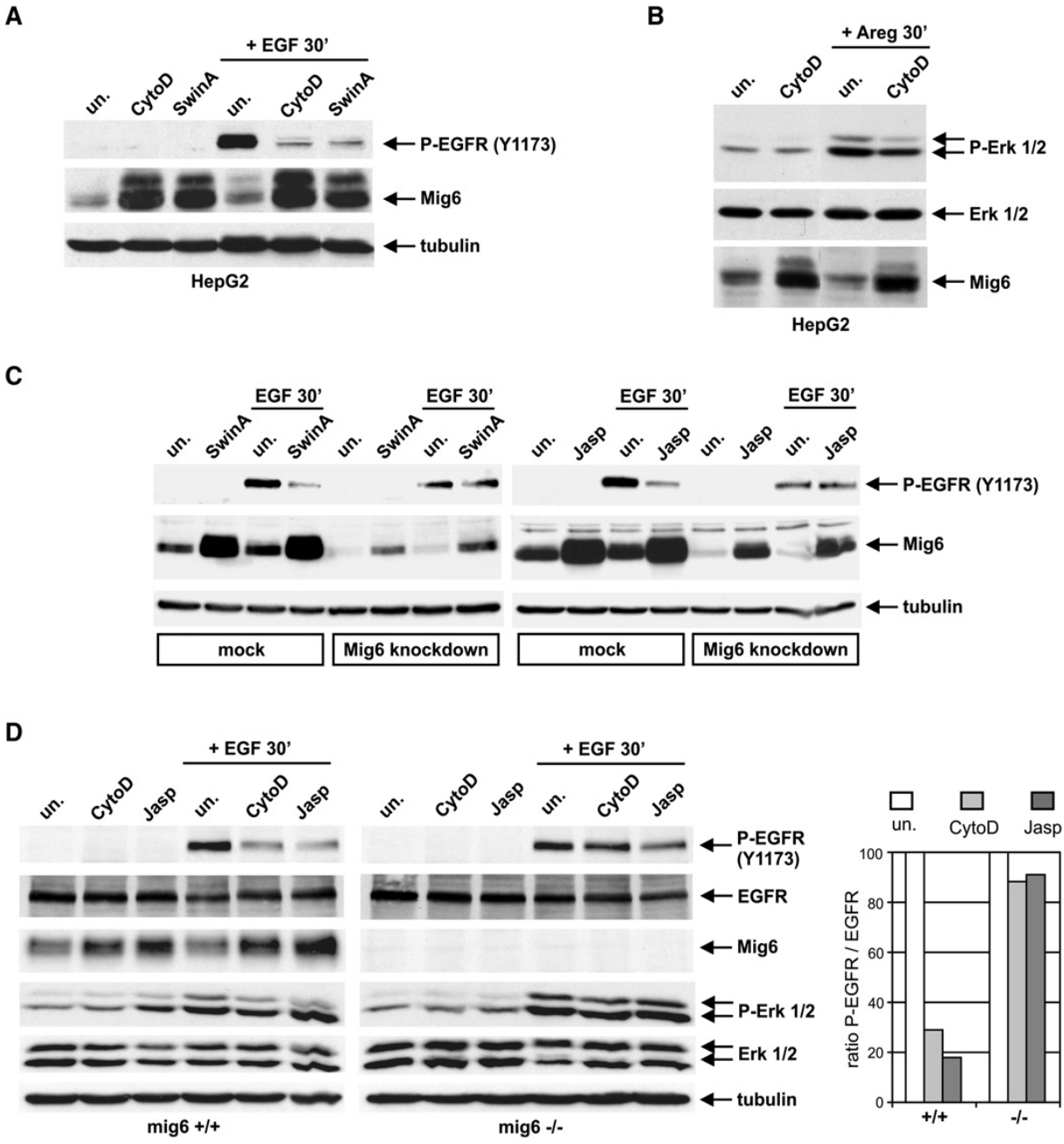


Figure 25. Actin-Mediated Mig6-Induction Regulates EGFR-MAPK Signalling

(A) Hepg2 cells were treated with cytochalasin D or swinholide A for 7 hr or left untreated (un.). Subsequently, they were stimulated with egf (25 ng/ml) for 30 min when indicated, and total lysates were subjected to immunoblotting with antibodies specific for tyrosine-phosphorylated egfr, mig6, or tubulin as a control. (b) cells were pretreated with actin drugs as in (a) and subsequently stimulated with amphiregulin (areg, 50 ng/ml). Erk activity was determined by immunoblotting with phosphospecific anti-erk and panerk antibodies. (c) cells stably transfected with mig6 shrna (knockdown) were treated with swinholide or jasplakinolide, followed by egf stimulation, and analyzed for mig6 expression and egfr phosphorylation. (d) mefs prepared from wt (mig6+/+) or mig6 knockout (mig6-/-) mice were treated with the indicated actin drugs for 7 hr prior to egf stimulation. The bar chart represents the relative egfr phosphorylation upon egf stimulation, as determined by densitometry.

3.9 Induction of Mig6 by serum or LPA is dependent on MAL

The next step was to determine the physiological stimuli which could induce Mig6 expression through MAL mediated transcription.

Serum stimulation induces Mig6 expression in NIH3T3 and MEFs as shown by immunofluorescence (Figure 26). Moreover Mig6 mRNA level is induced by the known activators of the Rho-Actin-Mal-Srf pathway LPA and S1P within the same range as by the actin binding drug Cytochalasin D (Figure 27 A). FCS which is a more complex stimulus induced more potently Mig6 expression. However, all stimuli were unable to stably induce Mig6 expression over the time. Nine hours after the stimulations Mig6 levels were back to background.

In order to show that MAL is necessary for LPA or FCS mediated induction of Mig6, two different approaches were used. First, Mig6 induction by LPA and serum could be repressed by two dominant negative MAL constructs (Figure 27 B). Second, knock down of MAL and MRTFB by shRNA impaired Cytochalasin D, LPA and serum induction of Mig6 mRNA in the same range as it does for a known target of the Rho-Actin-MAL-SRF pathway like Srf. The repression of another target Acta2, which is highly dependent on MRTFs-SRF, was much more pronounced (Figure 27 C). The MAL dependency for LPA or serum induction of Mig6 could be also observed at the protein level (Figure 27 D).

Thus, Mig6 is induced by physiological agonists of the Rho-Actin-MAL-SRF pathway like LPA on a MRTFs dependent manner.

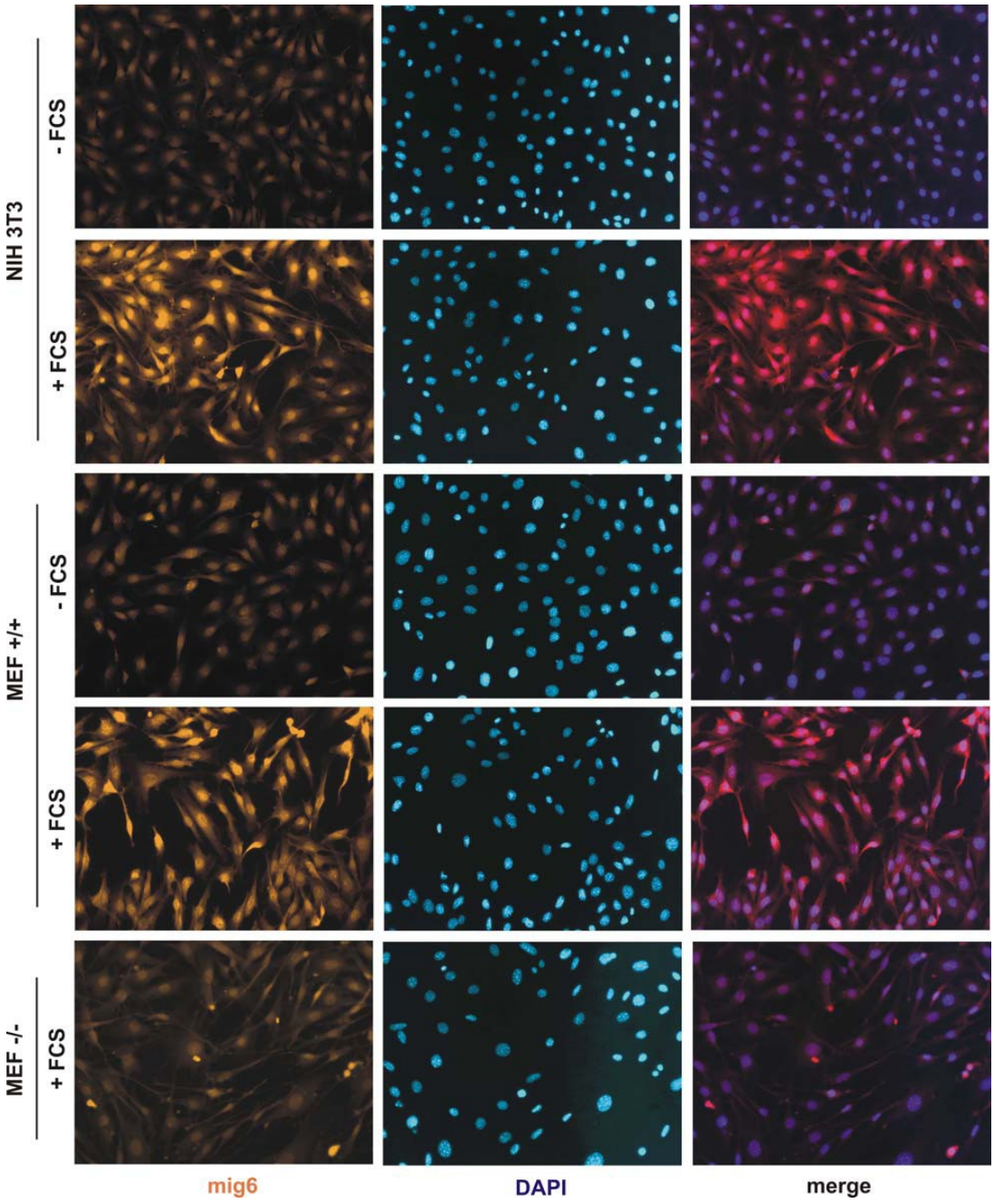


Figure 26. Serum Induces Mig6 Expression

Immunofluorescence micrographs showing the induction of Mig6 protein upon serum stimulation of NIH 3T3, wild-type (*+/+*), or mig6 knockout (*-/-*) MEF for 7 hr, in comparison to serum-starved control cells (-FCS). Mig6 protein, detected with the rabbit polyclonal antiserum #1573 against Mig6, is shown in red, control DAPI staining in blue, and the merged overlay.

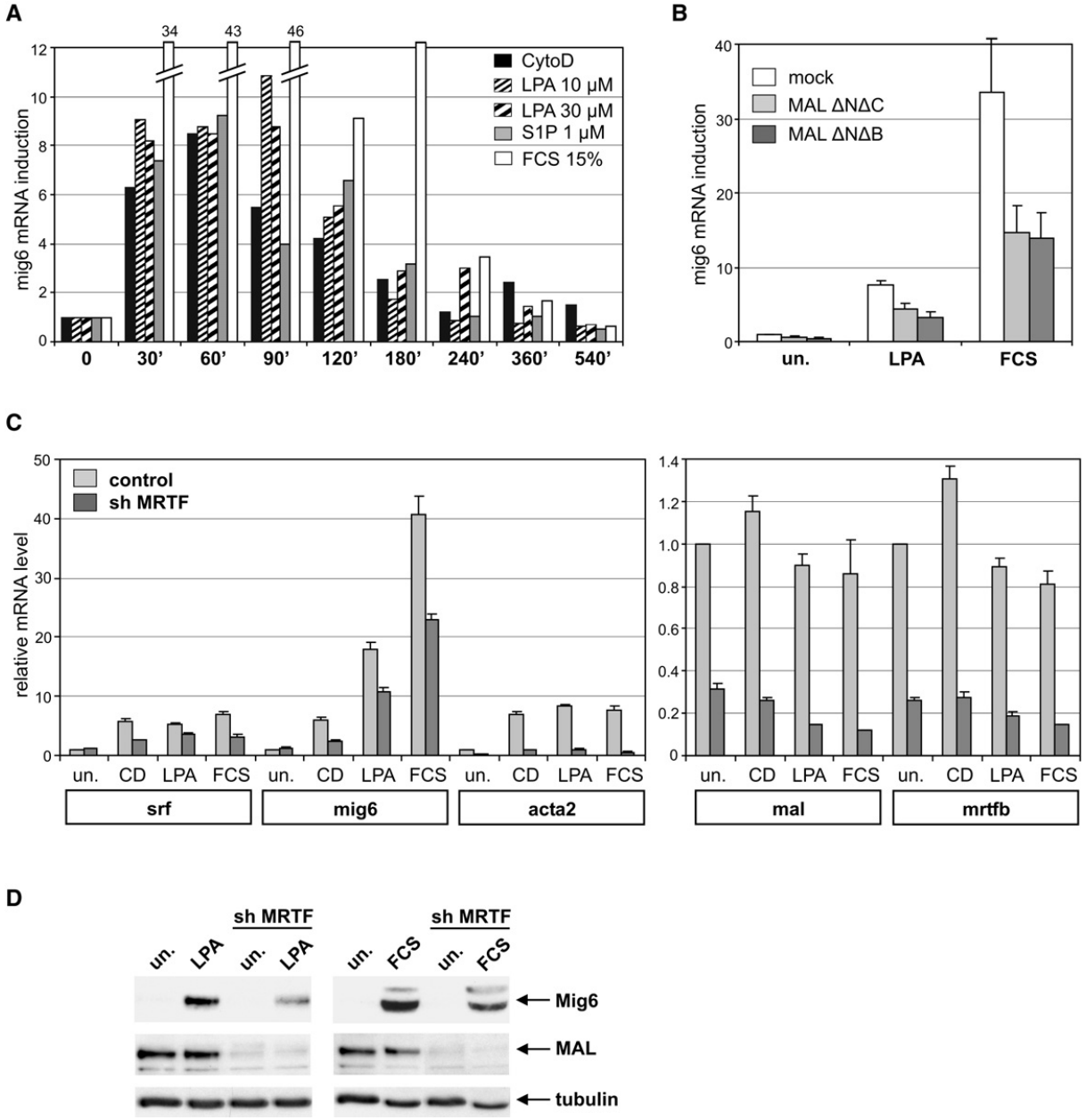


Figure 27. Serum-, LPA-, and S1P-Induced Mig6 Expression Involves MAL

(A) Transient induction of mig6 mRNA. NIH 3T3 cells were stimulated with cytochalasin D, the GPCR ligands LPA and S1P, and serum (FCS) for the indicated times in minutes. The mig6 mRNA was quantified by RT-PCR and normalized to hprt. (B) Dominant-negative MAL inhibits mig6 expression. Cells were transfected by electroporation with MAL ΔNΔC or MAL ΔNΔB, which are defective for transactivation and SRF binding, respectively. Following serum withdrawal for 24 hr, cells were stimulated with 50 mM LPA or 15% serum for 90 min. Shown is the average induction of endogenous gene expression compared to mock-transfected control cells. Error bars, SEM (n = 4). (C) Knockdown of MAL and MRTF-B affects inducibility of mig6. Cells were transiently infected with control retrovirus or virus expressing shRNA targeting both MAL and MRTF-B (sh MRTF). Four days postinfection, the relative mRNA amount of mig6 and the known MAL targets srf and acta2 in control and knockdown cells was quantified in triplicates by quantitative RT-PCR. Efficiency of the MAL and MRTF-B knockdown is shown on the right. Error bars, SEM (n = 3). (D) MAL/MRTF-B knockdown reduces Mig6 protein induction by LPA and serum for 7 hr. Total cell lysates were analyzed by western blotting using antibodies against Mig6 (Mig6 (PE-16)), MAL, and tubulin.

3.10 The small GTPase Rho and the MAPK cascade are necessary for MIG6 expression

In order to evaluate the role of Rho in the induction of Mig6, the cell permeable Rho inhibitor TAT-C3 (Sahai and Olson, 2006) was utilised. This inhibitor is composed of the HIV TAT leader sequence that permits transduction of the protein across the plasma membrane and the C3 enzyme that selectively catalyzes the ADP-ribosylation, and consequent inactivation, of RhoA, RhoB, and RhoC of the Rho GTPase protein family. Pretreatment of NIH3T3 cells with TAT-C3 impaired the induction of Mig6 mediated by LPA and FCS (Figure 28). Cytochalasin D induction was not decreased by TAT-C3 in accordance with the fact that Actin is downstream of Rho GTPases.

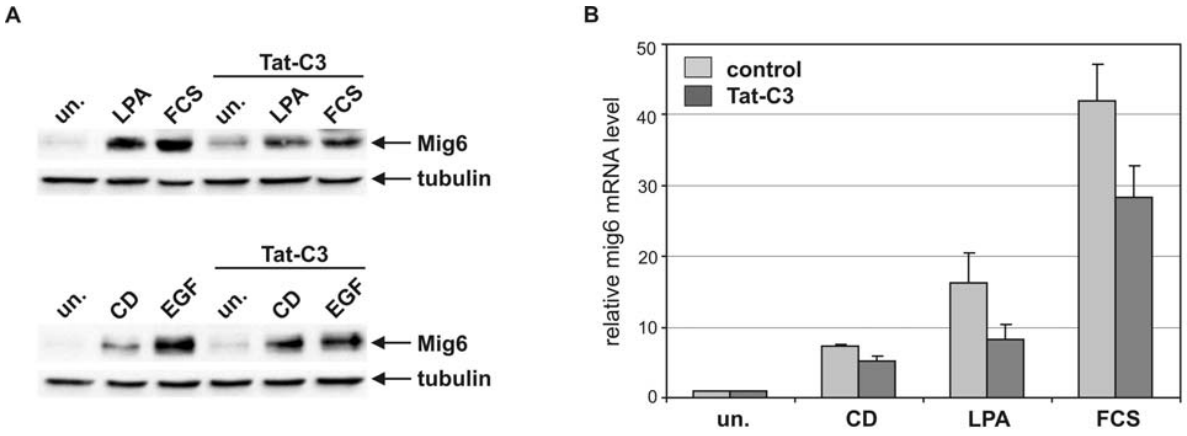


Figure 28. Involvement of the GTPase RhoA in Mig6 Induction

(A and B) NIH3T3 cells were serum starved for 15 hr in the presence or absence of the cell permeable Rho inhibitor TAT-C3 (3 µM) and then stimulated with LPA (50 µM), FCS (15%), cytochalasin D (CD, 5 µM), or EGF (100 ng/ml) as indicated. Following 7 hr incubation, total cell lysates were subjected to western blotting using antibodies specific for Mig6 and tubulin as a control (A). The mRNA was isolated after 90 min stimulation and quantified by qRT-PCR (B). Following normalization to hprt, the relative mig6 mRNA induction is shown. Error bars, SEM (n = 3).

In the course of the experiment the contribution of the MAP kinase cascade to the induction of Mig6 was evaluated. Surprisingly, inhibition of MEK, the kinase upstream of ERK, by the UO126 inhibitor strongly impaired Cytochalasin D mediated induction of Mig6 and consequently restored EGFRY1173 phosphorylation (Figure 29A).

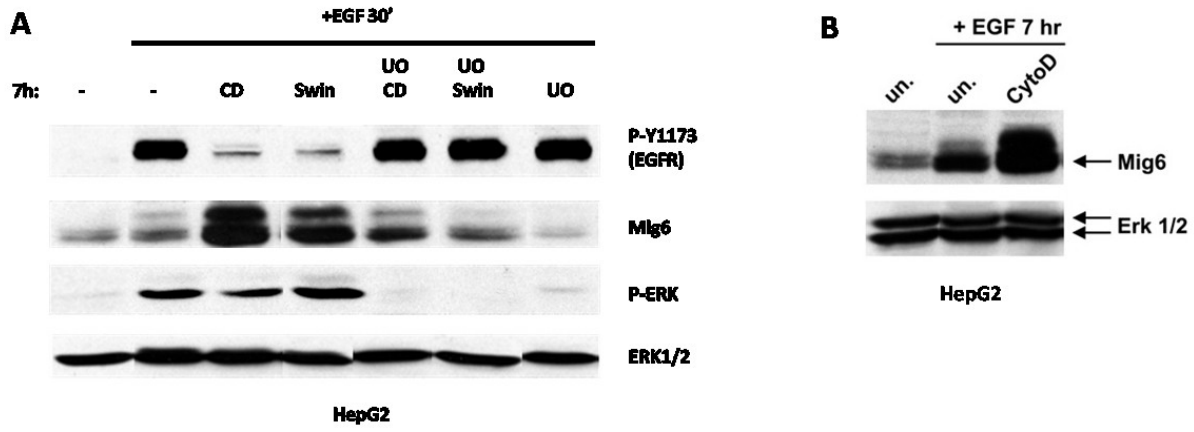


Figure 29. Role of the MAP Kinase Pathway in the Mig6 Induction

(A) Starved HepG2 cells were pretreated with the MEK inhibitor UO126 (UO, 10µM) for 30min and stimulated with cytochalasin D (CD, 5µM) or Swinholide A (Swin, 0,3µM) for 7 hours. Thirty minutes before lysis, the cells were stimulated with EGF (100nM) when indicated. (B) Induction of endogenous Mig6 by long-term stimulation (7 hr) with EGF alone or in combination with cytochalasin D. Lysates were immunoblotted with antibodies against Mig6, with panErk as a control.

Then, the induction of Mig6 by the EGF-MAP kinase pathway and the Actin-MAL pathway were compared (Figure 29B). Even though the MAP kinase pathway was fully activated by treatment with EGF, addition of Cytochalasin D induced more strongly Mig6 expression suggesting the presence of two pathways controlling Mig6 levels.

3.11 LPA impairs EGFR-MAPK signalling by a transactivation independent induction of Mig6

Induction of Mig6 by Actin binding drugs inhibits phosphorylation of EGFR. But, does a physiological stimulus like LPA induce the dephosphorylation of EGFR through the transcriptional upregulation of Mig6?

LPA stimulation was able to dramatically reduce the phosphorylation of EGFR in MEFs wild type but failed to do so in MEFs Mig6-/- showing the absolute requirement of Mig6 (Figure 30 A). Importantly, pretreatment of cells before LPA stimulation with the MMP inhibitor Batimastat (BB94) or the EGFR inhibitor AG1478 from the tyrphostine family didn't affect Mig6 induction singnificantly (Figure 30 B). This result demonstrate that the upregulation of Mig6 by LPA and consequent dephosphorylation of EGFR is independent from the transactivation of EGFRs by GPCRs (Daub et al., 1996) through Matrix Metalloprotease

(MMP) cleavage of the EGFR pro-ligand HB-EGF (Prenzel et al., 1999). In conclusion, on top of their transactivation function of EGFRs, LPA and GPCRs have a delayed inhibitory function of EGFRs through the transcriptional induction of Mig6.

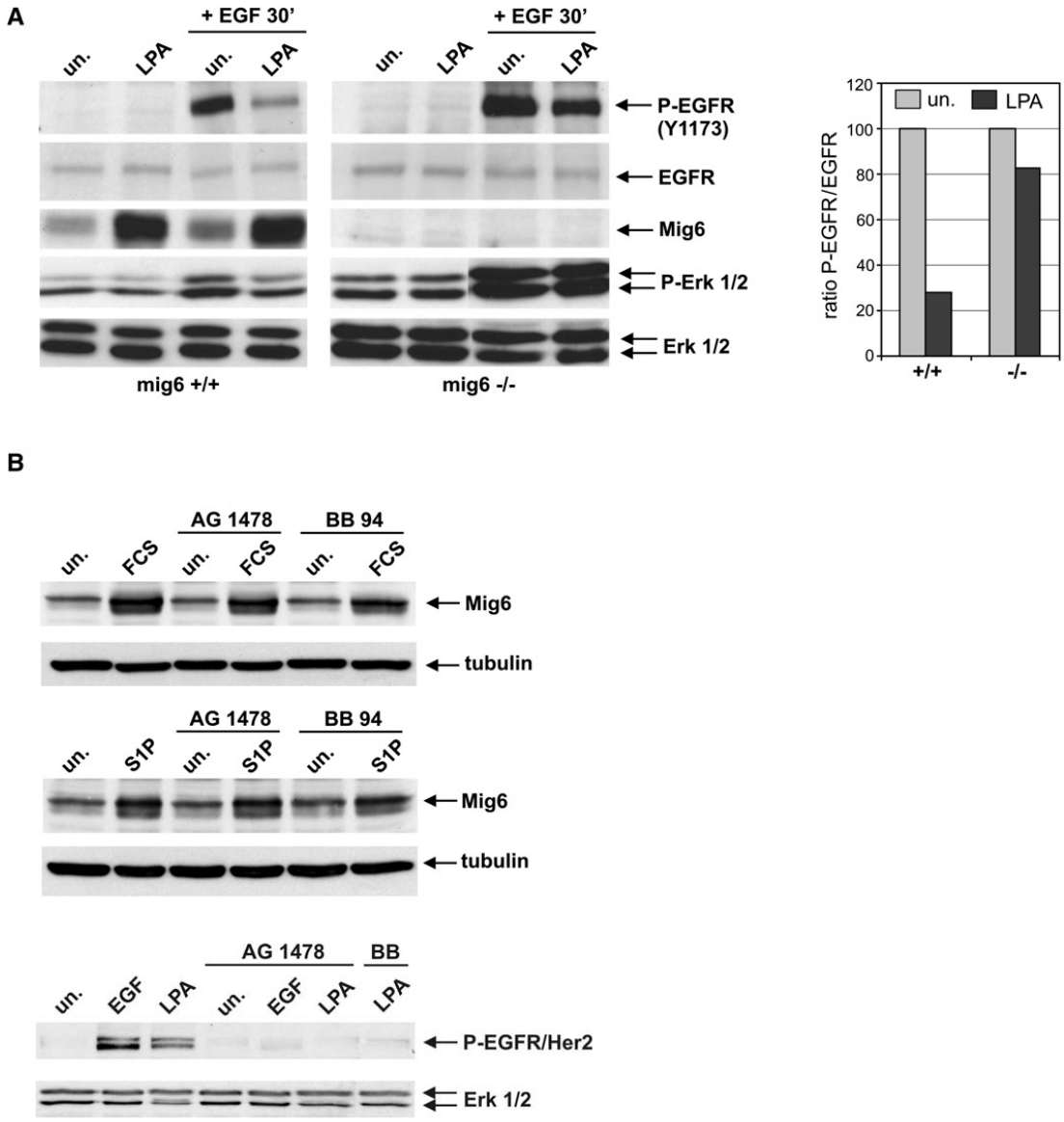


Figure 30. LPA Attenuates Signaling through EGFR and MAPK Erk by Transactivation-Independent Induction of Mig6

(A) WT or mig6 knockout MEFs were treated with LPA for 7 hr prior to EGF stimulation (40 ng/ml). Activation of EGFR and MAPK Erk was determined by immunoblotting with phosphospecific EGFR and Erk antibodies. Densitometric quantification of the relative EGFR phosphorylation upon EGF stimulation is shown by the bar chart. (B) Mig6 upregulation by serum or lipid agonists is largely independent of EGFR transactivation. MEFs were pretreated with the EGFR kinase inhibitor AG1478 or the metalloprotease inhibitor BB94 for 30 min, followed by stimulation with FCS or S1P for 7 hr. As a control for inhibitor functions, the phosphorylation of EGFR/Her2 was monitored.

3.12 MAL harbours antiproliferative features independent of Mig6

Mig6 by downregulating the EGFR MAP kinase cascade represses cell proliferation. Moreover, several other genes identified in the screen like Dusp5, ZFP36, Bok or Noxa are considered to be antiproliferative. Therefore, the role of MAL in cell proliferation was investigated.

Overexpression of MAL or MALΔN completely blocked proliferation of NIH3T3 cells compared to GFP or ELK1 another coactivator of SRF controlled by the MAP kinase pathway (Figure 31 A and B). The dominant negative construct gave even a slight proliferative advantage. Colony formation assay confirmed the antiproliferative properties of MAL and MALΔN (Figure 31 B and C).

The next step was to try to rescue the antiproliferative properties monitored for MAL by knocking down Mig6. Decreased level of Mig6 gave a proliferative advantage to the GFP expressing cells but didn't rescue the MAL antiproliferative effect (Figure 32). As control, a partial rescue of the antiproliferative effect of MAL was monitored in cells overexpressing shRNA against MRTFs.

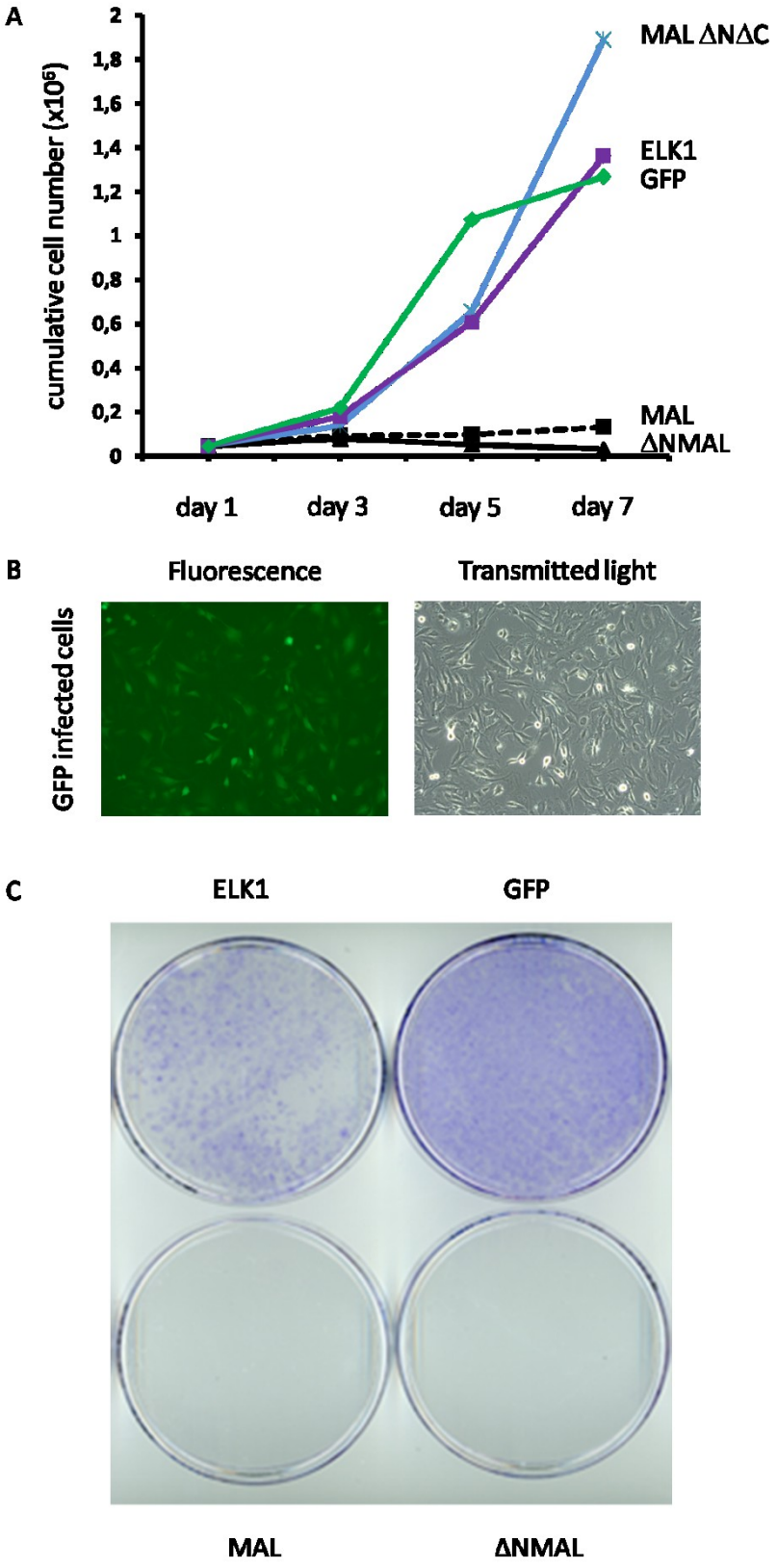


Figure 31. MAL Represses Cell Proliferation

(A) Proliferation curve of NIH3T3 cells were infected with the indicated constructs. (B) Penetrance of the infection shown by the GFP fluorescence. (C) Colony formation assay of NIH3T3 cells infected with the indicated constructs.

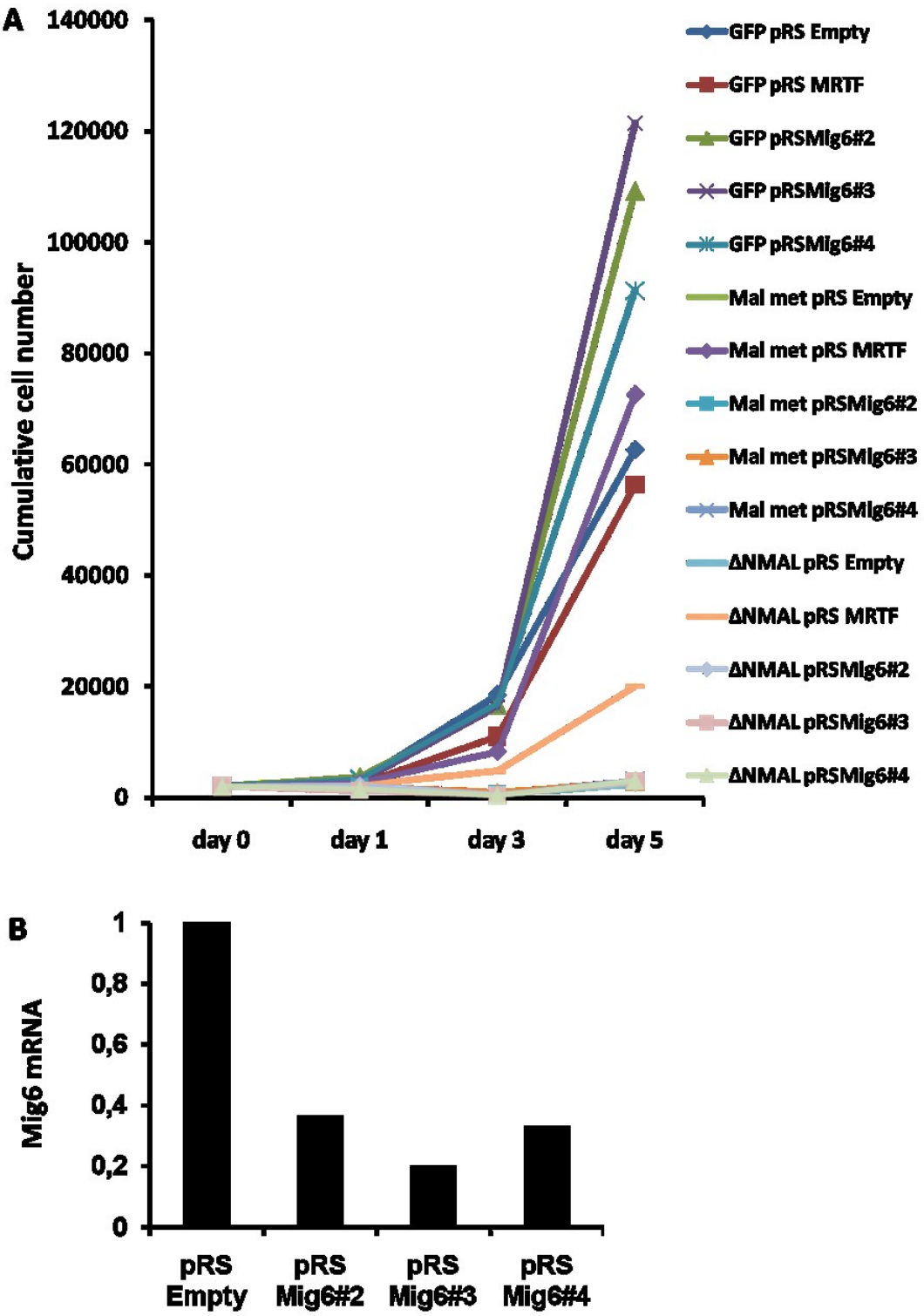


Figure 32. Mig6 Knockdown gives a Proliferative Advantage to NIH3T3 Cells but is Unable to Rescue for the Antiproliferative Effect of MAL.

(A) NIH3T3 cells were infected with pRS vectors coding shRNA against Mig#6 or MRTF and 5 days later with pLPCX vectors coding for GFP, MALmet or ΔNMAL. 1 day after the cells were seeded to monitor their proliferation. (B) Knock down efficiency of Mig6 in the pLPCX infected cells determined by qRT-PCR.

In conclusion, LPA or S1P signalling through GPCRs induce the Rho-Actin-MAL-SRF pathway and trigger the expression of Mig6 which inhibits the EGFR-MAP kinase axis. Mig6 induction is likely to contribute to the antiproliferative effect of MAL overexpression but is not the essential mediator as knock down of Mig6 could not rescue MAL antiproliferative effect. Finally, the transcriptional circuit presented here add a new level of interaction between the two pathways controlling SRF activity (Figure 33).

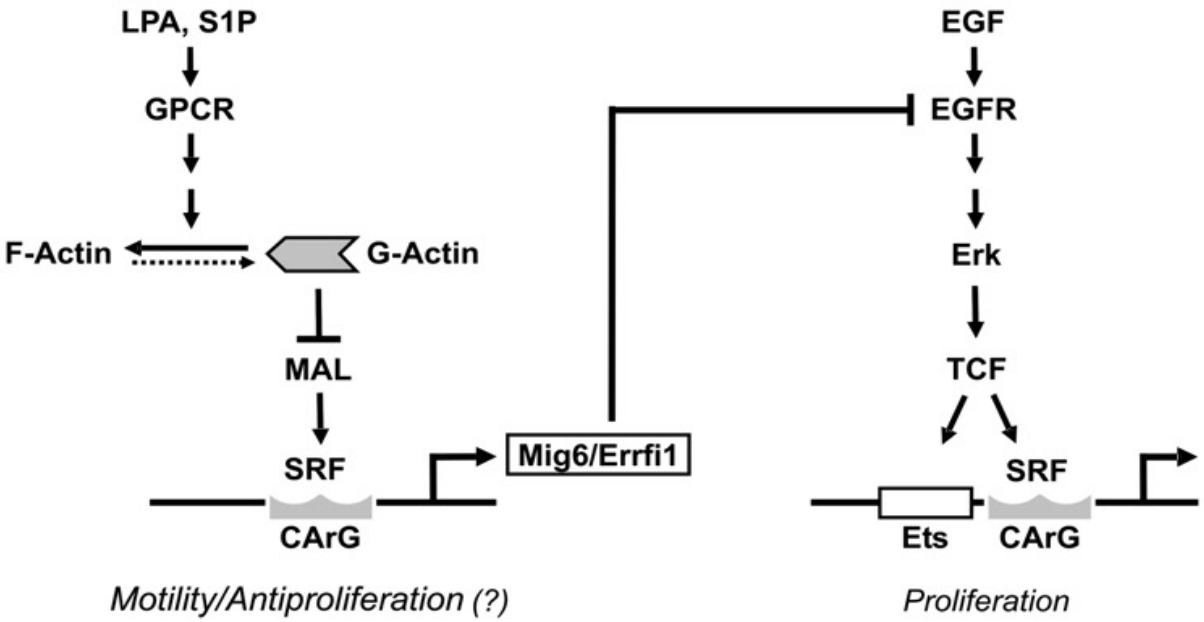


Figure 33. Model of a Negative Feedback from Actin-MAL Signaling on the EGFR-MAPK Pathway, Mediated by Mig6.

Lipid agonists, such as LPA and S1P, induce MAL-regulated expression of Mig6, which interferes with the MAPK Erk pathway at the level of the EGFR. The two pathways controlling distinct SRF target genes are thereby regulated antagonistically through a transcriptional circuit.

4 Results part II

4.1 MAL induces apoptosis

MAL overexpression represses cell proliferation dramatically. Therefore, apoptosis in MAL overexpressing cells was evaluated. The first readout employed was to monitor by FACS the Sub-G1 population of cells stained with propidium iodide after detergent permeabilization. Apoptotic cells with degraded DNA appear as cells with hypodiploid DNA content (Schmid et al., 1994). Cells overexpressing the isoform MALmet or the activated MALΔN harbour a huge increase in sub-G1 peak compared to cells overexpressing GFP, the dominant negative MAL construct MALΔNΔC or the TCF Elk1, coactivator of SRF but controlled by the MAP kinase pathway (Figure 34 A). The second readout was the activation of the effector caspase 3 by proteolytic cleavage. Caspase 3 can be cleaved by the complex formed by Apaf 1, Cytochrome C and Caspase 9 after activation of the intrinsic apoptotic pathway or by Caspase 8 or 10 after activation of the extrinsic pathway (Boatright and Salvesen, 2003). MALΔN infected cells harbour an activation of Caspase 3 compared to GFP, MALΔNΔC or the TCF Elk1. The activation of Caspase 3 in MALmet infected cells in this experiment was barely detectable (Figure 34 B). It is likely, in part, due to the fact that the cells were kept at a higher density, which may favour survival, than in the case of the Sub-G1 readout. Another explanation could be that the activation of Caspase 3 is only transient in MAL induced apoptosis and that the time point chosen was not the optimum. Induction of apoptosis by MAL overexpression could be confirmed by a third readout, by annexin V staining (Descot et al., 2009), which recognizes the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane which occurs during apoptosis (Fadok et al., 1998).

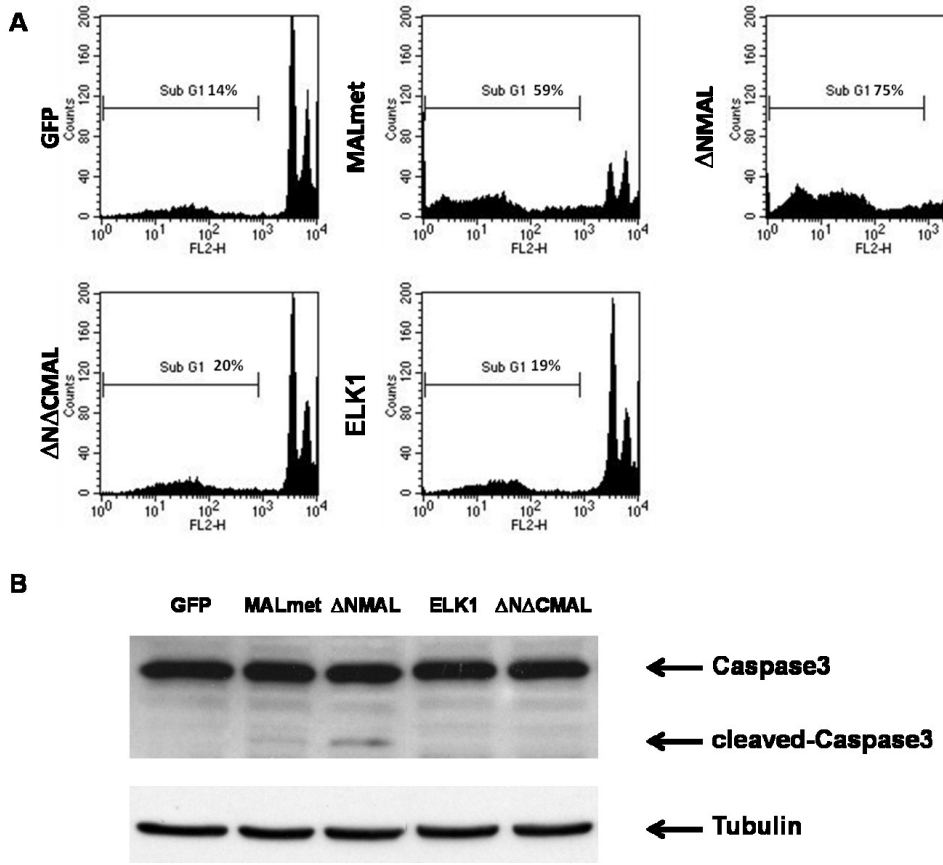


Figure 34. MAL Induces Apoptosis

Apotosis in NIH3T3 cells 3 days post infection with the indicated constructs monitored by (A) propidium iodide staining (q.v. 6.2.2.5 for details) or (B) monitoring caspase 3 cleavage in total cell lysates by immunobloting for Caspase 3 and Tubulin as loading control. N.B. The cell density was superior in (B) compared to (A).

4.2 Bok and Noxa are controlled by G-actin MAL-SRF signalling

Two characterised proapoptotic genes present in the screen, Bok and Noxa, could potentially mediate the MAL induced apoptosis. Bok and Noxa are pro-apoptotic members of the Bcl-2 family. The role of Noxa in response to death signals is to displace the proapototic member of the Bak/Bax family like Bok from an inhibitory complex with anti apoptotic proteins like Mcl1 (Ploner et al., 2008). Once released, Bok oligomerises as a channel in the mitochondria outer membrane and induces Cytochrome C release. Then, the cytochrome C assembles in the cytoplasm with Apaf1 and Caspase 9 to activate the effector caspase 3 and to trigger cell death.

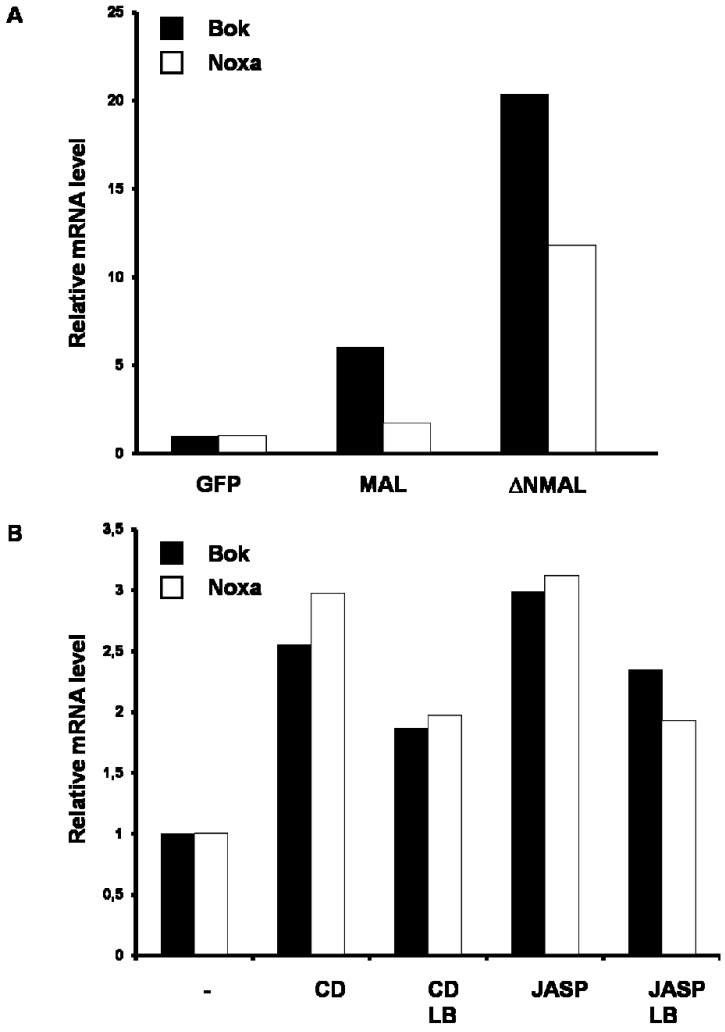


Figure 35. Bok and Noxa are Induced by Activators of MAL/SRF Mediated Transcription.

(A) mRNA levels of Bok and Noxa were measured by qRTPCR in NIH3T3 cells infected with the indicated constructs 3 days post infection or (B) in serum starved NIH3T3 cells pretreated with latrunculin B (LB, 5µM) for 15min and treated with cytochalasin D (CD, 5µM), or jasplakinolide (JASP, 0,5µM) for 90 min as indicated.

Bok and Noxa are induced by MAL and MALΔN (Figure 35 A) as well by the two activating actin binding drugs Cytochalasin D and Jasplakinolide which are supposed to activate MAL mediated transcription by two different modes. Latrunculin B pretreatment could impair slightly the induction mediated by the two actin binding drugs (Figure 35 B).

4.3 Bok and Noxa expression are not dependent on p53

As Bok and Noxa have been described as p53 targets (Yakovlev et al., 2004), their induction by activators of the Actin-MAL-SRF pathway was examined in MEFs p53-/-. The basal

expression of the two genes was unchanged in MEFS p53-/- versus p53+/+. Induction of Bok was even possible in MEFs p53-/- by the actin binding drugs while no significant induction of Bok and Noxa in MEFs p53+/+ at the mRNA level could be achieved (Figure 36 and not shown). These initial results await to be reproduced in further experiments but suggest that at least induction of Bok by actin signalling is independent from p53.

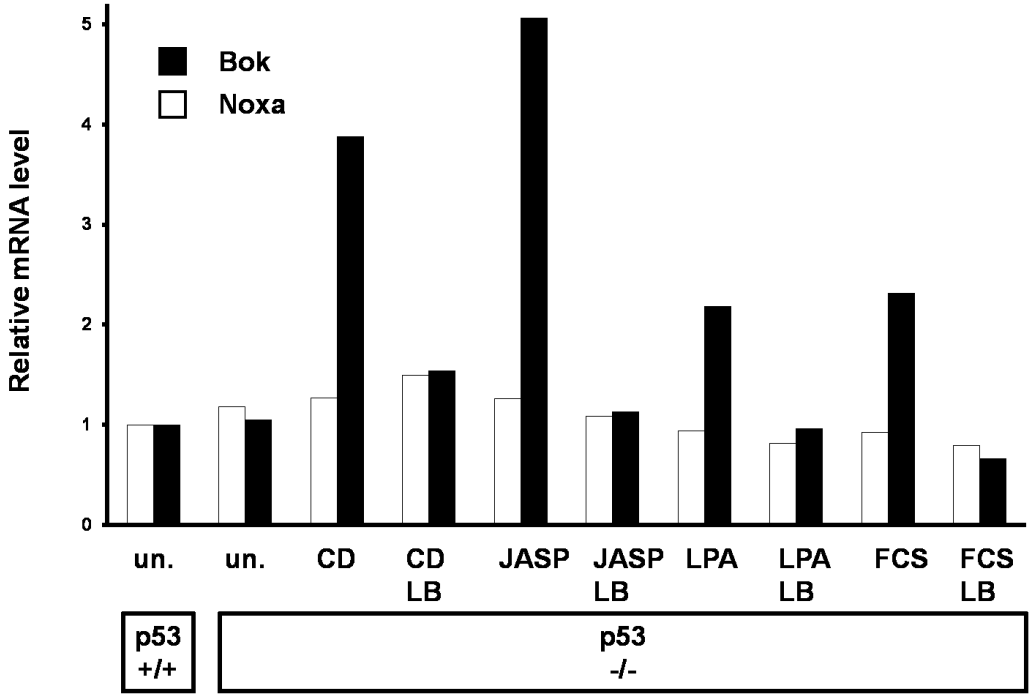


Figure 36. Bok and Noxa Levels are not Dependent on p53

Serum starved MEFs p53+/+ or p53-/- were pretreated with latrunculin B (LB, 5µM) for 15min and treated with cytochalasin D (CD, 5µM), or jasplakinolide (JASP, 0,5µM) for 90 min as indicated.

4.4 Bok and Noxa induction by inducers of the Rho-actin-MAL pathway is dependent on MAL

MAL and the activating actin binding drugs were able to induce Bok and Noxa expression (q.v. 4.2) meaning that activation of Actin-MAL signalling is sufficient to control Bok and Noxa expression. The next step was to investigate if MAL and MRTFB were necessary for the induction of Bok and Noxa by the actin binding drug Cytochalasin D but also by the known physiological activators of the Actin-MAL pathway, LPA and FCS. In MRTF depleted cells by shRNA, the induction of Bok and Noxa at the mRNA level were dramatically impaired (Figure 37). This initial result, which need to be confirmed, shows that induction of the two

proapoptotic genes Bok and Noxa by inducers of the Rho-Actin-MAL pathway is strongly dependent on MRTF.

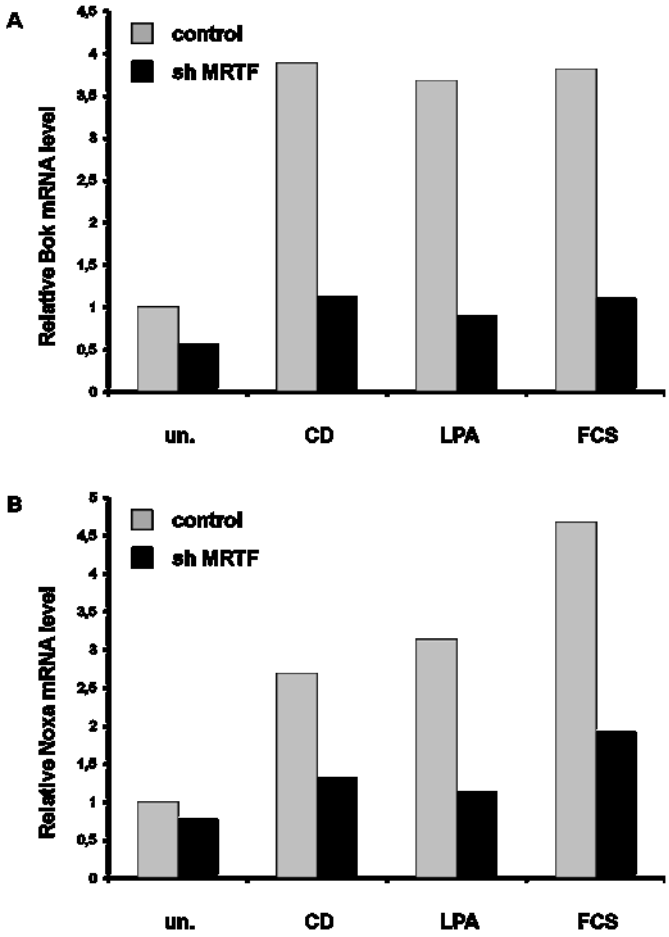


Figure 37. Bok and Noxa Inductions are Dependent on MRTF.

(A) Bok and (B) Noxa mRNA were monitored in serum starved NIH3T3 cells depleted or not of MRTF A and B by shRNA. The cells were stimulated with cytochalasin D (CD, 5µM), LPA (10µM) or FCS (15%) for 90 min as indicated.

4.5 Identification of a MAL/SRF response element in the Bok promoter

Then, in order to identify an Actin-MAL response element in the Bok and Noxa promoter, the proximal promoters of the two genes were cloned in a luciferase reporter. The Bok promoter ranging from -185bp to +127bp could be induced by Cytochalasin D, MALΔN and SRFVP16 but only very weakly by LPA and FCS. Mutation or deletion of a CARG box at –99bp impaired dramatically the Bok promoter inducibility (Figure 38). Moreover, the Bok proximal promoter was bound by MAL and SRF as shown by ChIP (Shaposhnikov et al., Unpublished). Therefore, it is likely that the MAL-SRF complex binds this element to induce Bok expression.

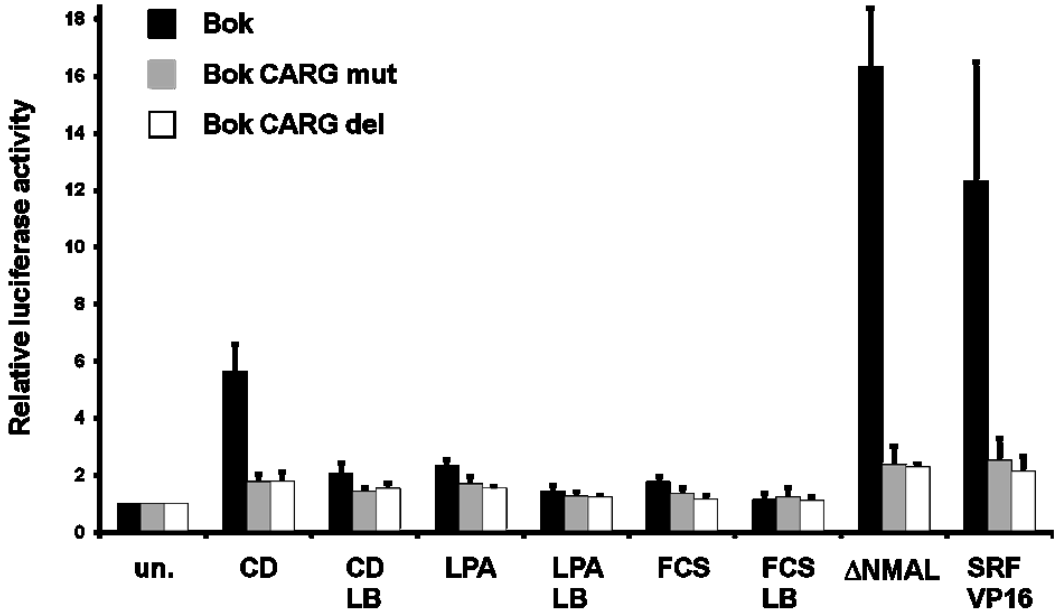


Figure 38. Identification of a MAL/SRF Response Element in the Bok Promoter

The Bok promoter fragment ranging from -185 to +127 was cloned in a luciferase reporter and cotransfected with activated ΔNMAL, SRF-VP16 or an empty vector in NIH3T3 cells. The cells were serum starved overnight, pretreated with latrunculin B (LB, 5µM) and treated with cytochalasin D (CD, 5µM), LPA (10µM) or FCS (15%) for 7 hours as indicated.

Concerning Noxa, the activity of the proximal promoter was not differentially regulated by the actin binding drugs. Moreover MALΔN or SRFVP16 could not induce either the different Noxa proximal promoter construct. However, the mouse Noxa genomic locus contains a CARG box in the first intron which has been conserved during evolution. As SRF has already been shown to bind response element in intronic regions of target genes (Mack and Owens, 1999; Sun et al., 2006a), the transcriptional activity of this intronic element was evaluated. The genomic DNA containing this candidate response element was cloned upstream of a TATA box controlling the expression of the luciferase gene. Only a slight differential regulation by the actin binding drugs Cytochalasin D and Latrunculin B could be monitored as well as an induction by SRFVP16 but not by MALΔN (Figure 39). Hence, this site remains a candidate site which needs validation by ChIP before drawing any conclusion.

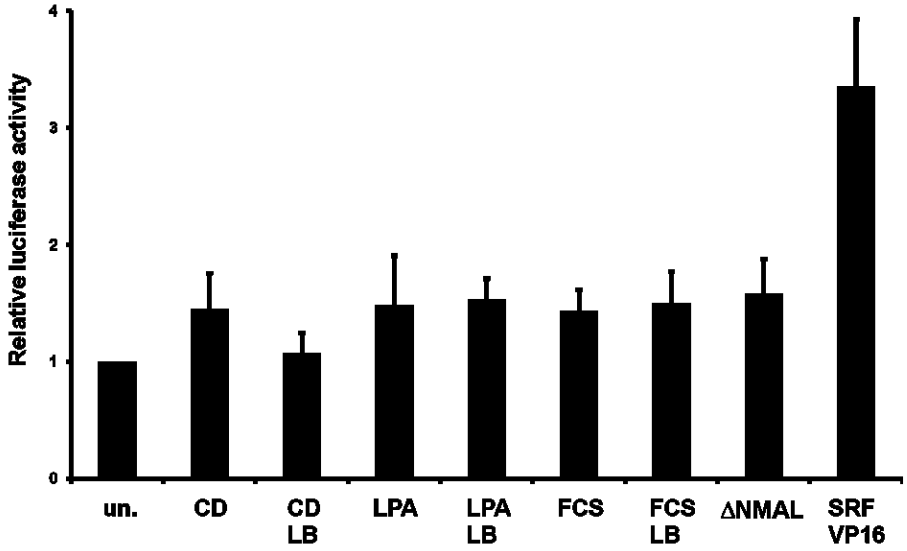


Figure 39. Transcriptional Activity of a Noxa Intronic Region

The Noxa intronic region ranging from + 1539 to + 1905 bp was cloned in front of a TATA box in a luciferase reporter and cotransfected with activated ΔNMAL, SRF-VP16 or an empty vector in NIH3T3 cells. The cells were serum starved, pretreated with latrunculin B (LB, 5µM) and treated with cytochalasin D (CD, 5µM), LPA (10µM) or FCS (15%) for 7 hours as indicated.

4.6 Overexpression of activated MAL leads to MCL1 degradation and Bcl2 overexpression

Once bound to Mcl1, Noxa triggers its proteasome mediated degradation (Ploner et al., 2008). Therefore, Mcl1 levels were monitored in cells overexpressing MAL or MALΔN. Cells infected by MALΔN showed a reduced Mcl1 level compared to GFP, SRFVP16, ELK1 or the dominant negative MAL construct MALΔNΔC (Figure 40). This result is preliminary and needs to be repeated.

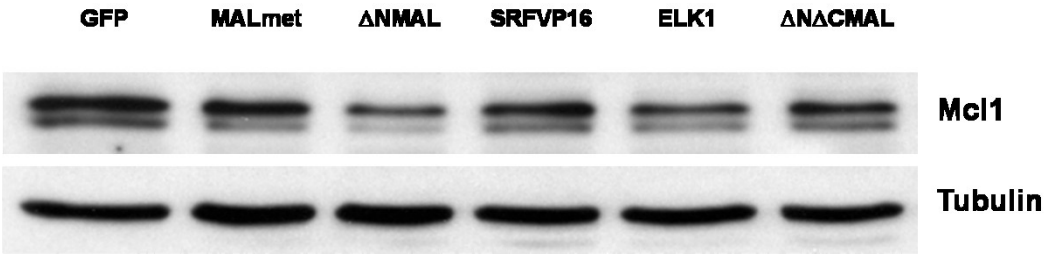


Figure 40. Over Expression of Activated ΔNMAL leads to a Degradation of Mcl1

NIH3T3 cells were infected with viruses coding for the indicated constructs. Three days post infections, levels of Mcl1 and tubulin were monitored by immunobloting.

As Mcl1 (Townsend et al., 1999; Vickers et al., 2004) and Bcl2 (Schratt et al., 2004) are controlled by SRF, the expression levels of these two genes were monitored in cells overexpressing MALΔN (Figure 41). Interestingly, Bcl2 but not Mcl1 was induced by MALΔN consistent with the fact that MCL1 is controlled by TCF-SRF signalling.

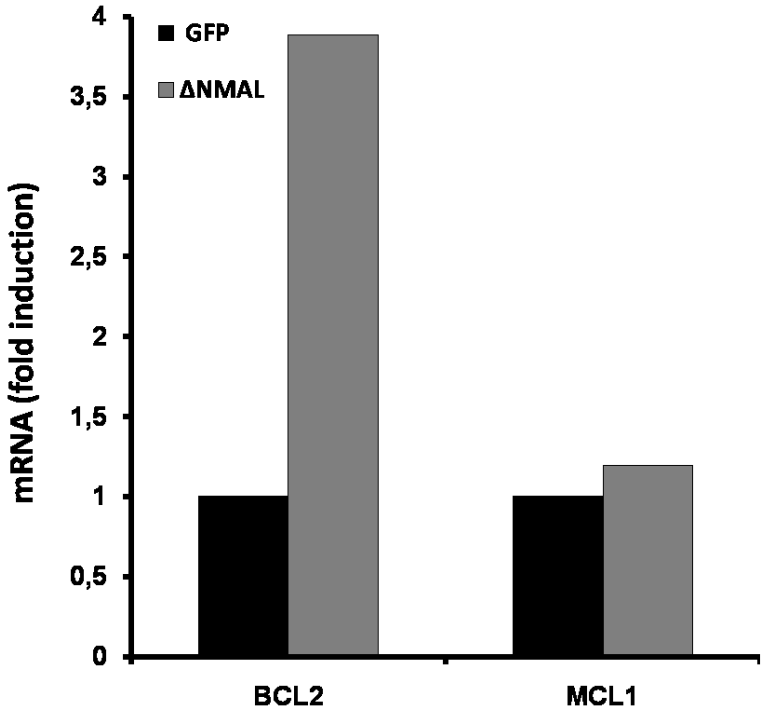


Figure 41. Over Expression of Activated ΔNMAL Leads to the Expression of Bcl2 but not Mcl1

NIH3T3 cells were infected with viruses coding for MALΔN or GFP as control. Three days post infections, levels of Bcl2 and Mcl1 mRNAs were monitored by qRTPCR.

4.7 MCL1 or BCL2 overexpression is not sufficient to rescue the antiproliferative effect of MAL

MCL1, but not BCL2, inhibits the proapoptotic BAK (Willis et al., 2005) which belongs to the same family as BOK. Therefore, the potential of MCL1 and BCL2 as control to rescue the antiproliferative and proapoptotic features of MAL was evaluated. If the inductions of Bok and Noxa are key in the inhibition of proliferation mediated by MAL, then MCL1, but not BCL2 overexpression should be able to rescue the proliferation of MAL infected cells.

Unfortunately, neither MCL1 nor BCL2 were able to rescue the antiproliferative effect of MAL (Figure 42).

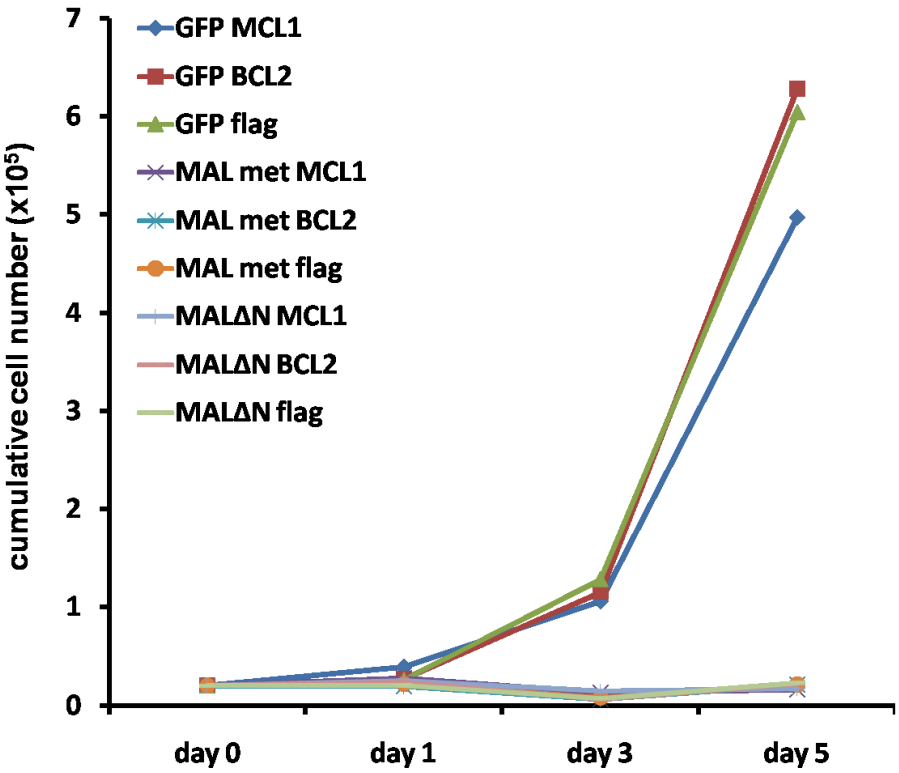


Figure 42. MCL1 or BCL2 Overexpression do not Rescue the Antiproliferative Properties of MAL

NIH3T3 CELLS were infected with pLPCX vectors coding for MCL1, BCL2 or the flag tag as control. Two days later these cells were infected a second time either with MALmet, MALΔN or GFP as control. One day later the cells were seeded for growth curve analysis.

4.8 Bok or Noxa overexpression in NIH3T3 cells do not induce apoptosis nor repress cell proliferation

Next, the impact of Bok and Noxa overexpression on the proliferation of NIH3T3 cells was evaluated. Neither Bok nor Noxa could reduce cell proliferation as measured by growth curve or colony formation assay (Figure 43).

Then, the effect of overexpression of Bok or Noxa on the sensitivity of NIH3T3 cells to apotosis inducers was analysed. Cells overexpressing BOK, NOXA or GFP as control were

treated with the aspecific PKC inhibitor Staurosporine, TNFα, or the DNA damaging agent Doxorubicine. Overexpression of NOXA or BOK alone had no effect on apoptosis and didn't synergise with any of the apoptosis stimuli with may be the exception of Bok with Doxorubicin (Figure 44). These experiments were done only once and need to be reproduced before drawing final conclusions.

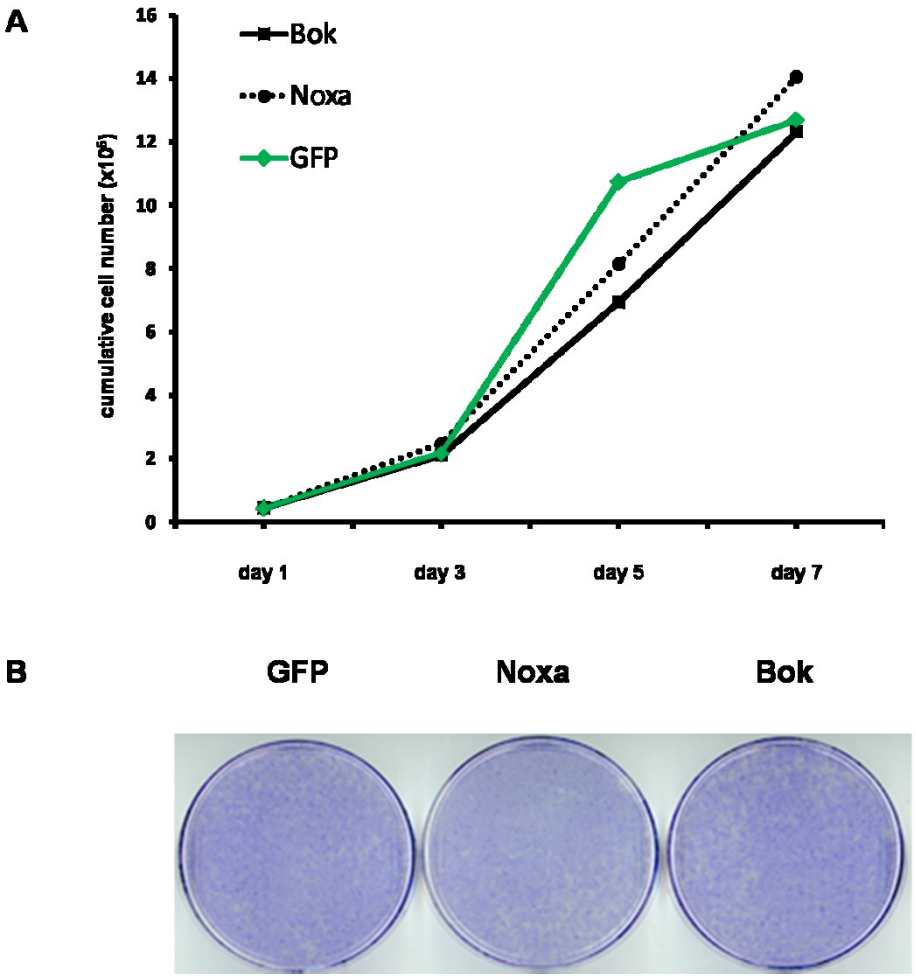


Figure 43. Overexpression of Bok or Noxa doesn't Impair the Proliferation of NIH3T3 cells

(A) Proliferation curve and (B) colony formation assay of NIH3T3 cells infected with viruses coding for Bok, Noxa or GFP as control.

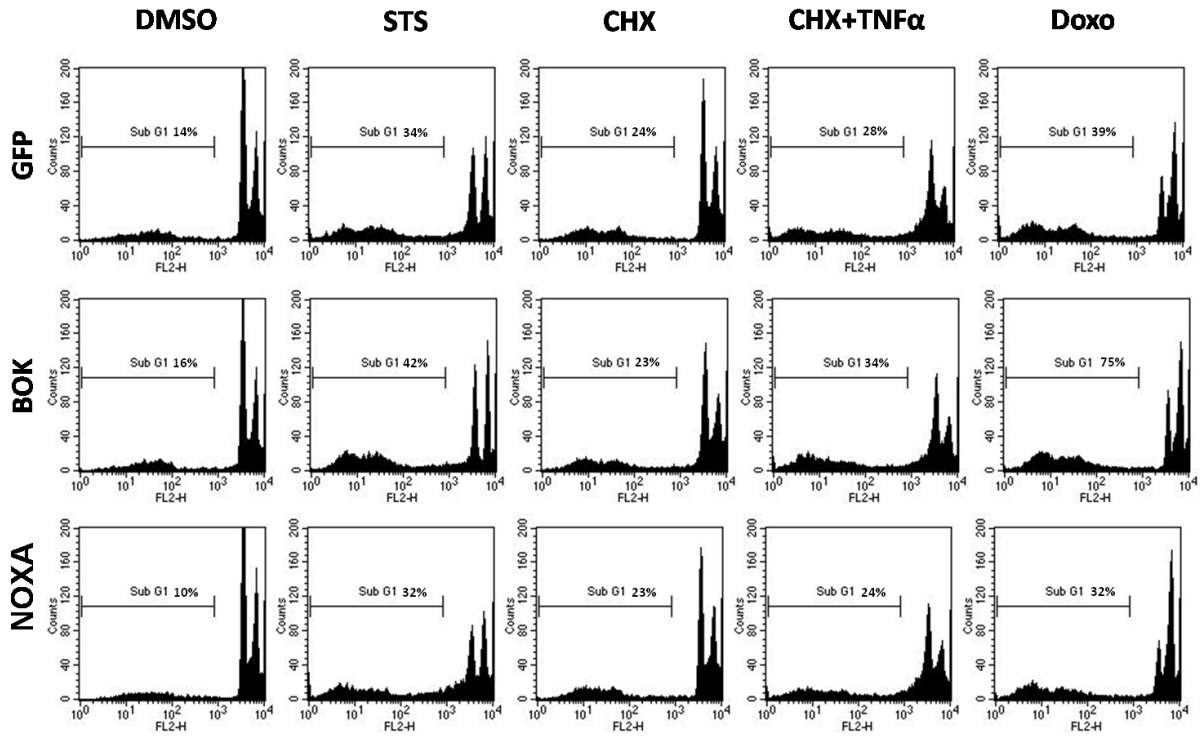


Figure 44. Overexpression of Noxa or Bok are Not Sufficient to Induce Apoptosis in NIH3T3 Cells

NIH3T3 cells were transiently infected with viruses coding for Bok, Noxa, or GFP as control and treated with DMSO (vehicle), staurosporine (STS, 500nM), cycloheximide (CHX, 5µg/ml), TNFα (1ng/ml), or doxorubicine (Doxo, 1µg/ml) for 14 hours.

4.9 The actin MAL-SRF signalling is activated by apoptosis inducers

MAL induces apoptosis and controls the expression of at least two known pro-apoptotic genes BOK and NOXA. In order to understand which external stimuli could trigger this transcriptional regulation, the hypothesis of a possible induction of MAL-SRF activity by apoptotic stimuli like Staurosporine, TNFα and Doxorubicine was tested. In all cases, SRF activity was induced. Importantly, SRF activity induction could be repressed by Latrunculin B treatment underscoring the role of the Actin-MAL pathway in SRF activity during apoptosis (Figure 45 A, B and C).

Induction of SRF activity was also monitored in the course of anoïkis, a kind of apoptosis triggered in epithelial cells when they lose attachment. In the rat bladder epithelial cell line NBT2, activity of SRF was increased when the cells were seeded on plates with low adherence properties compared to regular cell culture plates (Figure 45 D).

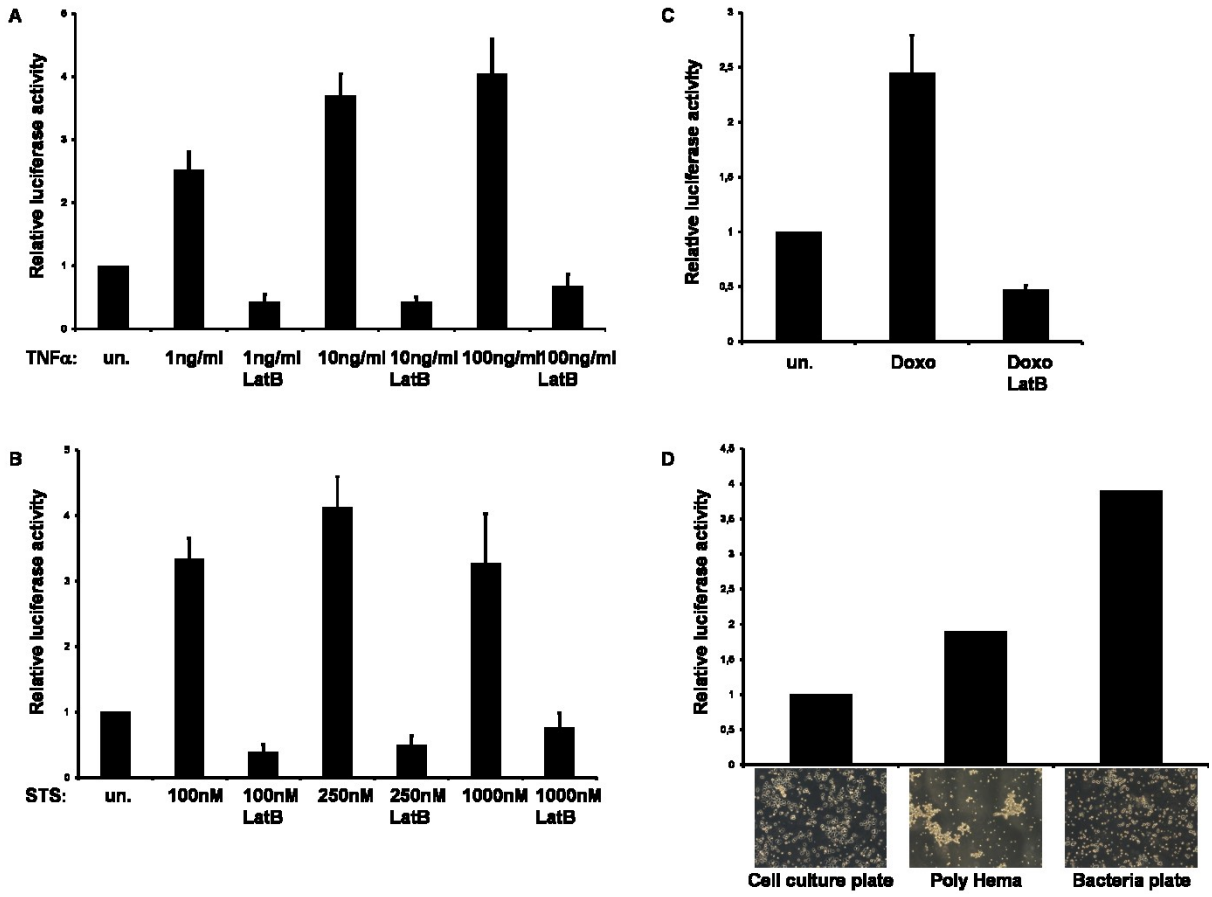


Figure 45. Induction of SRF Mediated Transcription by Apoptotic Inducers

SRF activity was monitored with the 3DA luciferase reporter (A and B) in starved NIH3T3 treated for 10 hours with the indicated concentrations of TNFα and Staurosporine (STS), (C) in non starved NIH3T3 treated for 24 hours with 1µg/ml of doxorubicine where indicated or (D) in starved NBT2 plated on regular cell culture plates coated or not with poly Hema or on plate used for bacteria culture. Where indicated, the cells were pretreated with 5µM Latrunculin B (LatB).

On one hand SRF by its association with MAL responds to apoptotic signals and controls the expression of proapoptotic genes like Bok and Noxa. On the other hand, SRF is also known to respond to survival signals by its association with TCFs controlled by the MAP kinase pathway. In this case, SRF triggers the expression of anti-apoptotic genes like Mcl1 which inhibits genes like Bok and is itself inhibited by NOXA. Hence, SRF could be at the centre of a transcriptional control of essential modulators of apoptosis (Figure 46).

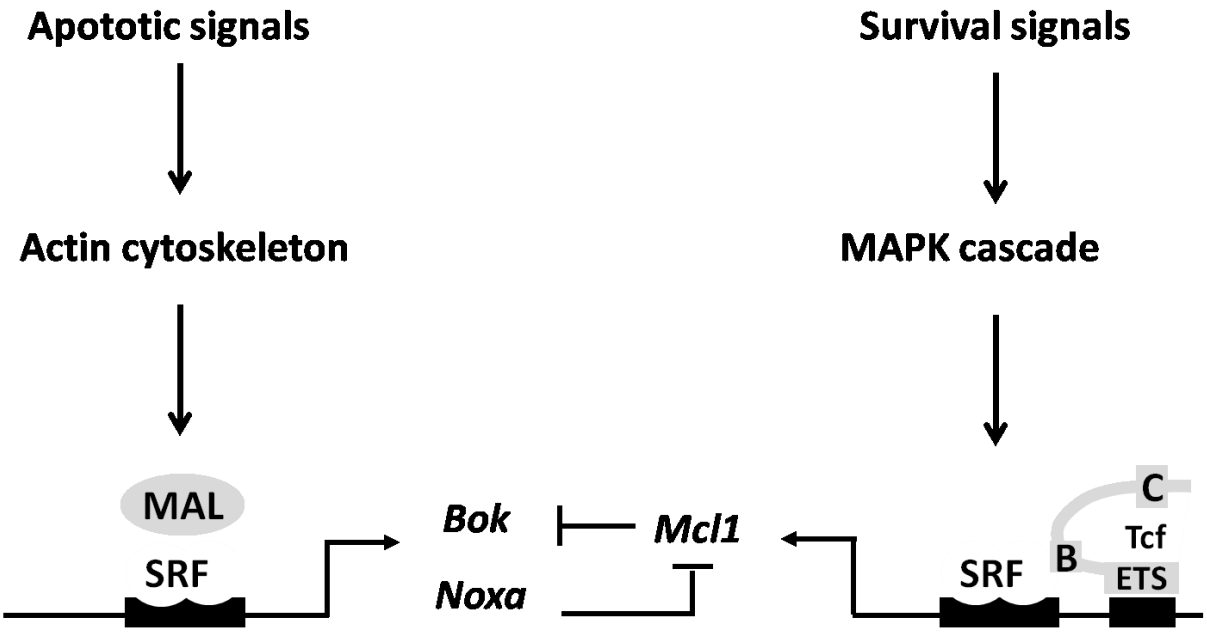


Figure 46. Model which Places SRF at the Centre of Transcriptional Control of Apoptosis

5 Discussion

5.1 MRTFs-SRF: the only set of transcription factors regulated by G-Actin?

The goal of this project was to identify on a genome wide basis the genes controlled by the Actin-MAL-SRF pathway in order to reveal its physiological role. Targeting this pathway at the Actin level may have lead to the identification of genes controlled by transcription factors regulated by Actin but independent of MAL or SRF. Unfortunately, no data supporting this hypothesis could be produced so far. The high percentage, superior to 30%, of genes in the screen already identified in other screen for SRF targets alleviates the hypothesis of Actin regulated SRF independent targets.

Yet, supporting arguments remain for the existence of genes regulated by Actin independently of MAL-SRF.

First, the group of genes which harbour the inversely regulated pattern, repressed by Cytochalasin D and induced by Latrunculin B (group 1, Annexe 1), was not characterised extensively. This group of genes was left on the side because of the generally weak induction by Latrunculin B. Members of this group like Arrdc3, Ccl2, Fam43A (BC022623), Gadd45b or the two histone 1 genes Hist1h1c, Hist1h4i could be the starting point for a search for new kinds of actin responsive promoter elements.

Second, other set of transcription factors regulated by monomeric actin may have different kinetics for the control of transcription than the complex MAL-SRF. Due to experimental limitations, only a single time point was used in the screen. This time point was determined by monitoring the expression of known targets of MAL-SRF after actin binding drug treatment. Hence, genes with a delayed response due to a slower or more complex regulation managed by different sets of transcription factors than MAL and SRF could have been missed.

Third, simple explanations would be that the hypothetical transcription factors controlled by actin are not expressed in the mouse NIH3T3 fibroblast cell line used for the screen or that they are not regulatable under serum starved conditions.

One alternative approach to identify G actin regulated genes independently from MAL and SRF would be to look in silico for proteins having an actin binding domain recognizing the same interface on actin as MAL and a transcription activation domain or a nuclear localisation signal. So far, no such screen has been performed.

A screen for proteins having an RPEL motif, the same actin binding domain as MAL, revealed the existence of three proteins named RPEL A, B and C proteins also known as PHosphatase and ACTin Regulators (PHACTR 1, 2 and 3) or Scapinin for RPEL C (Favot et al., 2005; Sagara et al., 2009). Overexpression of these proteins modified the cell shape but it is unclear if this effect was MAL independent as the three proteins have the potential to activate MAL mediated transcription by titrating MAL away from actin. The specific transcriptional potential, if it exists, of the RPEL proteins needs to be clarified.

Until now, no transcription factor or coactivator of transcription factor having a WH2 domain, which shares with RPEL motifs the same interaction surface on actin (Figure 5), has been characterised.

5.2 Were some regulators of the Rho Actin MAL SRF pathway identified in the screen?

One classical outcome of genome scale identification of genes targeted by a signalling pathway is the discovery of new positive or negative regulators. They may reinforce a signalling pathway important for a cell fate decision for example or provide a way to turn off rapidly the signal at the origin of their expression to maintain the cellular homeostasis.

5.2.1 Known regulators: FHL2, actin

In the screen, several known regulators of actin-MAL-SRF signalling like actin itself (Miralles et al., 2003), or FHL2, a negative regulator of the MAL-SRF complex (Philippar et al., 2004) were identified. Yet, new interesting candidate regulators were spotted.

5.2.2 LPAR4: the LPA receptor 4

Two probe sets of the LPA receptor 4 (Gpr23) which is a positive regulator of Rho signalling (Lee et al., 2008b; Yanagida et al., 2007) were differentially regulated by the actin binding drugs. Interestingly, on the opposite of LPA1, LPA4 represses cell motility (Lee et al., 2008b). Thus, LPA4 could be an interesting target to explain why MRTF represses cell motility in some cell types but promotes it in others (Leitner and Posern, Unpublished). Moreover, the

regulation of LPA receptors at the transcriptional level is poorly characterised which makes this target even more attractive.

5.2.3 RGS16: a negative regulator of GPCR signalling

Concerning as well the upstream part the Rho-Actin-MAL pathway, Rgs16 was identified in the screen. Rgs16 negatively regulates LPA mediated activation of Rho and SRF by inhibiting Gα13 independently of its GTPase activating protein (GAP) activity (Johnson et al., 2003). Rgs16 binds actually Gα13 and prevent its association with p115 Rho-GEF.

5.2.4 What about RhoJ?

Another target, RhoJ, may have a role in the modulation of the Rho-Actin-MAL pathway. This small GTPase regulates the clathrin dependent endocytosis and subsequent recycling of membrane receptors (de Toledo et al., 2003). It could be interesting to evaluate the impact of the knockdown or overexpression of RhoJ on LPA signalling.

5.2.5 Are Mig6 and Dusp5 positive regulators of MRTF mediated transcription?

Mig6 downregulates the MAP kinase pathway and therefore likely the TCFs like Elk1. As MRTFs and TCFs compete for SRF binding, the inhibition of TCFs by Mig6 induction may bring about an increase of MRTF mediated transcription. Another target identified in the screen, Dusp5, a specific phosphatase for Erk1/2 (Mandl et al., 2005), may increase as well MRTF mediated transcription by the same mechanism.

Actually, by decreasing ERK phosphorylation, Mig6 and DUSP5 could increase MRTF mediated transcription also by relieving MAL from the inhibition mediated by G-actin which is dependent on ERK (Muehlich et al., 2008).

Monitoring the transcription activity of the SM22 promoter, which is controlled by the competition Elk1/Myocardin, after overexpression of either Mig6 or Dusp5 should answer this question.

5.3 Mig6 regulation: open questions

The Rho-Actin-MAL-SRF pathway controls the EGF receptor inhibitor Mig6/Erffi1 expression in response to external stimuli. However, some points still remain to be clarified.

5.3.1 MAL-SRF response element in the Mig6 promoter

Mig6 is induced by actin binding drugs in mouse and human cell lines (q.v. 3.4). Yet, the identified ten bases long response element in the mouse Mig6 promoter contains two additional mutations in the human Mig6 promoter. Therefore, the activity of this element in the human promoter is questionable. It could be that the MAL-SRF control of the human Mig6 gene relies on a differently located response element. Cloning and analysis of the human Mig6 promoter should answer this question.

5.3.2 Why the cloned Mig6 promoter doesn't respond to LPA or FCS?

Seen the strong induction of Mig6 mRNA after LPA or FCS stimulation (q.v. 3.9), one would expect that a cloned Mig6 promoter would respond similarly. Unfortunately it is not the case even though MAL and SRF were shown to bind the proximal Mig6 promoter by ChIP (Descot et al., 2009). A first explanation would be that the response element identified is not the only MAL-SRF response element. Seen the size and complexity of the Mig6 genomic locus it could well be that different response elements need to cooperate. A second explanation could be that the transiently transfected Mig6 promoters in the cells don't have the proper chromatin environment to represent with fidelity the activity of an endogenous Mig6 promoter.

MAL full length or LPA alone were not able to activate the promoter of Mig6. However, they synergised to trigger Mig6 promoter activity (Figure 23). This could lead to two different hypotheses. Either the transfected number of Mig6 promoters is so high that it could dilute different factors binding to MAL which are required for the regulation of the Mig6 promoter. Second, the activity of the transfected Mig6 promoter is so high compared to the endogenous promoter that it is not possible to monitor any increase by physiological stimuli unless MAL is overexpressed.

5.3.3 Role of NFκB transcription factors in the Mig6 regulation

The MAL-SRF response element identified in the Mig6 promoter could be bound by NFκB transcription factors. Interestingly, other elements link Mig6 to NFκB. First, Mig6 has been identified as a target of TNF alpha signalling in chromaffin cells (Ait-Ali et al., 2008). Second, Mig6 activates NFκB transcription (Tsunoda et al., 2002). A cleaved N terminus fragment of Mig6 binds IκBα on its NFκB binding domain and then releases NFκB from the inhibition

mediated by IκBα. Third, disruption of the actin cytoskeleton by actin binding drugs promotes DNA binding of NFκB family members (Nemeth et al., 2004). All in all, the contribution of NFκB to the transcription of Mig6 was evaluated. In the absence of NFκB signalling, the basal expression of Mig6 is completely impaired but not the induction by actin binding drugs (q.v. 3.7). It could be very interesting to identify the NFκB response element in the Mig6 promoter and check if it is the same as for the MAL-SRF complex or not. In this strategy, monitoring the activity of the cloned Mig6 promoters in MEFs NEMO-/- compared to NEMO+/+ would be valuable.

5.3.4 Mig6 expression dependency on the MAP kinase pathway

The MAP kinase pathway, as well as the Rho-Actin pathway, is sufficient and necessary for the induction of Mig6. Previously characterised targets like Srf or Vinculin are also dependent to some extent (about 40 to 50%) on MEK activity for their induction by LPA and FCS (Gineitis and Treisman, 2001). Yet, the almost complete block of the actin binding drug induction of Mig6 by UO126 is surprising. One possible explanation could come from the fact that MAL phosphorylation by Erk has been shown to be necessary for the induction of MAL-SRF mediated transcription by BDNF or synaptic activity in neurons (Kalita et al., 2006). Therefore, it would be important to identify which sites on MKL1 are phosphorylated by ERK, to create some MAL mutants mimicking these phosphorylation sites and to analyse their transcriptional activity. Additionally, it could be interesting to monitor MAL binding to the Mig6 promoter by ChIP upon treatment with the MEK inhibitor UO126. This would clarify which step of MAL activation is inhibited as MAL can bind a promoter but stay inactive until it is released from actin (Vartiainen et al., 2007).

5.3.5 Mig6 induction by LPA and transactivation

LPA treatment activates EGFRs by transactivation in a transient fashion. After 75 min minutes, EGFR phosphorylation is back to the initial level in COS7 cells (Daub et al., 1997). Mig6 is likely to take part in the shutdown of EGFR signalling activated after transactivation. The LPA induction of Mig6 could be a way to prevent further activation of EGFRs. Therefore, it would be interesting to monitor the kinetics of the transactivation process in Mig6 depleted cells.

5.4 How does MAL actually repress proliferation?

In the screen several genes with antiproliferative or proapototic features that may contribute to the MAL induced repression of cell proliferation were found. Mig6, Bok and Noxa were already presented in the result sections. Here, I would like to discuss other interesting candidates from the screen.

Dusp5 is a specific phosphatase for Erk1/2 and therefore downregulates mitogenic signals (Mandl et al., 2005).

The Rassf3 and Rassf6 which mediate some of the antiproliferative properties of RAS appeared in the screen. Rassf6 overexpression induces apoptosis in different cell types (Allen et al., 2007; Ikeda et al., 2007). Interestingly, Rassf proteins functionally interact with MST1/2 kinases which are involved in apoptosis. First, Rassf6 and MST2 form a complex and inhibit each other upon static conditions. Upon activation of MST2, Rassf6 is released and can trigger apoptosis independently of MST kinases. Second, Rassf1 (and may be Rassf3) activate MST1 after stimulation with FAS.

Intriguingly, two other targets identified in the screen are or are likely to be controlled by MST1 in the Hippo pathway which represses cell proliferation and control apoptosis. The first target is Stk38l (NDR2) which is activated by Rassf1/MST1 in response to Fas receptor activation and promotes apoptosis (Vichalkovski et al., 2008). The second is Mobkl2A, a member of the Mob family of proteins involved in cell proliferation, cell death and cell polarity (Vitulo et al., 2007). Mobkl2a has not been shown directly to be controlled by MST1 but its homologs Mobkl1a and b are a substrate of MST1. Mobkl1a is activated after FAS ligand and is a coactivator of NRD (Vichalkovski et al., 2008). Finally, the MST kinases activity is linked to the status of the cytoskeleton (Densham et al., 2009). All in all, this antiproliferative module centered on MST kinases deserves follow up studies as a possible mediator of the antiproliferative effects of MAL. A first approach would be to block the two apoptotic pathways controlled by MSTs by knocking down NRD1/2 and Rassf6.

ZFP36 (Tristetrapolin) is a RNA binding protein controlling the stability of mRNA containing AU rich elements (ARE) like certain hematopoietic cell growth factors (e.g. granulocyte-monocyte colony stimulating factor, GM-CSF), interleukins, interferons, TNFα, and some

proto-oncogenes (e.g. c-fos, k-ras and pim-1). Overexpression of Zfp36 causes apoptosis (Johnson and Blackwell, 2002; Johnson et al., 2000).

Two ankyrin repeat domain containing proteins (Ankrd1 and Ankrd15) were also identified. Ankrd1 has a caspase-associated recruitment domain (CARD). Ankrd1 knockdown promoted cell proliferation while overexpression inhibited cell proliferation and promoted apoptosis (Liu et al., 2002). Ankrd15 also known as Kank1 (kidney ankyrin repeat containing protein) represses renal cell proliferation (Sarkar et al., 2002).

Jarid2, also known as Jumonji, contains a DNA-binding domain, called an AT-rich interaction domain (ARID). Jarid2 represses cell proliferation by binding and inhibiting the cyclin D1 promoter in cardiac cells and in neurons (Takahashi et al., 2007; Toyoda et al., 2003).

The Btg2 gene (also known as PC3/PIS21) is the founding member of the BTG/TOB family of antiproliferative genes. Btg2 impairs G1/S progression, either by a Rb-dependent pathway through inhibition of cyclin D1 transcription (Guardavaccaro et al., 2000), or in a Rb-independent fashion by cyclin E downregulation (Lim et al., 1998). Btg2 is assumed to mediate its function by interacting with the Protein Arginine N-Methyltransferase 1 as BTG2 growth inhibition is abrogated in PRMT1 depleted cells (Hata et al., 2007).

All these genes are likely to contribute to MAL induced repression of cell proliferation. However, is there one or a limited number of genes which are absolutely required? Successive or combinatorial knockdown of these candidate genes could be an approach. However, the critical genes responsible for the antiproliferative effects of MAL may not have appeared in the screen because of a delayed induction or because they are secondary targets. Therefore, an unbiased genome wide siRNA screen would be more appropriate to identify the genes or the signalling pathways mediating the repression of proliferation monitored after overexpression of MAL.

A less precise option would be to identify which known oncogene or cDNA sequence is able to rescue MAL mediated proliferation arrest. Again, this would lead us to some candidate cellular functions which have to be altered by MAL to block proliferation.

5.5 MAL and the induction of apoptosis

5.5.1 How to explain the discrepancy between the original finding that MAL was antiapoptotic and the results presented in this work?

MAL induces apoptosis. However, BSAC an isoform of MAL was discovered as anti apoptotic factor able to rescue the TNFα induced apoptosis (Sasazuki et al., 2002). Several explanations can be proposed to explain this discrepancy.

5.5.1.1 Role of Traf5 and the TRAF2 binding protein Tifa?

The identification of MAL as able to rescue TNFα induced apoptosis was done in TRAF2/TRAF5 double knockout (DKO) MEFs. These cells were supposed to be highly susceptible to TNF-induced cell death because of an impaired TNF-induced NFκB activation (Tada et al., 2001). Recent findings contradict this idea and show that Traf2 deficiency favours apoptosis by impairing the recruitment of antiapoptotic proteins to the TNF receptor independently of NFκB and that Traf5 contribution to the increased TNF α induced apoptosis observed in MEFs Traf2/Traf5 DKO compared to the MEFs Traf2 KO is marginal (Zhang et al., 2009).

In the screen, Traf5 and the Traf2 binding protein also known as TRAF-interacting protein with a forkhead-associated domain (Tifa) were identified as candidate targets of Actin-MAL-SRF signalling. Traf5 being absent in the MEFs DKO, it is not the reason for the antiapoptotic feature of MAL observed. However, Tifa by activating furthermore IκB kinase (IKK) (Ea et al., 2004) and consequently NFκB mediated induction of antiapoptotic genes like cIAP1/2, cFLIP, Bcl2 and XIAP could be a candidate for the observed anti-apoptotic features of MAL in MEFs DKO.

5.5.1.2 Induction of BCL2?

Even though not present in the screen, in the course of this work, Bcl2 was shown to be induced by activated MAL (Figure 41). BCL2 is a known target of SRF (Schratt et al., 2004) and represses TNFα induced apoptosis (Burow et al., 1998; Jaattela et al., 1995). Therefore, Bcl2 could be also a good candidate to explain MAL repression of apoptosis in MEFs Traf2/Traf5 DKO.

5.5.1.3 Role of the FOXOs inhibitor FKHL18?

The forkhead like 18 protein (Fkhl18 or Foxs1) is a repressor of the proapototic Foxo3a and Foxo4a transcription factors (Sato et al., 2008). Foxo3a has been shown to turn the tumour necrosis factor receptor signaling towards apoptosis by upregulation of genes related with TNF receptor signaling, such as TNF-alpha, TANK (TRAF-associated NF-kappaB activator), TTRAP (TRAF and TNF receptor-associated protein), or IκBRas1(Lee et al., 2008a). Likely as a consequence of the induction of these targets, cells overexpressing Fox3a harbours an activation of JNK and NFκB. Moreover, Fkhl18 is assumed to inhibit other FOXO transcription factors like FOXO1 which controls the tumour necrosis factor receptor-associated death domain (TRADD) (Rokudai et al., 2002). MAL mediated induction of Fkhl18 could hence repress death signals from the TNF receptor.

5.5.1.4 Induction of NFκB transcription by upregulation of Mig6?

Mig6 can activate in a non canonical fashion NFκB (Tsunoda et al., 2002) which is known to inhibit TNFα mediated apoptosis (Van Antwerp et al., 1996; Van Antwerp et al., 1998). Therefore, Mig6, as a MAL-SRF target could also mediate MAL mediated repression of TNFα induced apoptosis in MEFs Traf2/Traf5 DKO.

5.5.2 Control of Bok and Noxa by MRTFs

The identification of proapoptotic genes from the intrinsic pathway as targets of MAL is very interesting but raises several questions.

5.5.2.1 Why mitogenic signals like LPA and FCS induce Bok and Noxa?

A first step would be to understand why mitogenic signals like LPA and FCS induce the proapotptotic genes Bok and Noxa. Serum induction of Bok and Noxa have been actually already reported (Hershko and Ginsberg, 2004; Rodriguez et al., 2006) but was proposed to be mediated by E2F transcription factors. The induction of proapototic genes by serum in part through the Rho-Actin-MAL pathway could be a contribution to the high sensitivity observed for G1/S cells to apoptosis inducers. Having proapoptotic genes already expressed when cells enter the cell cycle could be considered as a safety mechanism: if something wrong happens during the cell cycle then the cell trigger its death program.

5.5.2.2 Candidate stimuli inducing Bok and Noxa on a MAL-SRF dependent manner

The canonical inducers of the Rho-Actin-Mal-SRF pathway, LPA and serum, induce Bok and Noxa. Do other stimuli of Mal mediated transcription induce Bok and Noxa? The first stimuli to consider would be the apoptotic inducers that activate the pathway like TNFα, DNA damage, staurosporine or anoïkis (q.v. 4.9). Another stimulus to consider could be the loss of epithelial cell-cell contact that we characterized as a MAL-SRF inducer (Busche et al., 2008). The loss of cell-cell contact is one step with the loss of adherence to the ECM leading to anoïkis. Finally, force, which activates MRTF (Zhao et al., 2007), could also induce Bok and Noxa to prepare the cell to apoptosis in case of damages due to mechanical stress.

5.5.2.3 Is there a competition between TCFs and MRTFs to control BOK and Noxa?

Concerning Bok and Noxa, another interesting point would be to evaluate the impact of the MAP kinase pathway on their expression. Basically, is there a competition on the Bok and Noxa promoter like on the SM22 promoter between TCFs and MRTFs? Prosurvival signals could use this mechanism to decrease the expression of these two proapoptotic genes.

5.5.3 A conserved MAL-SRF regulation of Bcl2 family members?

MAL and SRF can induce the Bok promoter in which a response element was identified. So far, no MAL-SRF response element in the Noxa proximal promoter was identified but Noxa is a probable direct target of MAL-SRF. Mcl1 has been shown to be controlled by SRF (Townsend et al., 1999; Vickers et al., 2004) like Bcl2 (Schratt et al., 2004). Moreover, MAL could trigger the expression of Bcl2 (Figure 41). Several key players of the control of apoptosis seem to be regulated by MAL and SRF. Therefore, it would be interesting to evaluate the possible impact of MAL-SRF on the expression of the other members of the Bcl2 family and to try to find a possible conserved MAL-SRF response element throughout the Bcl2 family.

5.5.4 Control of Bok and Noxa by MAL-SRF: a new way to couple the extrinsic and intrinsic apoptotic pathways?

Finally, the complex MAL-SRF is activated upon TNFα signalling and two proapoptotic genes, Bok and Noxa, are triggered by MAL. MRTF-SRF mediated transcription could be a new way, transcriptionally dependent, to couple the extrinsic and the intrinsic pathways controlling apoptosis. Normally, the coupling of the two pathways is done by tBid, the product of

caspase 8 cleavage of Bid (Billen et al., 2008). tBID migrates to the mitochondria where it induces permeabilization of the outer membrane that is dependent on the pro-apoptotic proteins Bax and/or Bak. However, Bid independent pathways to mediate TNFα induced apoptosis exist. For example, even though Bid-/- hepatocytes display a higher resistance to TNFα mediated apoptosis, they still died from apoptosis at later time points (Chen et al., 2007). An induction by TNFα of MAL mediated transcription could participate in the Bid independent TNFα apoptosis by inducing Bok and Noxa.

6 Material and methods

6.1 Materials

6.1.1 Chemicals

Acetic acid	Merck, Darmstadt
Acrylamid	Serva, Heidelberg
AG1478	Alexis Biochemicals,
Agar (Difco™)	BD Bioscience, Heidelberg
Agarose	Eurogentec, Cologne
Amphiregulin	Sigma, Taufkirchen
Ampicillin	Roche, Mannheim
Antipain	Fluka, Buchs
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Merck, Darmstadt
Batimastat	British Biotech, Oxford
BES (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid))	Merck, Darmstadt
Bisacrylamide	Roth, Karlsruhe
Bromophenol blue	Sigma, Taufkirchen
BSA (Bovine Serum Albumin)	Sigma, Taufkirchen
Calcium chloride	Merck, Darmstadt
Chloroquin	Biotrend Chemikalien, Cologne
CIAP	NEB, Frankfurt am Main
Cycloheximide	Sigma, Taufkirchen
Cytochalasin D	Calbiochem, Beeston, UK
Desoxyribonucleotide	Roche, Mannheim
DMEM	Gibco, Eggenstein
DMSO (Dimethylsulfoxide)	Sigma, Taufkirchen
DNA polymerase (Phusion Hot start or not)	NEB, Frankfurt am Main
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ecl plus Western Blotting Detection System	GE Healthcare, München
EDTA (Ethylenediaminetetraacetic acid)	Merck, Darmstadt
Ethanol	Riedel de Haen, Hanover
Ethidium bromide	Sigma, Taufkirchen
EGF	Sigma, Taufkirchen
L-glutamine	Gibco, Eggenstein
Glycerol 100%	Merck, Darmstadt
HCl (Hydrochloric acid),37%	Merck, Darmstadt
Jasplakinolide	Calbiochem, Beeston, UK
Isopropanol	Fluka, Buchs, Switzerland

Kanamycin	Sigma, Taufkirchen
KCl	Merck, Darmstadt
KH_2PO_4	Merck, Darmstadt
$K_2HPO_4,3H_2O$	Merck, Darmstadt
Latrunculin B	Calbiochem, Beeston, UK
Leupeptin	Roche Diagnostics, Basel
LPA	Sigma, Taufkirchen
MEM	Gibco, Eggenstein
beta-Mercaptoethanol	Merck, Darmstadt
Methanol	Fischer Scientific, Schwerte
MgCl2	Merck, Darmstadt
Moviol	Sigma-Aldrich, Taufkirchen
NaCl	Merck, Darmstadt
Na_2HPO_4	Merck, Darmstadt
NaOH	Merck, Darmstadt
Non essential amino acids	PAA, Cölbe
Non fat milk powder	Töpfer Naturaflor, Kempten
Penicilin	PAA, Cölbe
Pepstatin	Sigma-Aldrich, Taufkirchen
Paraformaldehyde	Sigma, Taufkirchen
PBS	Merck, Darmstadt
PIPES	Sigma, Taufkirchen
Phenol	Roth, Karlsruhe
PMSF (Phenylmethylsulfonylfluoride)	Sigma, Taufkirchen
Polybren (Hexadimethrinbromide)	Sigma, Taufkirchen
Ponceau S	Sigma, Taufkirchen
Precision Plus Proteinstandard	Biorad, München
Propidiumiodide	Sigma, Taufkirchen
Protein A or G sepharose	Pharmacia, Freiburg
Puromycin	Sigma, Taufkirchen
Restriction enzymes	NEB, Frankfurt am Main
S1P	Sigma, Taufkirchen
SDS (sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium bicarbonate	PAA, Cölbe
Sodium orthovanadate	Aldrich, Steinheim
Sodium pyruvate	PAA, Cölbe
Streptomycin	PAA, Cölbe
Swinholide A	Calbiochem, Beeston, UK
T4 DNA ligase	Roche, Mannheim
TEMED (N,N,N',N'-Tetraethylmethylendiamine)	Serva, Heidelberg
Tris base	Sigma, Taufkirchen

(2-amino-2-hydroxymethyl-propane-1,3-diol)	
Triton X-100	Serva, Heidelberg
Trypsine	PAA, Cölbe
Tryptone (Bacto™)	BD Bioscience, Heidelberg
Western Lightning® Western Blot Chemiluminescence Reagent Plus (Enhanced Luminol Reagent)	Perkin Elmer, Rodgau-Jügesheim
Western Lightning® Western Blot Chemiluminescence Reagent Plus (Oxidizing Reagent)	Perkin Elmer, Rodgau-Jügesheim
Xylene cyanol	Sigma, Taufkirchen
Yeast Extract (Bacto™)	BD Bioscience, Heidelberg

6.1.2 Solutions and buffers:

LB-Medium: 1,0% Trypton
0,5% Yeast Extract
1,0% NaCl
pH 7,2

TAE (10X): 400mM Tris/Acetate , pH 8,0
10mM EDTA

Annealing buffer for DNA oligonucleotides (10x):
100 mM Tris HCl pH 7.5
1 M NaCl
10 mM EDTA

HNTG lysis buffer: 50 mM HEPES pH 7.5
150 mM NaCl,
1% Triton X-100
1mM EDTA
10% glycerol
10 mM sodium pyrophosphate
2 mM sodium orthovanadate
10 mM sodium fluoride
1 mM phenylmethylsulphonyl fluoride
10 mg/ml aprotinin

6x SDS gel loading buffer:
125mM Tris-HCl pH 6.8
2% SDS
20% glycerol
0.2% bromophenol blue

RIPA buffer : Tris-HCl, pH8,0 20 mM
NaCl 150 mM
Glycerin 5% (v/v)
EDTA 1 mM
Triton X-100 1%
Deoxycholat 0,5%
SDS 0,1%

Lämmli buffer: 20% Glycerin
3% SDS
10 mM EDTA pH 8,0
0,05 % Bromophenol blue
5% Beta-Mercaptoethanol

Tris-Glycin-SDS (10x): 250 mM Tris/HCl pH 7,5
2 M Glycin
1% SDS

TBST: 20 mM Tris pH 7.5
150 mM NaCl
0.1 % Triton X-100

NET-gelatine: (10X) 150 Mm NaCl
5 mM EDTA
50 mMTris/HCl pH 7,4
0,05 % Triton X-100

Stripping solution: 62,5mM Tris/HCl pH 6,8
2% SDS
100mM Beta-Mercaptoethanol

PIPES buffer: 10mM PIPES
0.1M NaCl
2mM MgCl2
0.1 % Triton X-100
pH 6.8

Propidium iodide staining solution: 20mg of propidium iodide diluted in 1 litre of PIPES buffer
200µg/ml RNase A

6.1.3 Antibodies

6.1.3.1 Primary antibodies

Antibody	Supplier
Flag (M2)	Sigma, Taufkirchen
HA	Babco, Berkeley
HA-HRP (3F10)	Roche Diagnostics, Basel
Beta-Actin	Sigma, Taufkirchen
Tubulin	Sigma, Taufkirchen
Ki67	Transduction laboratories, Heidelberg
Mig6 (used for the majority of the experiments unless indicated)	Homemade (Hackel et al. 2001)
Mig6 (PE-16)	Sigma, Taufkirchen
Mig6 (rabbit polyclonal antiserum#1573)	Gift from Ingvar Ferby
EGFR	Biomol, Hamburg
P-EGFRY1173	Cell signaling,
ERK 1,2	Transduction laboratories, Heidelberg
P-ERK	Cell signaling, Danvers, MA
P-EGFRY1173/PHER2Y1248	Cell signaling, Danvers, MA
p38	Santa Cruz, Heidelberg
P-p38	Cell Signaling, Danvers, MA
JNK	Santa Cruz, Heidelberg
P-JNK	Cell Signaling, Danvers, MA
BOK	Cell Signaling, Danvers, MA
NOXA	Acris, Herford
Caspase3	Cell Signaling, Danvers, MA
P53	Santa Cruz, Heidelberg
BCL2	Transduction Laboratories, Heidelberg
MCL1	Abcam, Cambridge
MAL/MRTF#79	Homemade (Sina Pleiner)
MAL/MRTF	Santa Cruz, Heidelberg
Rhodamine/Phalloidin	Molecular probes,

6.1.3.2 Secondary antibodies

Antibody	Supplier
HRP coupled swine anti rabbit	Dako Cytomation, Hamburg
HRP coupled goat anti mouse	Dako Cytomation, Hamburg
TRITC coupled swine anti rabbit	Dako Cytomation, Hamburg

Antibody	Supplier
Alexa fluor 546 Rabbit	Invitrogen, Karlsruhe

6.1.4 Primers

6.1.4.1 Primers for quantitative real time PCR

The mRNA and gene structures were retrieved either from NCBI Entrez Gene or from Ensembl. If possible, the primers chosen were either spanning an intron larger than 1kb or one of the primer was binding a intron exon boundary to limit amplification from possible genomic DNA contaminants. The majority of the primers were designed with the Primer 3 software using the default parameters with the exceptions listed in the following table.

Parameter	Default values	Changed values	Example of Relaxed values
Product Size Ranges	-	105-250	105-350
GC content	20-80%	45-65%	40-70
Max Poly-X	5	3	4
Number of return	5	200	
Max Self complementarity	8	3	5
Max 3' stability	9	3	7

If no primer pair could be found, one or several parameters were then relaxed. As an example some relaxed values are given in the above table.

Target gene	Forward primer	Reverse primer
For mouse genes:		
Hprt1	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Mig6	TGGCCTACAATCTGAACTCCC	GACCACACTCTGCAAAGAAGT
Ctgf	CCCTAGCTGCCTACCGACT	CATTCCACAGGTCTTAGAACAGG
Itga5	GGTGACAGGACTCAGCAACTG	GCAGACTACGGCTCTCTTGG
Srf	GGCCGCGTGAAGATCAAGAT	CACATGGCCTGTCTCACTGG
Egr1	AGCGAACAACCCTATGAGCAC	TCGTTTGGCTGGGATAACTCG
c-Fos	TTCAACGCCGACTACGAG	CCACGGAGGAGACCAGAGT
MrtfA	CCAGGACCGAGGACTATTTG	CGAAGGAGGAACTGTCTGCTA
MrtfA	TCTCAGGCACCAAGACAGAG	GAAGGCGACCACTACCTCAC
MrtfB	CCCACCCCAGCAGTTTGTTGTT	TGCTGGCTGTCACTGGTTTCATC

Target gene	Forward primer	Reverse primer
Acta2	GGGAGTAATGGTTGGAATGG	CAGTGTCGGATGCTCTTCAG
Bok	GGCAAGGTAGTGTCCCTGTA	GCTCATCTCTCTGGCAACAAC
Noxa	CGCCAGTGAACCCAACG	GGCTCCTCATCCTGCTCTTT
Mcl1	GCTTCATCGAACCATTAGCA	TGATGCCGCCTTCTAGGTC
Dusp5	CGGTTGAAATCCTTCCCTTC	CCTCCTTCTTCCCTGACACA
Zfp36	CATCTACGAGAGCCTCCA	TGAGTAGGTCCGACAGAG
Bcl2	AGTACCTGAACCGGCATCTG	GCAGGGTCTTCAGAGACAGC
For human genes:		
MCL1	TGGTGCCTTTGTGGCTAAAC	TGCCAAACCAGCTCCTACTC

6.1.4.2 Primers to amplify gene promoters

Promoter	Forward primer	Reverse primer
Mig6 -1635bp	cggcGAGCTCagcaacccagcagcccctaca	gccgCTCGAGacgctccgcgcctcgcact
Mig6 -726bp	cggcGAGCTCctcacactgctcctcactgc	gccgCTCGAGacgctccgcgcctcgcact
Mig6 -330bp	cggcGGTACCcttggatccaagtgcatctc	gccgCTCGAGacgctccgcgcctcgcact
Mig6 -147bp	cggcGGTACCttcggtagcggcatcgcc	gccgCTCGAGacgctccgcgcctcgcact
Mig6 -329bp to -92bp	cggcGGTACCcttggatccaagtgcatctc	gccgGAGCTCcgcagccaatccgggcgggc
Bok	gccgGGTACCagaacttgtgctggcctttct	cggcGAGCTCagttctggtttcaggacccgc
Dusp5	cggcACGCGTcctgacactccaccggtagt	gccgGCTAGCcccagaaagctggggatt
Zfp36	gccgGAGCTCggtggcgcgaatggccttgg	gccgGGTACCgtttttctctcggcttctgg

6.1.4.3 Primers to mutate gene promoter constructs

Target	Forward primer	Reverse primer
Mig6 Del. CARG -260bp	AAAACTCGGTGTCCTTAGGCTA	TTGTGAGCGGCCCTGTTT
Mig6 Mut. CARG -260bp	TCGGGAAAACTCGGTGTCCTTA	GAGGGTTGTGAGCGGCCCT
Bok Del. CARG -99bp	CGAAGCCCTAAGCCTGGCTT	AAACGGCAACCCCCG
Bok Mut. CARG -99bp	GCCGGCGAAGCCCTAAGCCTGGCTT	GAGGGAAACGGCAACCCCCGG

6.1.4.4 Oligonucleotides used to create shRNA coding vectors

Target	Sequence (5'->3')
For mouse genes:	
Mig6 sh2FW	gatccccAAGGTCAAGCTTGCCCCCTttcaagagaAGGGGGCAAGCTTGACCTTtttttggaaa
Mig6 sh2RV	agcttttccaaaaaAAGGTCAAGCTTGCCCCCTtctcttgaaAGGGGGCAAGCTTGACCTTggg
Mig6 sh3FW	gatccccGAGGATCAAGTTATGTGTGttcaagagaCACACATAACTTGATCCTCtttttggaaa
Mig6 sh3RV	agcttttccaaaaaGAGGATCAAGTTATGTGTGtctcttgaaCACACATAACTTGATCCTCggg
Mig6 sh4FW	gatccccCCTCAAAGCCATCGCAGATttcaagagaATCTGCGATGGCTTTGAGGtttttggaaa
Mig6 sh4RV	agcttttccaaaaaCCTCAAAGCCATCGCAGATtctcttgaaATCTGCGATGGCTTTGAGGggg
Mrtf shFW	gatccccgCATGGAGCTGGTGGAGAAGAAttcaagagatTCTTCTCCACCAGCTCCATGtttttggaaa
Mrtf shRV	agcttttccaaaaaCATGGAGCTGGTGGAGAAGAAtctcttgaaTTCTTCTCCACCAGCTCCATGcggg
For human genes:	
MIG 6 sh1FW	gatccccggatcaagttgtatgtggtttcaagagaaccacatacaacttgatccttttttggaaa
MIG 6 sh1RV	agcttttccaaaaaggatcaagttgtatgtggttctcttgaaaccacatacaacttgatccgggg
MIG 6 sh2FW	gatccccggtggaattcctaactagcttcaagagagctagttaggaattccacctttttggaaa
MIG 6 sh2RV	agcttttccaaaaaggtggaattcctaactagctctcttgaagctagttaggaattccaccgggg

6.1.4.5 Primers used to clone coding sequences

Gene	Use	Sequence
Bcl2	Inner PCR FW	cggcGAATTCatggcgcaagccgggaga
	Inner PCR RV	gccgATCGATtcacttgtggcccaggtatg
	Outer PCR FW	tgcggaggaagtagactgat
	Outer PCR RV	tcgaccatttgcctgaatgt
	GSP-RT	tccatgaccacaggcacag
Bok	Inner PCR FW	cggcGAATTCatggaggtgctgcggcgct
	Inner PCR RV	gccgATCGATtcatctctctggcaacaacag
	Outer PCR FW	gggtttgaatggaagggtcta
	Outer PCR RV	tgagggaggtgctttgtagg
	GSP-RT	acaccgaccctgactttctg
Noxa	Inner PCR FW	cggcGAATTCatgcccgggagaaaggc
	Inner PCR RV	gccgATCGATtcaggttactaaattgaagagcttgg
	Outer PCR FW	gctggtgctgcctactgaag
	Outer PCR RV	agcgagcgtttctctcatca
	GSP-RT	ccttcatcatccctgctcag
Mig6	Inner PCR FW	cggcGAATTCatgtcaacagcaggagttgct

	Inner PCR RV	gccgATCGATttatggagaaaccacgtagga
	Outer PCR FW	aatttgaaggcatcccagag
	Outer PCR RV	ccagcataacagcacctcat
	GSP-RT	gctgttcctccagcttgttt
Elk1	Inner PCR FW	cggcGAATTCatggacccatctgtgacgct
	Inner PCR RV	gccgATCGATtcatggcttctggggccct
	Outer PCR FW	gcttctggttgctgcttctg
	Outer PCR RV	ggaggaaatgggtgagatgt
	GSP-RT	tggagagttcaggagcataga
SRFVP16	FW	cggcGAATTCatgttaccgacccaagctg
	RV	gccgATCGATctacccaccgtactcgtcaa
MCL1	FW	cggcGAATTCatgtttggcctcaaaagaaa
	RV	gccgATCGATctatcttattagatatgccaaacca

6.1.4.6 Sequencing and colony PCR primers

Primer name	**Priming in :**	**Sequence**
pRSpuroF1241	pSUPER retro puro	GGAAGCCTTGGCTTTTG
pRSpuroR1532	pSUPER retro puro	TCGCTATGTGTTCTGGGAAA
111_RTFFlucfor2	Luciferase gene	CGGTCGGTAAAGTTGTTCCA
GL2	pGL3	CTTTATGTTTTTGGCGTCTTCCA
RV3	pGL3	CTAGCAAAATAGGCTGTCCC
pLPCXF2844seq	pLPCX	AGCTCGTTTAGTGAACCGTCAGATC
pLPCXR3026seq	pLPCX	ACCTACAGGTGGGGTCTTTCATTCCC
pLPCXF2845col	pLPCX	GCTGGTTTAGTGAACCGTCA
pLPCXR2930col	pLPCX	GGCCTTAATGGCCTAACGA
pLPCXF2815col	pLPCX	CGTGTACGGTGGGAGGTCTA
pLPCXR2930col	pLPCX	GGCCTTAATGGCCTAACGA
EF+	pEF	TTCTCAAGCCTCAGACAGTGG
EF-corrected	pEF	TTGGACAGCAAGAAAGCGAGC
EF- original	pEF	TTGGACAGCAAGAAAGGCAGC
HRASfw460seq	HRAS	GGATGCCTTCTACACGTTGG
HRASrv154seq	HRAS	GCACGTCTCCCCATCAAT
RHOAfw439seq	RHOA	GGGTGCCTTGTCTTGTGAAT
RHOArv147seq	RHOA	ACTATCAGGGCTGTCGATGG
Diap1fw3477seq	Dia1	ACTGGAGAAGCAGCAGAAGC
Diap1rv958seq	Dia1	CGGATGTGAACTCGGAAGTC
Human MKL1rv205seq	MKL1	CATCGGCTAGTCTGGCTCTC
Human MKL1fw2651seq	MKL1	ACCTGGACAGCATGGACTG

6.2 Methods

6.2.1 Molecular biology methods

6.2.1.1 Preparation of electrocompetent bacteria

5 ml of LB medium were inoculated with a single, well isolated bacterial colony and incubated at 37°C overnight with shaking. Four 500 ml flasks containing 100 ml LB each were inoculated with 1 ml of the overnight culture and incubate at 37°C with shaking until the cell density reaches an optical density of 0.5 at 600 nm (2.5-3 h). The bacterial cultures were then chilled on ice for 20min and transferred afterwards into some chilled centrifuge bottles. The cells were collected by centrifugation in a pre-chilled centrifuge for 10 min at 1200 x g at 4°C (Beckmann Allegra 6KR: 2500 rpm). The supernatant was decanted and the cells washed by resuspension in the same amount of ice-cold sterile 10% glycerol amount (50 ml per 50 ml tube). The cells were incubated on ice for 20min. The cells were then pelleted as before. The supernatant was removed and the cells were gently resuspended in 5 ml of 10% glycerol per 50ml tube. The cells were unified into 2 tubes and incubated on ice for 20min. The cells were pelleted as before. The supernatant was removed and the cells gently resuspended in 2ml of 10% glycerol. The cells were then ready for electroporation. They were frozen in 50µl aliquots in liquid nitrogen and then stored at -80°C.

To test for transformation efficiency, one 50µl bacteria aliquot was thawed on ice and transferred into a chilled 2 mm electroporation cuvette. 0.1 ng of known control plasmid were added. The electroporation was done using a Biorad Gene Pulser at 2.5 kV, 25 µF, 200 Ohm (typical time 4 ms). Then, 500 µl of LB medium supplemented with 10mM MgCl2 were added to the cuvette to resuspend the bacteria. The bacteria were then left for 1 hour at 37°C and then plated on an agar plate containing antibiotics. The expected transformation rate should be 10^8-10^9 per µg DNA for ligations (lower rates only acceptable for retransformation of plasmids).

6.2.1.2 Plasmid DNA preparation

Plasmid DNA preparation from E. coli was done by using the Qiaprep spin Miniprep or Maxiprep according to manufacturer protocol (Qiagen). The selection marker was added shortly before use at a concentration of 100µg/ml for ampicillin and 50µg/ml for kanamycin.

6.2.1.3 Restriction digest of DNA and cloning

The following reagents were mixed:

5 µg DNA

3 µl 10x appropriate NEB buffer and 0.3 µl BSA (only if required by enzyme)

1 µl enzyme A

1 µl enzyme B

q.s. with H_2O to 30µl

The reaction was carried out for 2 hours at 37°C unless another reaction temperature was required.

To dephosphorylate a vector, 0,5 µl of Calf Intestine Alkaline Phosphatase (CIP, CIAP) were added to the reaction mix an placed for 30 min at 37 °C.

To blunt DNA ends, the digestion was carried out in a larger volume, 50µl, with a maximum of 2µl of enzyme. After 2 hours of digestion, 2µL of klenow and 1 µl of dNTPs (2mM each) were added. The reaction mix was then incubated at 25°C for 20min. The reaction was then stopped by adding 1µl of EDTA (500mM). The DNA was then purified with a Qiagen PCR purification kit and cut with a second enzyme.

Ligation

The digested plasmid and insert were purified by agarose gel electrophoresis. The DNA were extracted from the gels by using the Qiaquick Gel extraction kit according to manufacturer protocol. The concentration of recovered DNA was then measured with a spectrophotometer Nanodrop (Thermo Scientific).

For the ligation, the following reagents were mixed:

30 to 60 fm of plasmid

3 to 5 times more of insert than plasmid (molar ratio)

2 µl of 10x ligase buffer containing 10mM ATP

1µl T4 DNA ligase (400U/µl)

Q.S. with H_2O to 20µl

The ligation was then carried out at 16°C for at least 1 hour up to overnight. As control a ligation mix that doesn't contain any insert was set up.

Finally, 2 to 4 µl of the ligation mix without purification were electroporated in E. coli as described above.

6.2.1.4 Promoter cloning

The promoters were amplified by PCR from genomic DNA prepared from mouse liver.

Genomic DNA isolation

The livers from mouse C57BL/6 were minced in 25mg pieces, snap frozen and stored at -80°C until use. The isolation of the DNA was done by using the DNeasy kit according to manufacturer protocol (Qiagen).

PCR amplification of the promoter fragments

The primers used for the PCR were designed to amplify the consensus NCBI promoters or the promoters defined by the genomatix software. They possess restriction sites for subsequent the cloning in the pGL3 reporter vector.

The PCR reaction mix was composed of:

Genomic DNA: 200ng

Forward primer (10mM): 2,5µl (0,5µM final)

Reverse primer (10mM): 2,5µl (0,5µM final)

HF buffer: 10µl

10mM dNTPs: 1µL

Phusion polymerase (2U/µl):1µl

Q.S. H2O to: 50µL

The PCR program was:

Cycle step	Temp.	Time	Cycles
Initial denaturation	98°C	30s-3min	1
Denaturation	98°C	5-10s	36
Annealing	X°C *	10-30s	
Extension	72°C	15-30 s/kb	
Final extension	72°C	5-10min	1
	4°C	hold	

*: The annealing temperature was determined for each primer set with the nearest-neighbour method.

The PCR product was then purified by agarose gel electrophoresis and extracted with a Qiaquick gel extraction kit. The purified product was then digested with the proper digestion enzymes for cloning into the pGL3 basic vector.

6.2.1.5 Deletion analysis and mutagenesis of the promoter constructs

The long 5' deletions of the promoter constructs were done by using different 5' primers and using the longer promoter construct as a template for the PCR instead of the genomic DNA. The deletion or the mutagenesis of response elements in the promoter constructs were done by using phosphorylated primers as described on the Figure 47.

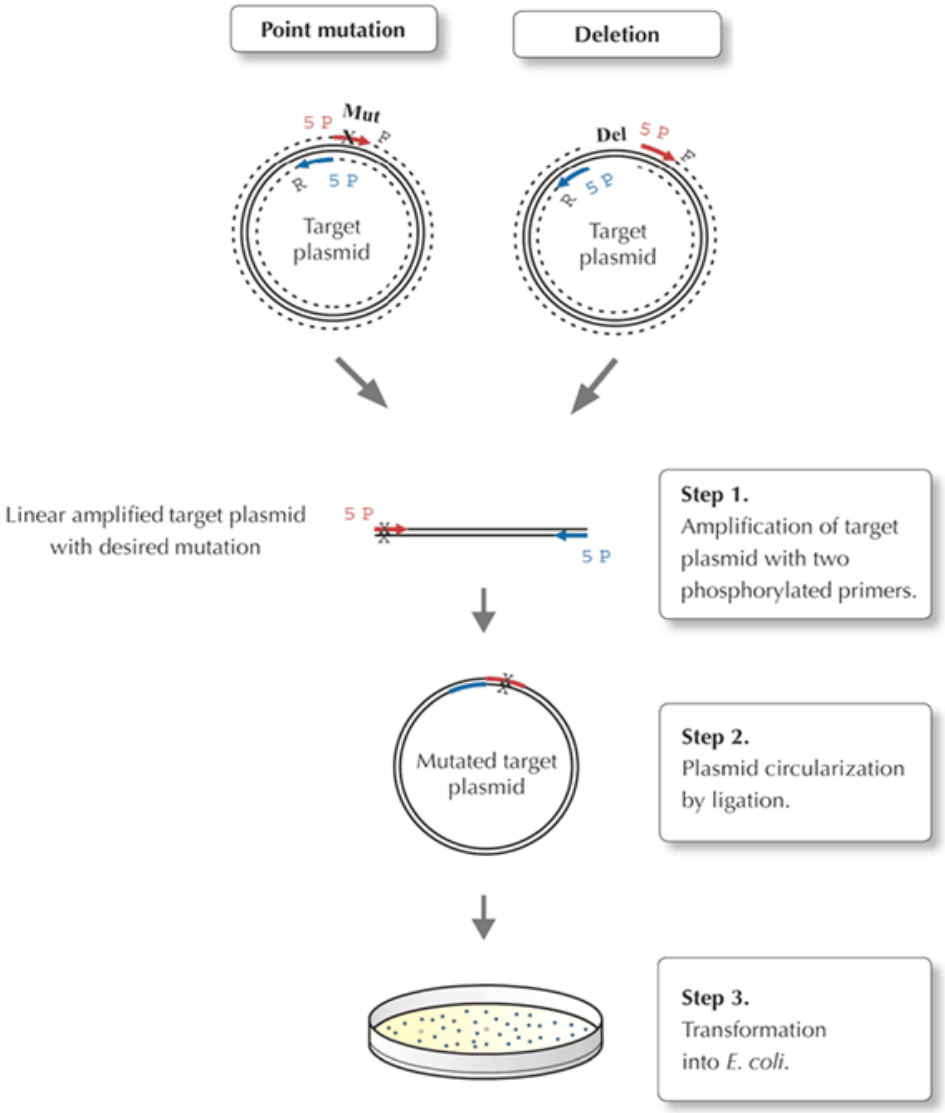


Figure 47. Mutagenesis Scheme of Promoter Constructs

(Modified from the Phusion DNA polymerase protocol)

6.2.1.6 Cloning of gene coding sequences

RNA preparation (QIAGEN) and first strand cDNA synthesis (ABgene) were done according to the manufacturers' protocol. For cDNA synthesis 1 µg of RNA and anchored oligo dT primers or gene specific primers were used. The cDNA of interest was subsequently amplified by nested PCR using the primers described in 6.1.4.5. The inner PCR primers harbour restriction sites for cloning in the expression vectors.

Usually, the outer PCR mix was composed of:

Template cDNA:	one fortieths of the RT reaction without purification (RT enhancer inactivated)
Forward primer (10mM) :	2,5µl (0,5µM final)
Reverse primer (10mM) :	2,5µl (0,5µM final)
HF buffer:	10µl
10mM dNTPs:	1µL
Phusion polymerase (2U/µl):	1µl
Q.S. H2O to:	50µL

The inner PCR mix was:

Template :	1µL of the outer PCR mix
Forward primer (10mM) :	2,5µl (0,5µM final)
Reverse primer (10mM) :	2,5µl (0,5µM final)
HF buffer:	10µl
10mM dNTPs:	1µL
Phusion polymerase (2U/µl):	1µl
Q.S. H2O to:	50µL

The PCR programs were similar as the one described in 6.2.1.4.

The product from the inner PCR was purified on a gel and subsequently digested with the proper restriction enzymes and ligated with the digested and dephosphorylated pLPCX retroviral vector as described in 6.2.1.3.

6.2.1.7 Cloning of shRNA coding vectors

Design of the shRNA

The sequence of the shRNA matching with the target mRNA was designed by using the online Dharmacon software. The overall design of the oligo nucleotides is shown on Figure 48.

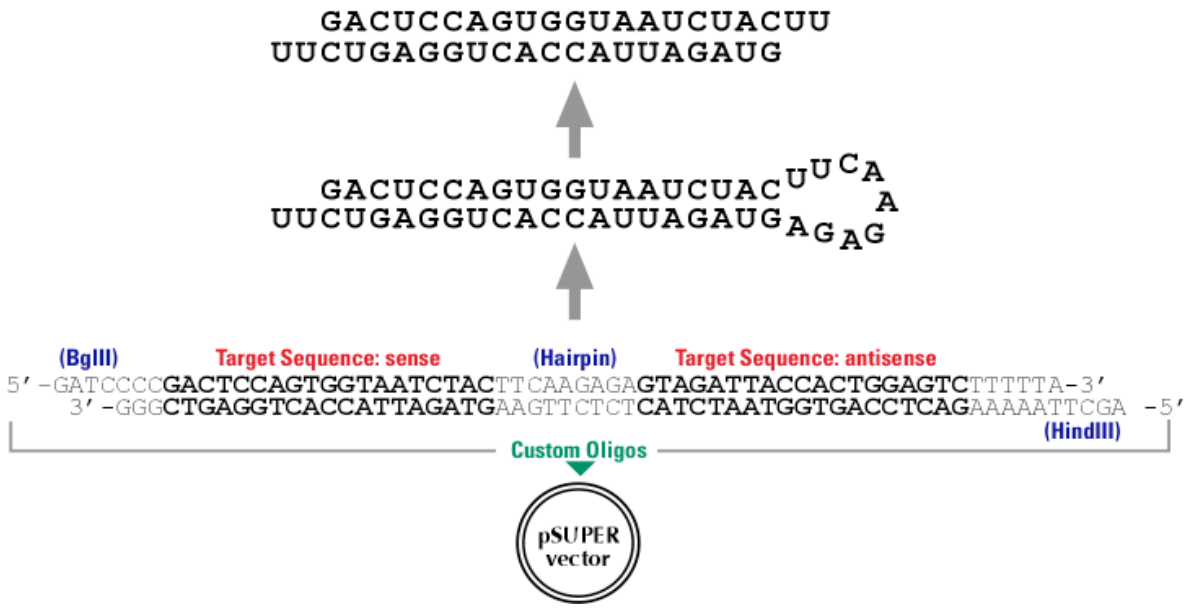


Figure 48. Transcription of the Designed 60 Bases Long Oligonucleotide to Hairpin RNA, Processed to Functional siRNA.

(From the pSUPER-retro-puro vector protocol, Oligoengine)

The oligos once annealed harbour on the 3'end a Hind3 site and on the 5'end a Bgl2 half-site. They are then ready for ligation. After ligation the Bgl2 site is then non functional anymore.

Annealing of the oligonucleotides

The forward and reverse oligos were resuspended in water at a concentration of 3µg/µl (approximately 150pmol/µl). 1µl of each oligos were mixed with 5µl of 10x annealing buffer and 43µl of water. This reaction mix was then heated to 99°C in a pcr machine for 5min. Afterwards the PCR machine was switched off. The mix was then left cooling down in the PCR machine for 3 hours. Efficiency of the annealing was then checked on a 4% agarose gel.

Ligation of the annealed oligos to pSUPER retro puro vector

The pSUPER retro puro vector was first digested with Bgl2 and Hind3 and purified on agarose gel. 100ng of the digested pSUPER retro puro vector, 400ng of annealed oligos, 2µl of T4DNA ligase, 0,5µl of BSA and water were mixed in a final volume of 20µl. The ligation was carried out overnight at 16°C. Before transformation, 1µl of Bgl2 restriction enzyme was added to the ligation mix. The Bgl2 digestion was carried at 37°C for 3 hours to decrease the number of false positive colonies due to undigested vector.

Colony screening by colony PCR

Single colonies were picked from agar plates and added directly to the colony pcr reaction mix composed of:

2 units of Taq polymerase

250 µM dNTPs

0,5 µM forward primer pRSpuroF1241

0,5 µM reverse primer pRSpuroR1532

1µL of 10x standard Taq buffer

8,25µl H_2O

The PCR program was:

Cycle step	Temp.	Time	Cycles
Initial denaturation	95°C	2min	1
Denaturation	95°C	50s	36
Annealing	50°C*	10s	
Extension	72°C	30s	
Final extension	72°C	5min	1
	4°C	hold	

The PCR products were then loaded on 3% agarose gel. Positive PCR products have a size of approximately 350bp compare to 290bp for negative ones.

The positive clones were checked by sequencing.

6.2.2 Cell biology methods

6.2.2.1 Cell lines

NIH3T3:	Immortalised mouse fibroblast (Todaro and Green, 1963)
HEK293:	Human Embryonic Kidney cells (Shaw et al., 2002)
MEF WT	Mouse Embryonic Fibroblast wild type
MEF WT im.	Mouse Embryonic Fibroblast wild type immortalized with the large T antigen of the Simian Vacuolating Virus 40 (SV40)
MEF Mig6 -/-	Mouse Embryonic Fibroblast isolated from embryos knock-out for Mig6
MEF p53 -/-	Mouse Embryonic Fibroblast isolated from embryos knock-out for p53
HepG2:	Hepatocellular carcinoma (Aden et al., 1979)
Hs817.T:	Hepatocellular carcinoma (ATCC # CRL-7549)
NBT2:	Rat urinary bladder carcinoma (Toyoshima et al., 1971)

Cell culture medium

Dulbecco's modified eagle medium (DMEM) : 4,5 mg/ml glucose, 2mM L-glutamine

Minimum essential medium (MEM): 2Mm L-glutamine

6.2.2.2 MEFs isolation and culture

MEFs Mig6 KO and WT were isolated from E13.5 embryos. Briefly, each embryo was separated from its placenta and surrounding membranes. The brain and dark organs were cut away. The embryos were then washed with PBS to remove as much blood as possible. The embryos were then finely minced with a razor blade, suspended in 2 ml of trypsine per embryo and placed in an incubator at 37°C for 15min. DMEM supplemented with FCS and amphotericin B was added. Each embryos was then centrifuged, resuspended in culture medium and plated on a 10cm dish. Genotyping was done by PCR.

6.2.2.3 Lipofection, electroporation and infection of mammalian cells

Lipofection

Transfections of NIH 3T3 cells were carried out with Lipofectamine (Invitrogen) according to the manufacturer's protocols, as described previously (Posern et al., 2004). For luciferase assays, 50000 cells/1-cm-diameter dish (12-well plate) were transfected with 25 ng of indicated luciferase reporter, 50 ng of pRL-TK together with the indicated amounts of plasmids in a total of 500 ng DNA. For protein expression analysis, $1x10^6$ cells were seeded in a 10-cm-diameter dish, transfected with 2µg of the indicated plasmids in a total of 5µg DNA starved in serum free DMEM for 24h and harvested. For mRNA analysis, $3,8x10^5$ cells were seeded in a 6-cm-diameter dish and transfected with 600ng of the indicated plasmids in a total of 2µg DNA. After transfection, cells were starved in DMEM supplemented with 0,5% FCS for 24h and harvested.

Electroporation:

Electroporation of NIH3T3 cells or MEFS were carried out with a GenePulser Xcell with CE and PC module (BioRad) using the exponential decay protocol (voltage, 250 V; pulse length, 60 ms) in a 4 mm GenePulser cuvette (Biorad). For protein expression analysis in MEFS, $8x10^6$ cells in 200µL of Optimem were electroporated with 20µg of DNA. 50% of the cells were then seeded in a 6-cm- diameter dish containing DMEM supplemented with 2% FCS. Twenty four hours later, cells were harvested.

Virus production and infection

pLPCX MAL constructs were generated by subcloning the XhoI/HindIII fragment from pEF MAL constructs into pLPCX (Clontech). The pLPCX GFP construct was generated by subcloning a Eco47III/ EcoRI fragment from pEFP-C2 (Clontech) into pLPCX. $6x10^6$ cells per 10-cm-diameter dish of the retroviral packaging cell line phoenix E were transfected with 20µg of DNA using the calcium phosphate precipitation method. One day later, the culture medium was changed. Two days later, the cell medium was harvested, filtered and concentrated on a Vivaspin 20 column and used to infect $1x10^5$ NIH3T3 cells seeded in a 2.5-cm-diameter dish in presence of Polybrene at a concentration of 8µg/ml. This procedure was repeated 10 hours later.

6.2.2.4 Microscopy and immunofluorescence

For immunofluorescence microscopy, cells were fixed with acetone/methanol for 5 minutes at -20°C, blocked with 10% FCS, 1% gelatine, 0.05% Triton X-100 in PBS. Staining conditions

were as follows: anti-Mig6 (gift from Ingwar Ferby) 1:1000 overnight at 4°C; Alexa Fluor 546 anti rabbit 1:1000 IgG (H+L) (Molecular Probes). Micrographs were taken using a Zeiss Axioplan 2 with MetaVue software (Molecular Devices).

6.2.2.5 Cell cycle analysis of unfixed, detergent-permeabilized cells stained with propidium iodide

Supernatant of adherent cells containing floating cells was first collected. Adherent cells were then washed with PBS that was then added to the floating cells previously collected. The adherent cells were then trypsinized and added to the floating cells to group all the cells and to neutralize the trypsine. The cells were then pelleted by centrifugation and resuspended in PBS at a cell density comprised between $1x10^6$ to $5x10^6$ cells /ml. Then, 0.2ml of this cell suspension was incubated with 2ml of propidium iodide solution (q.v. 6.1.2) for 20 min at room temperature. The cells were then analysed with a FACSCalibur flow cytometer from Becton Dickinson. Briefly, the cells were excited with an Argon Laser at 488nm. The signal went through a photomultiplier with a 585 nm bandpass filter to detect the propidium iodide emission which was recorded in the FL2-H channel. Hence, the x axis, labelled FL2-H, represents the intensity of PI staining. The y axis represents the number of cells.

6.2.3 Biochemical assays

6.2.3.1 Reporter Assays, western blotting

Transfections of NIH 3T3 were carried out using Lipofectamine (Invitrogen) according to the manufacturer's protocols, as described (Posern et al., 2004). For luciferase assays, 50,000 cells per 1 cm Ø dish (12 well plate) were transfected with 25 ng p3DA-Luc, 50 ng pRL-TK, together with the indicated amounts of plasmids in a total of 500 ng DNA. Luciferase activity was measured by dual luciferase assay kit (Promega) and normalised to pRL-TK luciferase. Figures show fold induction compared to control. Error bars usually indicate SEM of three independent experiments. Statistical analysis was done by unpaired student's t-test.

For visualising proteins in HNTG or RIPA lysates, anti-phospho-Erk (1:1000; Cell Signalling) anti-panErk (1:1000; Transduction Laboratories), anti-tubulin (1:10000; Sigma), anti-HA peroxidase conjugate (1:700; Roche), anti-phospho-EgfrY1173 (1:1000; Cell Signalling), anti-phospho-Her2Y1248 (1:1000; Cell Signalling), anti-Egfr (1:2000; Biomol) and anti-Mig6

(1:1000; homemade) antibodies were used, subsequently to SDS-PAGE and western blotting according to standard protocols.

6.2.3.2 Quantitative RT-PCR

RNA preparation (QIAGEN) and first strand cDNA synthesis (ABgene) were done according to the manufacturer's protocol. For cDNA synthesis 1 µg of RNA and anchored oligo dT primers were used. For cDNA quantitation two real time PCR machine were used. When using the LightCycler (Roche), one fortieths of the RT reaction was mixed with gene specific primers (0.5 µM), MgCl2 (3 mM) and LightCycler® FastStart DNA Master SYBR Green I mix (1.5 µl; Roche) to a total volume of 15.5 µl. When using the StepOnePlus (Applied Biosystem), one fortieths of the RT reaction was mixed with gene specific primers (0.250 µM), and LightCycler® FastStart DNA Master SYBR Green I mix (1.5 µl; Roche) to a total volume of 15.5 µl.

The PCR was carried out on a LightCycler instrument (Roche) or on a StepOnePlus (Applied Biosystem) according to the manufacturer's instructions.

Calculations were done using the $\Delta\Delta$Ct method.

6.2.4 Microarray analysis

All experiments were performed in triplicate. Total cellular RNA was labelled and hybridized to Affymetrix Murine Genome 430 2.0 arrays (monitoring more than 45000 transcripts) as recommended by the manufacturer. Raw fluorescence intensity files were generated with Affymetrix Microarraysuite version 5 software. Data were processed and analysed with R and Bioconductor (Gentleman et al., 2004). Arrays were assessed for quality, GCRMA- or RMA-normalized, filtered for low and invariant expression, and analyzed using an empirical Bayes moderated t-test for paired samples.

''Quality assessment'' consisted of RNA degradation plots, Affymetrix quality control metrics, sample cross-correlation, data distributions, and probe-level visualizations.

''Normalization'' incorporated (separately for each RNA type data set) background correction, normalization, and probe-level summation by GCRMA or RMA.

Differentially expressed genes were identified by a permutation-based method (Tusher et al., 2001). Briefly, to control for multiple testing, a false discovery rate (FDR) (Benjamini and Hochberg, 1995) was calculated as the percentage of genes falsely detected as differentially expressed among all genes detected as differentially expressed. A score is assigned to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than an adjustable threshold, permutations of the repeated measurements are used to estimate the percentage of genes identified by chance, which is the FDR. The *q*-value is the lowest FDR at which the gene is called significant. Genes were considered to be differentially expressed if they were detected at a false discovery rate of 5,47% for the GCRMA analysis and 5,17% for the RMA analysis.

7 Abbreviations

Acta2: alpha 2, smooth muscle, aorta

Actb: actin, beta

APS: Ammoniumperoxodisulfate

bp: base pair

Bok: BCL2-related ovarian killer

BSA: bovine serum albumin

CytoD: cytocalasine D

cDNA: complementary DNA

cFOS: FBJ (Finkel, Biskis, and Jinkins) osteosarcoma oncogene

ChIP: Chromatin Immunoprecipitation

CHX: Cycloheximide

CTGF: connective tissue growth factor

DAPI: 4´,6-diamidino-2-phenylindole

DMSO: Dimethylsulfoxide

DTT: dithiothreitol

ECL: enhanced chemiluminescence

EDTA: ethylenedinitrilotetraacetic acid

EGFR: epidermal growth factor receptor

Egr1: early growth response 1

ErbB : avian erythroblastosis oncogene B

Errfi1: ErbB receptor feedback inhibitor 1

FACS: Fluorescence Activated Cell Sorting

FCS: foetal calf serum

FHL2: four and a half LIM domains 2

FOXO: forkhead box O

GAP: GTPase activating protein

GEF: guanosine exchange factor

GFP: green fluorescent protein

GPCR: G protein-coupled receptor

HGF: hepatocyte growth factor

Hop: homeodomain-only protein

HPRT: hypoxanthine phosphoribosyltransferase

Ig: Immunoglobulin

IP: immunoprecipitation

Jasp: Jasplakinolide

LatB: Latrunculin B

LPA: Lysophosphatidic acid

LPAR: LPA receptor

MAPK: Mitogen-activated protein kinase

MAL: megakaryocytic acute leukaemia

MCL1: myeloid cell leukaemia sequence 1

MEF: Mouse embryonic fibroblast

Mef2: Myocyte enhancing factor 2

Mig6: mitogen-inducible gene 6

MRTF: Myocardin-related transcription factor

MKL1: megakaryoblastic leukaemia 1

MMP: Matrix metalloproteinase

NFκB: nuclear factor kappa B

kb: kilo base

kDa: kilo dalton

KO: knock-out

OTT: One twenty two

PAGE:polyacrylamid gel electrophoresis

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PMAIP1: Phorbol-12-myristate-13-acetate-induced protein 1

PMSF: phenylmethylsulfonyl fluoride

pRS: pRetroSuper vector

RNA: ribonucleic acid

RT: room temperature

RBM15: RNA binding motif protein 15

Rgs16: regulator of G-protein signaling 16

SCAI: suppressor of cancer cell invasion

SDS-PAGE: sodium dodecylsulfate polyacrylamid gelelectrophoresis

siRNA: small interference ribonucleic acid

SRF: serum response factor

shRNA: short hairpin RNA

SwinA: swinholide A

TCF: Ternary complex factor

TEMED: Tetramethylethylenediamine

TGFβ: Transforming growth factor beta

Traf: TNF-receptor-associated factor

TNFα: Tumour necrosis factor α

TSA: Trichostatine A

WT: wild-type

8 References

Aden, D.P., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B.B. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature *282*, 615-616.

Ait-Ali, D., Turquier, V., Tanguy, Y., Thouennon, E., Ghzili, H., Mounien, L., Derambure, C., Jegou, S., Salier, J.P., Vaudry, H., Eiden, L.E., and Anouar, Y. (2008). Tumor necrosis factor (TNF)-alpha persistently activates nuclear factor-kappaB signaling through the type 2 TNF receptor in chromaffin cells: implications for long-term regulation of neuropeptide gene expression in inflammation. Endocrinology *149*, 2840-2852.

Allen, N.P., Donninger, H., Vos, M.D., Eckfeld, K., Hesson, L., Gordon, L., Birrer, M.J., Latif, F., and Clark, G.J. (2007). RASSF6 is a novel member of the RASSF family of tumor suppressors. Oncogene *26*, 6203-6211.

Anastasi, S., Baietti, M.F., Frosi, Y., Alema, S., and Segatto, O. (2007). The evolutionarily conserved EBR module of RALT/MIG6 mediates suppression of the EGFR catalytic activity. Oncogene *26*, 7833-7846.

Anastasi, S., Fiorentino, L., Fiorini, M., Fraioli, R., Sala, G., Castellani, L., Alema, S., Alimandi, M., and Segatto, O. (2003). Feedback inhibition by RALT controls signal output by the ErbB network. Oncogene *22*, 4221-4234.

Aravind, L., and Koonin, E.V. (2000). SAP - a putative DNA-binding motif involved in chromosomal organization. Trends Biochem Sci *25*, 112-114.

Arsenian, S., Weinhold, B., Oelgeschlager, M., Ruther, U., and Nordheim, A. (1998). Serum response factor is essential for mesoderm formation during mouse embryogenesis. EMBO J *17*, 6289-6299.

Badorff, C., Seeger, F.H., Zeiher, A.M., and Dimmeler, S. (2005). Glycogen synthase kinase 3beta inhibits myocardin-dependent transcription and hypertrophy induction through site-specific phosphorylation. Circ Res *97*, 645-654.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. Roy. Statist. Soc. Ser*, 289–300.

Billen, L.P., Shamas-Din, A., and Andrews, D.W. (2008). Bid: a Bax-like BH3 protein. Oncogene *27 Suppl 1*, S93-104.

Boatright, K.M., and Salvesen, G.S. (2003). Mechanisms of caspase activation. Curr Opin Cell Biol *15*, 725-731.

Bolos, V., Peinado, H., Perez-Moreno, M.A., Fraga, M.F., Esteller, M., and Cano, A. (2003). The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci *116*, 499-511.

References

Brandt, D.T., Baarlink, C., Kitzing, T.M., Kremmer, E., Ivaska, J., Nollau, P., and Grosse, R. (2009a). SCAI acts as a suppressor of cancer cell invasion through the transcriptional control of beta1-integrin. Nat Cell Biol *11*, 557-568.

Brandt, D.T., Xu, J., Steinbeisser, H., and Grosse, R. (2009b). Regulation of myocardin-related transcriptional coactivators through cofactor interactions in differentiation and cancer. Cell Cycle *8*.

Bubb, M.R., Senderowicz, A.M., Sausville, E.A., Duncan, K.L., and Korn, E.D. (1994). Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. J Biol Chem *269*, 14869-14871.

Bubb, M.R., Spector, I., Beyer, B.B., and Fosen, K.M. (2000). Effects of jasplakinolide on the kinetics of actin polymerization. An explanation for certain in vivo observations. J Biol Chem *275*, 5163-5170.

Burow, M.E., Weldon, C.B., Tang, Y., Navar, G.L., Krajewski, S., Reed, J.C., Hammond, T.G., Clejan, S., and Beckman, B.S. (1998). Differences in susceptibility to tumor necrosis factor alpha-induced apoptosis among MCF-7 breast cancer cell variants. Cancer Res *58*, 4940-4946.

Busche, S., Descot, A., Julien, S., Genth, H., and Posern, G. (2008). Epithelial cell-cell contacts regulate SRF-mediated transcription via Rac-actin-MAL signalling. J Cell Sci *121*, 1025-1035.

Busche, S., and Posern, G. (Submitted).

Cao, D., Wang, Z., Zhang, C.L., Oh, J., Xing, W., Li, S., Richardson, J.A., Wang, D.Z., and Olson, E.N. (2005). Modulation of smooth muscle gene expression by association of histone acetyltransferases and deacetylases with myocardin. Mol Cell Biol *25*, 364-376.

Chen, F., Kook, H., Milewski, R., Gitler, A.D., Lu, M.M., Li, J., Nazarian, R., Schnepp, R., Jen, K., Biben, C., Runke, G., Mackay, J.P., Novotny, J., Schwartz, R.J., Harvey, R.P., Mullins, M.C., and Epstein, J.A. (2002a). Hop is an unusual homeobox gene that modulates cardiac development. Cell *110*, 713-723.

Chen, J., Kitchen, C.M., Streb, J.W., and Miano, J.M. (2002b). Myocardin: a component of a molecular switch for smooth muscle differentiation. J Mol Cell Cardiol *34*, 1345-1356.

Chen, X., Ding, W.X., Ni, H.M., Gao, W., Shi, Y.H., Gambotto, A.A., Fan, J., Beg, A.A., and Yin, X.M. (2007). Bid-independent mitochondrial activation in tumor necrosis factor alpha-induced apoptosis and liver injury. Mol Cell Biol *27*, 541-553.

Cheng, E.C., Luo, Q., Bruscia, E.M., Renda, M.J., Troy, J.A., Massaro, S.A., Tuck, D., Schulz, V., Mane, S.M., Berliner, N., Sun, Y., Morris, S.W., Qiu, C., and Krause, D.S. (2009). Role for MKL1 in megakaryocytic maturation. Blood *113*, 2826-2834.

Cooper, S.J., Trinklein, N.D., Nguyen, L., and Myers, R.M. (2007). Serum response factor binding sites differ in three human cell types. Genome Res *17*, 136-144.

Creemers, E.E., Sutherland, L.B., Oh, J., Barbosa, A.C., and Olson, E.N. (2006). Coactivation of MEF2 by the SAP domain proteins myocardin and MASTR. Mol Cell *23*, 83-96.

Daub, H., Wallasch, C., Lankenau, A., Herrlich, A., and Ullrich, A. (1997). Signal characteristics of G protein-transactivated EGF receptor. EMBO J *16*, 7032-7044.

Daub, H., Weiss, F.U., Wallasch, C., and Ullrich, A. (1996). Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature *379*, 557-560.

de Toledo, M., Senic-Matuglia, F., Salamero, J., Uze, G., Comunale, F., Fort, P., and Blangy, A. (2003). The GTP/GDP cycling of rho GTPase TCL is an essential regulator of the early endocytic pathway. Mol Biol Cell *14*, 4846-4856.

Densham, R., O'Neill, E., Munro, J., Konig, I., Anderson, K., Kolch, W., and Olson, M.F. (2009). MST kinases monitor actin cytoskeletal integrity and signal via JNK stress-activated kinase to regulate p21Waf1/Cip1 stability. Mol Cell Biol.

Descot, A., Hoffmann, R., Shaposhnikov, D., Reschke, M., Ullrich, A., and Posern, G. (2009). Negative regulation of the EGFR-MAPK cascade by actin-MAL-mediated Mig6/Errfi-1 induction. Mol Cell *35*, 291-304.

Descot, A., Rex-Haffner, M., Courtois, G., Bluteau, D., Menssen, A., Mercher, T., Bernard, O.A., Treisman, R., and Posern, G. (2008). OTT-MAL is a deregulated activator of serum response factor-dependent gene expression. Mol Cell Biol *28*, 6171-6181.

Du, K.L., Ip, H.S., Li, J., Chen, M., Dandre, F., Yu, W., Lu, M.M., Owens, G.K., and Parmacek, M.S. (2003). Myocardin is a critical serum response factor cofactor in the transcriptional program regulating smooth muscle cell differentiation. Mol Cell Biol *23*, 2425-2437.

Dudoit, S., Yan, Y.H., Callow, M.J., and Speed, T.P. (2002). Statistical methods for identifying differentially expressed genes in replicated cdna microarray experiments. Statistica Sinica *12*, 111-139.

Ea, C.K., Sun, L., Inoue, J., and Chen, Z.J. (2004). TIFA activates IkappaB kinase (IKK) by promoting oligomerization and ubiquitination of TRAF6. Proc Natl Acad Sci U S A *101*, 15318-15323.

Fadok, V.A., Bratton, D.L., Frasch, S.C., Warner, M.L., and Henson, P.M. (1998). The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death Differ *5*, 551-562.

Fan, L., Sebe, A., Peterfi, Z., Masszi, A., Thirone, A.C., Rotstein, O.D., Nakano, H., McCulloch, C.A., Szaszi, K., Mucsi, I., and Kapus, A. (2007). Cell contact-dependent regulation of

epithelial-myofibroblast transition via the rho-rho kinase-phospho-myosin pathway. Mol Biol Cell *18*, 1083-1097.

Fassler, R., Pfaff, M., Murphy, J., Noegel, A.A., Johansson, S., Timpl, R., and Albrecht, R. (1995). Lack of beta 1 integrin gene in embryonic stem cells affects morphology, adhesion, and migration but not integration into the inner cell mass of blastocysts. J Cell Biol *128*, 979-988.

Favot, L., Gillingwater, M., Scott, C., and Kemp, P.R. (2005). Overexpression of a family of RPEL proteins modifies cell shape. FEBS Lett *579*, 100-104.

Fiorentino, L., Pertica, C., Fiorini, M., Talora, C., Crescenzi, M., Castellani, L., Alema, S., Benedetti, P., and Segatto, O. (2000). Inhibition of ErbB-2 mitogenic and transforming activity by RALT, a mitogen-induced signal transducer which binds to the ErbB-2 kinase domain. Mol Cell Biol *20*, 7735-7750.

Geneste, O., Copeland, J.W., and Treisman, R. (2002). LIM kinase and Diaphanous cooperate to regulate serum response factor and actin dynamics. J Cell Biol *157*, 831-838.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A.J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J.Y., and Zhang, J. (2004). Bioconductor: open software development for computational biology and bioinformatics. Genome Biol *5*, R80.

Gineitis, D., and Treisman, R. (2001). Differential usage of signal transduction pathways defines two types of serum response factor target gene. J Biol Chem *276*, 24531-24539.

Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S.E., Scheffner, M., and Del Sal, G. (1999). Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. EMBO J *18*, 6462-6471.

Guardavaccaro, D., Corrente, G., Covone, F., Micheli, L., D'Agnano, I., Starace, G., Caruso, M., and Tirone, F. (2000). Arrest of G(1)-S progression by the p53-inducible gene PC3 is Rb dependent and relies on the inhibition of cyclin D1 transcription. Mol Cell Biol *20*, 1797-1815.

Guettler, S., Vartiainen, M.K., Miralles, F., Larijani, B., and Treisman, R. (2008). RPEL motifs link the serum response factor cofactor MAL but not myocardin to Rho signaling via actin binding. Mol Cell Biol *28*, 732-742.

Hackel, P.O., Gishizky, M., and Ullrich, A. (2001). Mig-6 is a negative regulator of the epidermal growth factor receptor signal. Biol Chem *382*, 1649-1662.

Hajra, K.M., Chen, D.Y., and Fearon, E.R. (2002). The SLUG zinc-finger protein represses E-cadherin in breast cancer. Cancer Res *62*, 1613-1618.

Hata, K., Nishijima, K., and Mizuguchi, J. (2007). Role for Btg1 and Btg2 in growth arrest of WEHI-231 cells through arginine methylation following membrane immunoglobulin engagement. Exp Cell Res *313*, 2356-2366.

Hershko, T., and Ginsberg, D. (2004). Up-regulation of Bcl-2 homology 3 (BH3)-only proteins by E2F1 mediates apoptosis. J Biol Chem *279*, 8627-8634.

Hill, C.S., and Treisman, R. (1995). Differential activation of c-fos promoter elements by serum, lysophosphatidic acid, G proteins and polypeptide growth factors. EMBO J *14*, 5037-5047.

Hill, C.S., Wynne, J., and Treisman, R. (1995). The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell *81*, 1159-1170.

Hofmann, W.A., Stojiljkovic, L., Fuchsova, B., Vargas, G.M., Mavrommatis, E., Philimonenko, V., Kysela, K., Goodrich, J.A., Lessard, J.L., Hope, T.J., Hozak, P., and de Lanerolle, P. (2004). Actin is part of pre-initiation complexes and is necessary for transcription by RNA polymerase II. Nat Cell Biol *6*, 1094-1101.

Ikeda, M., Hirabayashi, S., Fujiwara, N., Mori, H., Kawata, A., Iida, J., Bao, Y., Sato, Y., Iida, T., Sugimura, H., and Hata, Y. (2007). Ras-association domain family protein 6 induces apoptosis via both caspase-dependent and caspase-independent pathways. Exp Cell Res *313*, 1484-1495.

Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U., and Speed, T.P. (2003). Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics *4*, 249-264.

Iwasaki, K., Hayashi, K., Fujioka, T., and Sobue, K. (2008). Rho/Rho-associated kinase signal regulates myogenic differentiation via myocardin-related transcription factor-A/Smad-dependent transcription of the Id3 gene. J Biol Chem *283*, 21230-21241.

Iyer, D., Belaguli, N., Fluck, M., Rowan, B.G., Wei, L., Weigel, N.L., Booth, F.W., Epstein, H.F., Schwartz, R.J., and Balasubramanyam, A. (2003). Novel phosphorylation target in the serum response factor MADS box regulates alpha-actin transcription. Biochemistry *42*, 7477-7486.

Iyer, D., Chang, D., Marx, J., Wei, L., Olson, E.N., Parmacek, M.S., Balasubramanyam, A., and Schwartz, R.J. (2006). Serum response factor MADS box serine-162 phosphorylation switches proliferation and myogenic gene programs. Proc Natl Acad Sci U S A *103*, 4516-4521.

Jaattela, M., Benedict, M., Tewari, M., Shayman, J.A., and Dixit, V.M. (1995). Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. Oncogene *10*, 2297-2305.

Johnson, B.A., and Blackwell, T.K. (2002). Multiple tristetraprolin sequence domains required to induce apoptosis and modulate responses to TNFalpha through distinct pathways. Oncogene *21*, 4237-4246.

Johnson, B.A., Geha, M., and Blackwell, T.K. (2000). Similar but distinct effects of the tristetraprolin/TIS11 immediate-early proteins on cell survival. Oncogene *19*, 1657-1664.

Johnson, E.N., Seasholtz, T.M., Waheed, A.A., Kreutz, B., Suzuki, N., Kozasa, T., Jones, T.L., Brown, J.H., and Druey, K.M. (2003). RGS16 inhibits signalling through the G alpha 13-Rho axis. Nat Cell Biol *5*, 1095-1103.

Kalita, K., Kharebava, G., Zheng, J.J., and Hetman, M. (2006). Role of megakaryoblastic acute leukemia-1 in ERK1/2-dependent stimulation of serum response factor-driven transcription by BDNF or increased synaptic activity. J Neurosci *26*, 10020-10032.

Knoll, B., Kretz, O., Fiedler, C., Alberti, S., Schutz, G., Frotscher, M., and Nordheim, A. (2006). Serum response factor controls neuronal circuit assembly in the hippocampus. Nat Neurosci *9*, 195-204.

Kook, H., Lepore, J.J., Gitler, A.D., Lu, M.M., Wing-Man Yung, W., Mackay, J., Zhou, R., Ferrari, V., Gruber, P., and Epstein, J.A. (2003). Cardiac hypertrophy and histone deacetylase-dependent transcriptional repression mediated by the atypical homeodomain protein Hop. J Clin Invest *112*, 863-871.

Lee, H.Y., Youn, S.W., Kim, J.Y., Park, K.W., Hwang, C.I., Park, W.Y., Oh, B.H., Park, Y.B., Walsh, K., Seo, J.S., and Kim, H.S. (2008a). FOXO3a turns the tumor necrosis factor receptor signaling towards apoptosis through reciprocal regulation of c-Jun N-terminal kinase and NF-kappaB. Arterioscler Thromb Vasc Biol *28*, 112-120.

Lee, Z., Cheng, C.T., Zhang, H., Subler, M.A., Wu, J., Mukherjee, A., Windle, J.J., Chen, C.K., and Fang, X. (2008b). Role of LPA4/p2y9/GPR23 in negative regulation of cell motility. Mol Biol Cell *19*, 5435-5445.

Leitner, L., and Posern, G. (Unpublished).

Li, S., Chang, S., Qi, X., Richardson, J.A., and Olson, E.N. (2006). Requirement of a myocardin-related transcription factor for development of mammary myoepithelial cells. Mol Cell Biol *26*, 5797-5808.

Li, S., Czubryt, M.P., McAnally, J., Bassel-Duby, R., Richardson, J.A., Wiebel, F.F., Nordheim, A., and Olson, E.N. (2005). Requirement for serum response factor for skeletal muscle growth and maturation revealed by tissue-specific gene deletion in mice. Proc Natl Acad Sci U S A *102*, 1082-1087.

Lim, I.K., Lee, M.S., Ryu, M.S., Park, T.J., Fujiki, H., Eguchi, H., and Paik, W.K. (1998). Induction of growth inhibition of 293 cells by downregulation of the cyclin E and cyclin-dependent kinase 4 proteins due to overexpression of TIS21. Mol Carcinog *23*, 25-35.

Liu, B., Liu, Y., Chen, J., Wei, Z., Yu, H., Zhen, Y., Lu, L., and Hui, R. (2002). CARP is a novel caspase recruitment domain containing pro-apoptotic protein. Biochem Biophys Res Commun *293*, 1396-1404.

Liu, Z.P., Wang, Z., Yanagisawa, H., and Olson, E.N. (2005). Phenotypic modulation of smooth muscle cells through interaction of Foxo4 and myocardin. Dev Cell *9*, 261-270.

Loots, G.G., and Ovcharenko, I. (2004). rVISTA 2.0: evolutionary analysis of transcription factor binding sites. Nucleic Acids Res *32*, W217-221.

Ma, X., Renda, M.J., Wang, L., Cheng, E.C., Niu, C., Morris, S.W., Chi, A.S., and Krause, D.S. (2007). Rbm15 modulates Notch-induced transcriptional activation and affects myeloid differentiation. Mol Cell Biol *27*, 3056-3064.

Ma, Z., Morris, S.W., Valentine, V., Li, M., Herbrick, J.A., Cui, X., Bouman, D., Li, Y., Mehta, P.K., Nizetic, D., Kaneko, Y., Chan, G.C., Chan, L.C., Squire, J., Scherer, S.W., and Hitzler, J.K. (2001). Fusion of two novel genes, RBM15 and MKL1, in the t(1;22)(p13;q13) of acute megakaryoblastic leukemia. Nat Genet *28*, 220-221.

Mack, C.P., and Owens, G.K. (1999). Regulation of smooth muscle alpha-actin expression in vivo is dependent on CArG elements within the 5' and first intron promoter regions. Circ Res *84*, 852-861.

Mandl, M., Slack, D.N., and Keyse, S.M. (2005). Specific inactivation and nuclear anchoring of extracellular signal-regulated kinase 2 by the inducible dual-specificity protein phosphatase DUSP5. Mol Cell Biol *25*, 1830-1845.

Medjkane, S., Perez-Sanchez, C., Gaggioli, C., Sahai, E., and Treisman, R. (2009). Myocardin-related transcription factors and SRF are required for cytoskeletal dynamics and experimental metastasis. Nat Cell Biol *11*, 257-268.

Mercher, T., Coniat, M.B., Monni, R., Mauchauffe, M., Nguyen Khac, F., Gressin, L., Mugneret, F., Leblanc, T., Dastugue, N., Berger, R., and Bernard, O.A. (2001). Involvement of a human gene related to the Drosophila spen gene in the recurrent t(1;22) translocation of acute megakaryocytic leukemia. Proc Natl Acad Sci U S A *98*, 5776-5779.

Miano, J.M. (2003). Serum response factor: toggling between disparate programs of gene expression. J Mol Cell Cardiol *35*, 577-593.

Milyavsky, M., Shats, I., Cholostoy, A., Brosh, R., Buganim, Y., Weisz, L., Kogan, I., Cohen, M., Shatz, M., Madar, S., Kalo, E., Goldfinger, N., Yuan, J., Ron, S., MacKenzie, K., Eden, A., and Rotter, V. (2007). Inactivation of myocardin and p16 during malignant transformation contributes to a differentiation defect. Cancer Cell *11*, 133-146.

Miralles, F., Posern, G., Zaromytidou, A.I., and Treisman, R. (2003). Actin dynamics control SRF activity by regulation of its coactivator MAL. Cell *113*, 329-342.

Miralles, F., and Visa, N. (2006). Actin in transcription and transcription regulation. Curr Opin Cell Biol *18*, 261-266.

Morita, T., Mayanagi, T., and Sobue, K. (2007). Dual roles of myocardin-related transcription factors in epithelial mesenchymal transition via slug induction and actin remodeling. J Cell Biol *179*, 1027-1042.

Mouilleron, S., Guettler, S., Langer, C.A., Treisman, R., and McDonald, N.Q. (2008). Molecular basis for G-actin binding to RPEL motifs from the serum response factor coactivator MAL. EMBO J *27*, 3198-3208.

Muehlich, S., Wang, R., Lee, S.M., Lewis, T.C., Dai, C., and Prywes, R. (2008). Serum-induced phosphorylation of the serum response factor coactivator MKL1 by the extracellular signal-regulated kinase 1/2 pathway inhibits its nuclear localization. Mol Cell Biol *28*, 6302-6313.

Muller, S., Berger, M., Lehembre, F., Seeler, J.S., Haupt, Y., and Dejean, A. (2000). c-Jun and p53 activity is modulated by SUMO-1 modification. J Biol Chem *275*, 13321-13329.

Nakagawa, K., and Kuzumaki, N. (2005). Transcriptional activity of megakaryoblastic leukemia 1 (MKL1) is repressed by SUMO modification. Genes Cells *10*, 835-850.

Nemeth, Z.H., Deitch, E.A., Davidson, M.T., Szabo, C., Vizi, E.S., and Hasko, G. (2004). Disruption of the actin cytoskeleton results in nuclear factor-kappaB activation and inflammatory mediator production in cultured human intestinal epithelial cells. J Cell Physiol *200*, 71-81.

Oh, J., Richardson, J.A., and Olson, E.N. (2005). Requirement of myocardin-related transcription factor-B for remodeling of branchial arch arteries and smooth muscle differentiation. Proc Natl Acad Sci U S A *102*, 15122-15127.

Oh, J., Wang, Z., Wang, D.Z., Lien, C.L., Xing, W., and Olson, E.N. (2004). Target gene-specific modulation of myocardin activity by GATA transcription factors. Mol Cell Biol *24*, 8519-8528.

Olsen, J.V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., and Mann, M. (2006). Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell *127*, 635-648.

Philippar, U., Schratt, G., Dieterich, C., Muller, J.M., Galgoczy, P., Engel, F.B., Keating, M.T., Gertler, F., Schule, R., Vingron, M., and Nordheim, A. (2004). The SRF target gene Fhl2 antagonizes RhoA/MAL-dependent activation of SRF. Mol Cell *16*, 867-880.

Pipes, G.C., Creemers, E.E., and Olson, E.N. (2006). The myocardin family of transcriptional coactivators: versatile regulators of cell growth, migration, and myogenesis. Genes Dev *20*, 1545-1556.

Ploner, C., Kofler, R., and Villunger, A. (2008). Noxa: at the tip of the balance between life and death. Oncogene *27 Suppl 1*, S84-92.

Posern, G., Miralles, F., Guettler, S., and Treisman, R. (2004). Mutant actins that stabilise F-actin use distinct mechanisms to activate the SRF coactivator MAL. EMBO J *23*, 3973-3983.

Posern, G., Sotiropoulos, A., and Treisman, R. (2002). Mutant actins demonstrate a role for unpolymerized actin in control of transcription by serum response factor. Mol Biol Cell *13*, 4167-4178.

Posern, G., and Treisman, R. (2006). Actin' together: serum response factor, its cofactors and the link to signal transduction. Trends Cell Biol *16*, 588-596.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999). EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. Nature *402*, 884-888.

Qiu, P., Ritchie, R.P., Fu, Z., Cao, D., Cumming, J., Miano, J.M., Wang, D.Z., Li, H.J., and Li, L. (2005). Myocardin enhances Smad3-mediated transforming growth factor-beta1 signaling in a CArG box-independent manner: Smad-binding element is an important cis element for SM22alpha transcription in vivo. Circ Res *97*, 983-991.

Rodriguez, J.M., Glozak, M.A., Ma, Y., and Cress, W.D. (2006). Bok, Bcl-2-related Ovarian Killer, Is Cell Cycle-regulated and Sensitizes to Stress-induced Apoptosis. J Biol Chem *281*, 22729-22735.

Rodriguez, M.S., Desterro, J.M., Lain, S., Midgley, C.A., Lane, D.P., and Hay, R.T. (1999). SUMO-1 modification activates the transcriptional response of p53. EMBO J *18*, 6455-6461.

Rokudai, S., Fujita, N., Kitahara, O., Nakamura, Y., and Tsuruo, T. (2002). Involvement of FKHR-dependent TRADD expression in chemotherapeutic drug-induced apoptosis. Mol Cell Biol *22*, 8695-8708.

Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., and Grosschedl, R. (2001). PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. Genes Dev *15*, 3088-3103.

Sagara, J., Arata, T., and Taniguchi, S. (2009). Scapinin, the protein phosphatase 1 binding protein, enhances cell spreading and motility by interacting with the actin cytoskeleton. PLoS One *4*, e4247.

Sahai, E., and Olson, M.F. (2006). Purification of TAT-C3 exoenzyme. Methods Enzymol *406*, 128-140.

Sampath, P., and Pollard, T.D. (1991). Effects of cytochalasin, phalloidin, and pH on the elongation of actin filaments. Biochemistry *30*, 1973-1980.

Sarkar, S., Roy, B.C., Hatano, N., Aoyagi, T., Gohji, K., and Kiyama, R. (2002). A novel ankyrin repeat-containing gene (Kank) located at 9p24 is a growth suppressor of renal cell carcinoma. J Biol Chem *277*, 36585-36591.

Sasazuki, T., Sawada, T., Sakon, S., Kitamura, T., Kishi, T., Okazaki, T., Katano, M., Tanaka, M., Watanabe, M., Yagita, H., Okumura, K., and Nakano, H. (2002). Identification of a novel transcriptional activator, BSAC, by a functional cloning to inhibit tumor necrosis factor-induced cell death. J Biol Chem *277*, 28853-28860.

Sato, Y., Baba, T., Zubair, M., Miyabayashi, K., Toyama, Y., Maekawa, M., Owaki, A., Mizusaki, H., Sawamura, T., Toshimori, K., Morohashi, K., and Katoh-Fukui, Y. (2008). Importance of forkhead transcription factor Fkhl18 for development of testicular vasculature. Mol Reprod Dev *75*, 1361-1371.

Schmid, I., Uittenbogaart, C.H., and Giorgi, J.V. (1994). Sensitive method for measuring apoptosis and cell surface phenotype in human thymocytes by flow cytometry. Cytometry *15*, 12-20.

Schmidt-Supprian, M., Bloch, W., Courtois, G., Addicks, K., Israel, A., Rajewsky, K., and Pasparakis, M. (2000). NEMO/IKK gamma-deficient mice model incontinentia pigmenti. Mol Cell *5*, 981-992.

Schratt, G., Philippar, U., Berger, J., Schwarz, H., Heidenreich, O., and Nordheim, A. (2002). Serum response factor is crucial for actin cytoskeletal organization and focal adhesion assembly in embryonic stem cells. J Cell Biol *156*, 737-750.

Schratt, G., Philippar, U., Hockemeyer, D., Schwarz, H., Alberti, S., and Nordheim, A. (2004). SRF regulates Bcl-2 expression and promotes cell survival during murine embryonic development. EMBO J *23*, 1834-1844.

Selvaraj, A., and Prywes, R. (2004). Expression profiling of serum inducible genes identifies a subset of SRF target genes that are MKL dependent. BMC Mol Biol *5*, 13.

Shaposhnikov, D., Descot, A., and Posern, G. (Unpublished).

Shats, I., Milyavsky, M., Cholostoy, A., Brosh, R., and Rotter, V. (2007). Myocardin in tumor suppression and myofibroblast differentiation. Cell Cycle *6*, 1141-1146.

Shaw, G., Morse, S., Ararat, M., and Graham, F.L. (2002). Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. FASEB J *16*, 869-871.

Shin, C.H., Liu, Z.P., Passier, R., Zhang, C.L., Wang, D.Z., Harris, T.M., Yamagishi, H., Richardson, J.A., Childs, G., and Olson, E.N. (2002). Modulation of cardiac growth and development by HOP, an unusual homeodomain protein. Cell *110*, 725-735.

Shiota, J., Ishikawa, M., Sakagami, H., Tsuda, M., Baraban, J.M., and Tabuchi, A. (2006). Developmental expression of the SRF co-activator MAL in brain: role in regulating dendritic morphology. J Neurochem *98*, 1778-1788.

Sinha, S., Hoofnagle, M.H., Kingston, P.A., McCanna, M.E., and Owens, G.K. (2004). Transforming growth factor-beta1 signaling contributes to development of smooth muscle cells from embryonic stem cells. Am J Physiol Cell Physiol *287*, C1560-1568.

Sotiropoulos, A., Gineitis, D., Copeland, J., and Treisman, R. (1999). Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. Cell *98*, 159-169.

Spector, I., Shochet, N.R., Kashman, Y., and Groweiss, A. (1983). Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. Science *219*, 493-495.

Stern, S., Debre, E., Stritt, C., Berger, J., Posern, G., and Knoll, B. (2009). A nuclear actin function regulates neuronal motility by serum response factor-dependent gene transcription. J Neurosci *29*, 4512-4518.

Stritt, C., Stern, S., Harting, K., Manke, T., Sinske, D., Schwarz, H., Vingron, M., Nordheim, A., and Knoll, B. (2009). Paracrine control of oligodendrocyte differentiation by SRF-directed neuronal gene expression. Nat Neurosci *12*, 418-427.

Sun, H.Q., Yamamoto, M., Mejillano, M., and Yin, H.L. (1999). Gelsolin, a multifunctional actin regulatory protein. J Biol Chem *274*, 33179-33182.

Sun, Q., Chen, G., Streb, J.W., Long, X., Yang, Y., Stoeckert, C.J., Jr., and Miano, J.M. (2006a). Defining the mammalian CArGome. Genome Res *16*, 197-207.

Sun, Q., Taurin, S., Sethakorn, N., Long, X., Imamura, M., Wang, D.Z., Zimmer, W.E., Dulin, N.O., and Miano, J.M. (2009). Myocardin-dependent activation of the carg box-rich smooth muscle gamma actin gene: Preferential utilization of a single CArG element through functional association with the NKX3.1 homeodomain protein. J Biol Chem.

Sun, Y., Boyd, K., Xu, W., Ma, J., Jackson, C.W., Fu, A., Shillingford, J.M., Robinson, G.W., Hennighausen, L., Hitzler, J.K., Ma, Z., and Morris, S.W. (2006b). Acute myeloid leukemia-associated Mkl1 (Mrtf-a) is a key regulator of mammary gland function. Mol Cell Biol *26*, 5809-5826.

Tada, K., Okazaki, T., Sakon, S., Kobarai, T., Kurosawa, K., Yamaoka, S., Hashimoto, H., Mak, T.W., Yagita, H., Okumura, K., Yeh, W.C., and Nakano, H. (2001). Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death. J Biol Chem *276*, 36530-36534.

Takahashi, M., Kojima, M., Nakajima, K., Suzuki-Migishima, R., and Takeuchi, T. (2007). Functions of a jumonji-cyclin D1 pathway in the coordination of cell cycle exit and migration during neurogenesis in the mouse hindbrain. Dev Biol *303*, 549-560.

Tang, R.H., Zheng, X.L., Callis, T.E., Stansfield, W.E., He, J., Baldwin, A.S., Wang, D.Z., and Selzman, C.H. (2008). Myocardin inhibits cellular proliferation by inhibiting NF-kappaB(p65)-dependent cell cycle progression. Proc Natl Acad Sci U S A *105*, 3362-3367.

Taurin, S., Sandbo, N., Yau, D.M., Sethakorn, N., Kach, J., and Dulin, N.O. (2009). Phosphorylation of myocardin by extracellular signal regulated protein kinase. J Biol Chem.

Todaro, G.J., and Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol *17*, 299-313.

Todaro, G.J., Wolman, S.R., and Green, H. (1963). Rapid Transformation of Human Fibroblasts with Low Growth Potential into Established Cell Lines by Sv40. J Cell Physiol *62*, 257-265.

Townsend, K.J., Zhou, P., Qian, L., Bieszczad, C.K., Lowrey, C.H., Yen, A., and Craig, R.W. (1999). Regulation of MCL1 through a serum response factor/Elk-1-mediated mechanism links expression of a viability-promoting member of the BCL2 family to the induction of hematopoietic cell differentiation. J Biol Chem *274*, 1801-1813.

Toyoda, M., Shirato, H., Nakajima, K., Kojima, M., Takahashi, M., Kubota, M., Suzuki-Migishima, R., Motegi, Y., Yokoyama, M., and Takeuchi, T. (2003). jumonji downregulates cardiac cell proliferation by repressing cyclin D1 expression. Dev Cell *5*, 85-97.

Toyoshima, K., Ito, N., Hiasa, Y., Kamamoto, Y., and Makiura, S. (1971). Tissue culture of urinary bladder tumor induced in a rat by N-butyl-N-(-4-hydroxybutyl)nitrosamine: establishment of cell line, Nara Bladder Tumor II. J Natl Cancer Inst *47*, 979-985.

Tsunoda, T., Inokuchi, J., Baba, I., Okumura, K., Naito, S., Sasazuki, T., and Shirasawa, S. (2002). A novel mechanism of nuclear factor kappaB activation through the binding between inhibitor of nuclear factor-kappaBalpha and the processed NH(2)-terminal region of Mig-6. Cancer Res *62*, 5668-5671.

Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci U S A *98*, 5116-5121.

Van Antwerp, D.J., Martin, S.J., Kafri, T., Green, D.R., and Verma, I.M. (1996). Suppression of TNF-alpha-induced apoptosis by NF-kappaB. Science *274*, 787-789.

Van Antwerp, D.J., Martin, S.J., Verma, I.M., and Green, D.R. (1998). Inhibition of TNF-induced apoptosis by NF-kappa B. Trends Cell Biol *8*, 107-111.

Vartiainen, M.K., Guettler, S., Larijani, B., and Treisman, R. (2007). Nuclear actin regulates dynamic subcellular localization and activity of the SRF cofactor MAL. Science *316*, 1749-1752.

Velu, T.J., Beguinot, L., Vass, W.C., Willingham, M.C., Merlino, G.T., Pastan, I., and Lowy, D.R. (1987). Epidermal-growth-factor-dependent transformation by a human EGF receptor proto-oncogene. Science *238*, 1408-1410.

Vichalkovski, A., Gresko, E., Cornils, H., Hergovich, A., Schmitz, D., and Hemmings, B.A. (2008). NDR kinase is activated by RASSF1A/MST1 in response to Fas receptor stimulation and promotes apoptosis. Curr Biol *18*, 1889-1895.

Vickers, E.R., Kasza, A., Kurnaz, I.A., Seifert, A., Zeef, L.A., O'Donnell, A., Hayes, A., and Sharrocks, A.D. (2004). Ternary complex factor-serum response factor complex-regulated gene activity is required for cellular proliferation and inhibition of apoptotic cell death. Mol Cell Biol *24*, 10340-10351.

Vitulo, N., Vezzi, A., Galla, G., Citterio, S., Marino, G., Ruperti, B., Zermiani, M., Albertini, E., Valle, G., and Barcaccia, G. (2007). Characterization and evolution of the cell cycle-associated mob domain-containing proteins in eukaryotes. Evol Bioinform Online *3*, 121-158.

Wang, D., Chang, P.S., Wang, Z., Sutherland, L., Richardson, J.A., Small, E., Krieg, P.A., and Olson, E.N. (2001). Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. Cell *105*, 851-862.

Wang, D.Z., Li, S., Hockemeyer, D., Sutherland, L., Wang, Z., Schratt, G., Richardson, J.A., Nordheim, A., and Olson, E.N. (2002). Potentiation of serum response factor activity by a family of myocardin-related transcription factors. Proc Natl Acad Sci U S A *99*, 14855-14860.

Wang, J., Li, A., Wang, Z., Feng, X., Olson, E.N., and Schwartz, R.J. (2007). Myocardin sumoylation transactivates cardiogenic genes in pluripotent 10T1/2 fibroblasts. Mol Cell Biol *27*, 622-632.

Wang, Z., Wang, D.Z., Hockemeyer, D., McAnally, J., Nordheim, A., and Olson, E.N. (2004). Myocardin and ternary complex factors compete for SRF to control smooth muscle gene expression. Nature *428*, 185-189.

Willis, S.N., Chen, L., Dewson, G., Wei, A., Naik, E., Fletcher, J.I., Adams, J.M., and Huang, D.C. (2005). Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. Genes Dev *19*, 1294-1305.

Witke, W., Sharpe, A.H., Hartwig, J.H., Azuma, T., Stossel, T.P., and Kwiatkowski, D.J. (1995). Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. Cell *81*, 41-51.

Wu, Z., Irizarry, R.A., Gentleman, R., Murillo, F.M., and Spencer, F. (2004). A Model Based Background Adjustment for Oligonucleotide Expression Arrays. Johns Hopkins University, Dept. of Biostatistics Working Papers. Working Paper 1. .

Xie, P., Fan, Y., Zhang, H., Zhang, Y., She, M., Gu, D., Patterson, C., and Li, H. (2009). CHIP represses myocardin-induced smooth muscle cell differentiation via ubiquitin-mediated proteasomal degradation. Mol Cell Biol *29*, 2398-2408.

Xu, W., Baribault, H., and Adamson, E.D. (1998). Vinculin knockout results in heart and brain defects during embryonic development. Development *125*, 327-337.

Yakovlev, A.G., Di Giovanni, S., Wang, G., Liu, W., Stoica, B., and Faden, A.I. (2004). BOK and NOXA are essential mediators of p53-dependent apoptosis. J Biol Chem *279*, 28367-28374.

Yanagida, K., Ishii, S., Hamano, F., Noguchi, K., and Shimizu, T. (2007). LPA4/p2y9/GPR23 mediates rho-dependent morphological changes in a rat neuronal cell line. J Biol Chem *282*, 5814-5824.

Yin, F., and Herring, B.P. (2005). GATA-6 can act as a positive or negative regulator of smooth muscle-specific gene expression. J Biol Chem *280*, 4745-4752.

Zaromytidou, A.I., Miralles, F., and Treisman, R. (2006). MAL and ternary complex factor use different mechanisms to contact a common surface on the serum response factor DNA-binding domain. Mol Cell Biol *26*, 4134-4148.

Zhang, L., Blackwell, K., Thomas, G.S., Sun, S., Yeh, W.C., and Habelhah, H. (2009). TRAF2 suppresses basal IKK activity in resting cells and TNFalpha can activate IKK in TRAF2 and TRAF5 double knockout cells. J Mol Biol *389*, 495-510.

Zhang, M., Fang, H., Zhou, J., and Herring, B.P. (2007a). A novel role of Brg1 in the regulation of SRF/MRTFA-dependent smooth muscle-specific gene expression. J Biol Chem *282*, 25708-25716.

Zhang, X., Pickin, K.A., Bose, R., Jura, N., Cole, P.A., and Kuriyan, J. (2007b). Inhibition of the EGF receptor by binding of MIG6 to an activating kinase domain interface. Nature *450*, 741-744.

Zhao, X.H., Laschinger, C., Arora, P., Szaszi, K., Kapus, A., and McCulloch, C.A. (2007). Force activates smooth muscle alpha-actin promoter activity through the Rho signaling pathway. J Cell Sci *120*, 1801-1809.

Zhou, J., Zhang, M., Fang, H., El-Mounayri, O., Rodenberg, J.M., Imbalzano, A.N., and Herring, B.P. (2009). The SWI/SNF chromatin remodeling complex regulates myocardin-induced smooth muscle-specific gene expression. Arterioscler Thromb Vasc Biol *29*, 921-928.

9 Curriculum Vitae

Arnaud Descot

Date and place of birth: 03.05.1978, La Teste de Buch, France

Education

2004-2009: **PhD thesis** in the department of molecular biology directed by Axel Ullrich at the Max Planck Institute for Biochemistry (MPIB), Munich, Germany under the supervision of Guido Posern. Official supervisor: Stefan Jentsch.

2002-2003: •DEA Biologie-Santé **(Master's degree in molecular, cellular, and structural biology**) at *Ecole doctorale des science chimiques et biologiques pour la santé de Montpellier*.

•Awarded Ingénieur chimiste généraliste (**Master's degree in Chemistry**) at *Ecole Nationale Supérieure de Chimie de Montpellier* (ENSCM): the National Higher School of Chemistry, Montpellier, France, is a "Grande Ecole", i.e. a highly selective academic Institute of Science and Technology.

2001-2002: Sandwich year: 3 internships in organic synthesis, molecular biology and enzymology.

1999-2001: Third and fourth academic years at the ENSCM.

1996-1999: Three years of post-'A' level intensive education in highly selective Preparatory Classes in Pau (France) leading to the competitive examination to enter the National Institutes of Science and Technology.

Experience

2004-2009: Identification and functional characterization of genes controlled by monomeric actin on a genome wide basis by microarray like the EGFR inhibitor Mig6 or the proapoptotic Bok and Noxa. Characterisation of the leukemogenic fusion protein OTT-MAL (RBM15-MKL1). Tools and techniques: mammalian cell culture, cloning

(cDNA and promoter), infection, electroporation and lipofection of mammalian cells, quantitative real time PCR (Light cycler, StepOnePlus), immunoprecipitation, chromatin immunoprecipitation, western blotting, immunofluorescence, knock down experiment (shRNA), FACS.

Supervised by Guido Posern (Department of Molecular Biology, MPIB, Munich).

March-July 2003: SiRNA mediated Knock-Down and controlled-over expression of Protein aRginine Methyl Transferases (PRMT). Supervised by Claude Sardet and Eric Fabbrizio (Institute of Molecular Genetics, Montpellier).

Sept 2002 – Feb 2003: Production, purification of the Ligand Binding Domain (LBD) of a nuclear receptor (RARα), NMR study of the interaction between this LBD and a coactivator. Supervised by William Bourguet and Helene Demene (Center of structural biochemistry, Montpellier).

May-July 2002: Production of a chimeric fluorescent protein (N-Cadherin). Supervised by Philippe Huber and Marie-Hélène Prandini (Laboratory of Endothelium Development and Aging, CEA, Grenoble).

Oct 2001-March 2002: Asymmetric synthesis of potentially antibiotic molecules and NMR (1D, 2D) analysis. Supervised by Txusmari Aizpurua and Claudio Palomo (Faculty of Chemistry, San Sebastian, Spain).

Summer 2001: Enzymatic tests of inhibitors of the glutathione redox cycle. Supervised by Claudius D'Silva (Manchester Metropolitan University, UK).

Summer 2000: Antibody screening and identification of neuropeptides by immunochemistry methods. Localisation of mRNAs by in situ hybridisation (Neuroscience laboratory, University of Bordeaux).

Sept-Oct 1999: Solid phase synthesis of oligopeptides and modification of their lateral reactivity.

10 Publications

Epithelial cell-cell contacts regulate SRF-mediated transcription via Rac-actin-MAL signalling. (2008)
Busche S, Descot A, Julien S, Genth H, Posern G.(2008)
J Cell Sci. 121(Pt 7):1025-35.

OTT-MAL is a deregulated activator of serum response factor-dependent gene expression. (2008)
Descot A, Rex-Haffner M, Courtois G, Bluteau D, Menssen A, Mercher T, Bernard OA, Treisman R, Posern G.
Mol Cell Biol. 28(20):6171-81.

Negative regulation of the EGFR-MAPK cascade by actin-MAL-mediated Mig6/Errfi-1 induction. (2009)
Descot A, Hoffmann R, Shaposhnikov D, Reschke M, Ullrich A, Posern G.
Mol Cell. 35(3):291-304.

Transcriptional Regulation of the EPLIN-α gene by G-actin and MAL/MRTF coactivators.
Leitner L, Shaposhnikov D, Descot A, Hoffmann R, Posern G.
Submitted.

11 Acknowledgments

Here, I would like to express my gratitude to the different persons who helped me to complete this work.

My first thanks go to Guido Posern for the great opportunity given to accomplish this PhD thesis in an excellent institute, the supervision and all the support over the time. I would like also to thank Axel Ullrich for all the support, the stimulating scientific environment provided especially during the Ringberg retreats where I could see how one individual is able to impact several areas of drug development.

I am very thankful to Stephan Jentsch who has very kindly accepted to be my official supervisor.

I would like to thank here Monika Rex-Haffner for all her friendship, support and help. Sylvia, si tu lis ces quelques lignes un jour, merci beaucoup pour ton aide et ta présence durant une partie de cette thèse. Mr Johannes Schilling, it was a really great pleasure to work with you. Good luck for the future.

I am very thankful as well to Markus Reschke for his friendly help and collaboration, to my labmates Sina Pleiner, Dmitry Shaposhnikov, Laura Leitner, Stephan Busche, Anne-Marie Schmidt, Carolin Killi, Heidwig Kresse, Caroline Schaechterle, Arul Sahadevan and to all the members of the Ullrich department past and present.

I would like to thank as well Reinhard Fässler to have welcomed our group in his departemental seminar but also Olaf Stemmann, Herman Silljé, Pjotr Knyazev, Thorsten Berg, Michael Sixt, Marc Schmidt-Supprian, and Reinhard Hoffmann for help and discussion.

My last acknowledgments go to my parents Bernard and Marie-Thérèse Descot and to Rimma Fomina who supported me with all their affection throughout this time. Thank you immensely!

Finally, I would like to dedicate this work to the memory of my grandmothers Reine Descot and Madeleine Destabeau, and to the memory of Jean-Baptiste Andrault, Landry Bourely and Jean Seigne.

12 Annexe 1: Complete list of genes significantly regulated after actin binding drug treatment grouped by unsupervised clustering

Group 1 **Group 1:34 genes, 39 probes**

Probe.Set.ID	Gene.Title	Gene.Symbol	+CytoD	+CytoD+LatB	q-value(%)
1428942_at	metallothionein 2	Mt2	0,75	0,87	2,7
1428417_at	RIKEN cDNA 3110050N22 gene	3110050N22Rik	0,71	0,75	5,4
1434896_at	zinc finger protein 422, related sequence 1	MGI:3028594	0,71	0,83	3,8
1415996_at	thioredoxin interacting protein	Txnip	0,70	0,90	2,7
1429499_at	F-box only protein 5	Fbxo5	0,68	0,78	2,0
1423350_at	suppressor of cytokine signaling 5	Socs5	0,68	0,85	0,0
1419247_at	regulator of G-protein signaling 2	Rgs2	0,66	0,59	3,0
1434364_at	mitogen-activated protein kinase kinase kinase 14	Map3k14	0,66	0,89	3,0
1449972_s_at	zinc finger protein 97 /// cDNA sequence BC018101	Zfp97 /// BC018101	0,65	0,81	3,0
1447643_x_at	snail homolog 2 (Drosophila)	Snai2	0,64	0,73	3,75
1426471_at	zinc finger protein 52	Zfp52	0,62	0,81	4,71
1418673_at	snail homolog 2 (Drosophila)	Snai2	0,62	0,74	3,01
1447830_s_at	regulator of G-protein signaling 2	Rgs2	0,60	0,62	3,01
1451550_at	Eph receptor B3	Ephb3	0,58	0,73	2,74
1448494_at	growth arrest specific 1	Gas1	0,58	0,63	2,74
1419239_at	zinc finger protein 54	Zfp54	0,55	0,69	1,99
1452769_at	RIKEN cDNA 3732413I11 gene	3732413I11Rik	0,55	0,62	4,71
1427433_s_at	homeo box A3	Hoxa3	0,53	0,58	2,74
1449414_at	zinc finger protein 53	Zfp53	0,52	0,75	1,14
1419602_at	homeo box A2	Hoxa2	0,51	0,59	2,74
1425058_at	zinc finger protein 472	Zfp472	0,49	0,79	3,75
1435462_at	hypothetical LOC433022	LOC433022	0,48	0,41	5,43
1433465_a_at	expressed sequence AI467606	AI467606	0,48	0,68	3,01
1416101_a_at	histone 1, H1c	Hist1h1c	0,47	0,60	3,01
1424854_at	histone 1, H4i	Hist1h4i	0,43	0,48	1,14
1450971_at	growth arrest and DNA-damage-inducible 45 beta	Gadd45b	0,42	0,54	3,01
1451924_a_at	endothelin 1	Edn1	0,42	0,17	3,01
1442873_at	Fidgetin (Fign), mRNA	Fign	0,40	0,46	3,01
1436994_a_at	histone 1, H1c	Hist1h1c	0,40	0,56	5,43
1437868_at	cDNA sequence BC023892	BC023892	0,38	0,49	2,74
1449773_s_at	growth arrest and DNA-damage-inducible 45 beta	Gadd45b	0,38	0,55	0,00
1418072_at	histone 1, H2bc	Hist1h2bc	0,37	0,49	5,43
1429693_at	disabled homolog 2 (Drosophila)	Dab2	0,32	0,32	0,00
1427216_at	interferon zeta	Ifnz	0,30	0,29	1,14
1420380_at	chemokine (C-C motif) ligand 2	Ccl2	0,29	0,48	3,01
1426734_at	cDNA sequence BC022623	BC022623	0,27	0,62	3,01
1446326_at	procollagen, type I, alpha 2	Col1a2	0,22	0,27	5,43
1460056_at	RIKEN cDNA 1700109F18 gene	1700109F18Rik	0,18	0,22	3,01
1454617_at	arrestin domain containing 3	Arrdc3	0,16	0,42	3,01

Group 2 **Group 2:10 genes, 14 probes**

Probe.Set.ID	Gene.Title	Gene.Symbol	+CytoD	+CytoD+LatB	q-value(%)
1450295_s_at	poliovirus receptor	Pvr	2,62	2,54	3,01
1416601_a_at	Down syndrome critical region homolog 1 (human)	Dscr1	2,38	2,99	1,99
1417488_at	fos-like antigen 1	Fosl1	2,37	2,36	3,75
1448325_at	myeloid differentiation primary response gene 116	Myd116	2,26	2,11	2,74
1423905_at	poliovirus receptor	Pvr	2,25	2,11	0,00
1417487_at	fos-like antigen 1	Fosl1	2,21	2,38	1,99
1423903_at	poliovirus receptor	Pvr	2,16	2,13	0,00
1423904_a_at	poliovirus receptor	Pvr	1,84	1,87	5,43
1449300_at	CTTNBP2 N-terminal like	Cttnbp2nl	1,76	1,68	1,99
1428487_s_at	RIKEN cDNA 1500041J02 gene	1500041J02Rik	1,53	1,44	4,71
1436069_at	inhibitor of growth family, member 5	Ing5	1,52	1,51	1,14
1425966_x_at	ubiquitin C	Ubc	1,42	1,37	3,75
1415779_s_at	actin, gamma, cytoplasmic 1	Actg1	1,39	1,33	0,00
1423799_at	eukaryotic translation initiation factor 1	Eif1	1,24	1,29	0,00

Group 3 **Group 3:66 genes, 76 probes**

Probe.Set.ID	Gene.Title	Gene.Symbol	+CytoD	+CytoD+LatB	q-value(%)
1427735_a_at	actin, alpha 1, skeletal muscle	Acta1	60,85	2,92	0,00
1456658_at	actin, alpha 2, smooth muscle, aorta	Acta2	31,27	2,54	0,00
1429915_at	RIKEN cDNA 4930426L09 gene	4930426L09Rik	18,99	4,09	2,74
1418687_at	activity regulated cytoskeletal-associated protein	Arc	17,39	2,35	0,00
1425503_at	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	Gcnt2	16,50	3,14	0,00
1429183_at	plakophilin 2	Pkp2	12,85	2,08	0,00
1452670_at	myosin, light polypeptide 9, regulatory	Myl9	12,48	3,34	2,74
1422340_a_at	actin, gamma 2, smooth muscle, enteric	Actg2	11,91	1,48	0,00
1418250_at	ADP-ribosylation factor 4-like	Arfl4	11,71	2,30	1,99
1416689_at	tuftelin 1	Tuft1	10,28	2,12	3,01
1417917_at	calponin 1	Cnn1	8,07	1,29	0,00
1423072_at	RIKEN cDNA 6720475J19 gene	6720475J19Rik	7,85	1,07	1,14
1459552_at	---	---	7,47	1,50	3,75
1441768_at	RIKEN cDNA 9430051O21 gene	9430051O21Rik	7,29	1,14	0,00
1449799_s_at	plakophilin 2	Pkp2	7,07	1,64	1,14
1429262_at	Ras association (RalGDS/AF-6) domain family 6	Rassf6	7,01	1,40	1,14
1442001_at	protein kinase, AMP-activated, beta 2 non-catalytic subunit	Prkab2	6,65	1,79	0,00
1437820_at	forkhead-like 18 (Drosophila)	Fkhl18	6,62	1,22	5,43
1428384_at	DNA segment, Chr 4, Brigham & Women's Genetics 0951 expressed	D4Bwg0951e	6,43	1,66	0,00
1420991_at	ankyrin repeat domain 1 (cardiac muscle)	Ankrd1	6,12	1,55	0,00
1421415_s_at	glucosaminyl (N-acetyl) transferase 2, I-branching enzyme	Gcnt2	6,02	1,29	1,14
1442077_at	RIKEN cDNA 2310076G05 gene	2310076G05Rik	5,95	1,04	3,01
1444105_at	actin, alpha 2, smooth muscle, aorta	Acta2	5,79	1,06	0,00
1452521_a_at	urokinase plasminogen activator receptor	Plaur	5,39	1,66	3,01
1423268_at	integrin alpha 5 (fibronectin receptor alpha)	Itga5	5,39	1,84	3,75
1420992_at	ankyrin repeat domain 1 (cardiac muscle)	Ankrd1	5,08	1,48	0,00
1423948_at	Bcl2-associated athanogene 2	Bag2	4,65	1,66	0,00
1424408_at	LIM and senescent cell antigen like domains 2	Lims2	4,53	1,22	1,14
1452424_at	G protein-coupled receptor 23	Gpr23	4,28	1,00	3,01
1426037_a_at	regulator of G-protein signaling 16	Rgs16	4,25	1,33	3,01
1417872_at	four and a half LIM domains 1	Fhl1	4,18	1,06	0,00
1423267_s_at	integrin alpha 5 (fibronectin receptor alpha)	Itga5	4,09	1,55	0,00
1416953_at	connective tissue growth factor	Ctgf	4,02	1,50	0,00
1426575_at	transmembrane protein 23	Tmem23	3,98	1,38	3,01
1421073_a_at	prostaglandin E receptor 4 (subtype EP4)	Ptger4	3,96	1,46	3,01
1423049_a_at	tropomyosin 1, alpha	Tpm1	3,87	1,56	3,75
1435649_at	nexilin	Nexn	3,82	1,48	3,01
1420580_at	RIKEN cDNA 4930429B21 gene	4930429B21Rik	3,70	0,94	3,75
1426501_a_at	Traf2 binding protein	MGI:2182965	3,43	1,47	1,14
1437424_at	synapse defective 1, Rho GTPase, homolog 2 (C. elegans)	Syde2	3,31	1,32	1,14
1423071_x_at	hypothetical gene supported by BC019681; BC027236	LOC270335	3,20	1,10	2,74
1432509_at	RIKEN cDNA 5033430I15 gene	5033430I15Rik	3,18	1,25	3,75
1418492_at	gremlin 2 homolog, cysteine knot superfamily (Xenopus laevis)	Grem2	3,10	1,23	1,14
1422053_at	inhibin beta-A	Inhba	3,10	1,27	5,43
1433742_at	ankyrin repeat domain 15	Ankrd15	3,08	1,11	0,00
1426576_at	transmembrane protein 23	Tmem23	3,04	1,39	0,00
1417040_a_at	Bcl-2-related ovarian killer protein	Bok	2,92	1,26	0,00
1436499_at	transmembrane protein 23	Tmem23	2,92	1,38	3,75
1452803_at	GLI pathogenesis-related 2	Glipr2	2,91	1,22	3,75
1428301_at	similar to hypothetical protein LOC67055	LOC544986	2,90	1,23	0,00
1422698_s_at	jumonji, AT rich interactive domain 2	Jarid2	2,87	1,15	5,43
1428897_at	RIKEN cDNA 2610029I01 gene	2610029I01Rik	2,83	1,15	1,14
1452731_x_at	similar to hypothetical protein LOC67055LOC67055	LOC544986	2,83	1,35	3,01
1418892_at	ras homolog gene family, member J	Rhoj	2,82	1,37	3,75
1434186_at	G protein-coupled receptor 23	Gpr23	2,76	0,98	0,00
1434458_at	Follistatin (Fst), mRNA	Fst	2,64	0,84	5,43
1439665_at	G protein-coupled receptor 23	Gpr23	2,55	0,95	1,14
1440007_at	solute carrier family 43, member 1	Slc43a1	2,35	1,15	5,43
1431894_at	RIKEN cDNA 4833424O12 gene	4833424O12Rik	2,30	1,08	0,00
1425673_at	LIM domain containing preferred translocation partner in lipoma	Lpp	2,25	1,01	2,74
1433468_at	RIKEN cDNA 6430527G18 gene	6430527G18Rik	2,23	1,26	2,74
1456544_at	transmembrane protein 38B	Tmem38b	2,18	1,25	1,14
1442382_at	microtubule associated serine/threonine kinase family member 4	Mast4	2,12	1,11	1,99
1438210_at	G protein-coupled receptor 149	Gpr149	2,10	1,10	3,75
1454256_s_at	RIKEN cDNA 1700113I22 gene	1700113I22Rik	2,01	1,22	2,74
1451140_s_at	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	Prkag2	1,98	1,15	1,14
1428336_at	1-acylglycerol-3-phosphate O-acyltransferase 1 (lysophosphatidic acid acyltransferase, delta)	Agpat4	1,94	1,12	1,14
1455685_at	hypothetical protein 9530064J02	9530064J02	1,86	1,08	2,74
1423942_a_at	calcium/calmodulin-dependent protein kinase II gamma	Camk2g	1,80	1,13	5,43
1450173_at	receptor (TNFRSF)-interacting serine-threonine kinase 2	Ripk2	1,76	1,08	4,71
1420994_at	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	B3gnt5	1,70	1,01	1,14
1418509_at	carbonyl reductase 2	Cbr2	1,67	1,08	3,01
1427302_at	ectonucleotide pyrophosphatase/phosphodiesterase 3	Enpp3	1,59	1,03	3,75
1417357_at	emerin	Emd	1,55	1,11	1,14
1424541_at	transmembrane protein 70	Tmem70	1,53	1,03	1,14
1451999_at	LIM domain binding 3	Ldb3	1,24	1,00	2,74

Group 4 **Group 4:65 genes, 82 probes**

Probe.Set.ID	Gene.Title	Gene.Symbol	+CytoD	+CytoD+LatB	q-value(%)
1450791_at	natriuretic peptide precursor type B	Nppb	10,37	3,14	3,75
1423505_at	transgelin	Tagln	9,13	2,85	3,01
1459372_at	neuronal PAS domain protein 4	Npas4	8,76	4,50	1,14
1418256_at	serum response factor	Srf	8,71	3,40	0,00
1436229_at	PREDICTED: hypothetical protein XP_488897	---	8,37	2,83	0,00
1437199_at	gene model 337, (NCBI)	Gm337	8,18	3,16	0,00
1438221_at	RIKEN cDNA C130065N10 gene	C130065N10Rik	8,09	2,98	0,00
1456320_at	cDNA sequence BC049806	BC049806	7,79	3,09	0,00
1456257_at	RIKEN cDNA C130065N10 gene	C130065N10Rik	7,48	3,19	0,00
1427228_at	RIKEN cDNA 2410003B16 gene	2410003B16Rik	7,37	2,53	3,01
1419816_s_at	ERBB receptor feedback inhibitor 1	Errfi1	7,29	2,70	0,00
1433768_at	RIKEN cDNA 2410003B16 gene	2410003B16Rik	7,20	2,92	0,00
1418203_at	phorbol-12-myristate-13-acetate-induced protein 1	Pmaip1	6,04	2,38	1,14
1435875_at	protein kinase, AMP-activated, beta 2 non-catalytic subunit	Prkab2	5,24	2,79	1,99
1425654_a_at	DNA segment, Chr 15, ERATO Doi 366, expressed	D15Ertd366e	5,21	2,43	1,14
1416129_at	ERBB receptor feedback inhibitor 1	Errfi1	5,02	2,33	0,00
1417263_at	prostaglandin-endoperoxide synthase 2	Ptgs2	4,73	2,53	4,71
1419739_at	tropomyosin 2, beta	Tpm2	4,70	1,93	4,71
1435091_at	expressed sequence C80731	C80731	4,42	2,32	0,00
1448546_at	Ras association (RalGDS/AF-6) domain family 3	Rassf3	4,38	1,96	3,01
1425810_a_at	cysteine and glycine-rich protein 1	Csrp1	4,29	1,98	0,00
1448861_at	Tnf receptor-associated factor 5	Traf5	4,17	2,20	1,14
1416454_s_at	actin, alpha 2, smooth muscle, aorta	Acta2	4,16	2,00	1,14
1436507_at	interleukin-1 receptor-associated kinase 2	Irak2	4,11	2,41	0,00
1435727_s_at	DNA segment, Chr 15, ERATO Doi 366, expressed	D15Ertd366e	3,98	2,38	1,99
1419149_at	serine (or cysteine) peptidase inhibitor, clade E, member 1	Serpine1	3,98	2,13	3,01
1449566_at	NK2 transcription factor related, locus 5 (Drosophila)	Nkx2-5	3,94	1,94	2,74
1434388_at	MOB1, Mps One Binder kinase activator-like 2A (yeast)	Mobkl2a	3,87	2,26	0,00
1450629_at	DNA segment, Chr 15, ERATO Doi 366, expressed	D15Ertd366e	3,71	2,08	0,00
1423721_at	tropomyosin 1, alpha	Tpm1	3,68	1,60	0,00
1417613_at	immediate early response 5	Ier5	3,58	1,67	5,43
1426346_at	prolyl endopeptidase-like	Prepl	3,52	2,10	1,14
1422499_at	DNA segment, Chr 15, ERATO Doi 366, expressed	D15Ertd366e	3,51	2,17	0,00
1426345_at	prolyl endopeptidase-like	Prepl	3,46	2,13	1,14
1419431_at	epiregulin	Ereg	3,33	1,55	1,99
1433943_at	cDNA sequence BC063749	BC063749	3,26	1,89	3,01
1425811_a_at	cysteine and glycine-rich protein 1	Csrp1	3,25	1,81	0,00
1431734_a_at	DnaJ (Hsp40) homolog, subfamily B, member 4	Dnajb4	3,12	1,86	0,00
1452034_at	prolyl endopeptidase-like	Prepl	3,02	1,79	0,00
1419354_at	Kruppel-like factor 7 (ubiquitous)	Klf7	3,01	1,78	1,14
1419356_at	Kruppel-like factor 7 (ubiquitous)	Klf7	2,92	1,55	3,01
1418136_at	transforming growth factor beta 1 induced transcript 1	Tgfb1i1	2,86	1,82	1,14
1418932_at	nuclear factor, interleukin 3, regulated	Nfil3	2,76	1,53	0,00
1420514_at	transmembrane protein 47	Tmem47	2,69	1,56	0,00
1449885_at	transmembrane protein 47	Tmem47	2,62	1,44	1,14
1433671_at	RIKEN cDNA A130022J15 gene	A130022J15Rik	2,58	1,62	1,14
1416156_at	vinculin	Vcl	2,57	1,58	4,71
1428861_at	RIKEN cDNA 4631422O05 gene	4631422O05Rik	2,55	1,49	5,43
1435878_at	serine/threonine kinase 38 like	Stk38l	2,46	1,40	0,00
1452045_at	zinc finger protein 281	Zfp281	2,45	1,61	0,00
1451177_at	DnaJ (Hsp40) homolog, subfamily B, member 4	Dnajb4	2,44	1,63	1,14
1423831_at	protein kinase, AMP-activated, gamma 2 non-catalytic subunit	Prkag2	2,39	1,32	4,71
1429623_at	zinc finger protein 644	Zfp644	2,39	1,46	2,74
1417612_at	immediate early response 5	Ier5	2,35	1,54	1,14
1415791_at	ring finger protein 34	Rnf34	2,27	1,38	1,99
1434492_at	RIKEN cDNA A130022J15 gene	A130022J15Rik	2,24	1,32	0,00
1437763_at	DCN1, defective in cullin neddylation 1, domain containing 3 (S. cerevisiae)	Dcun1d3	2,18	1,41	0,00
1416084_at	zinc finger, A20 domain containing 2	Za20d2	2,18	1,52	3,01
1419184_a_at	four and a half LIM domains 2	Fhl2	2,18	1,39	3,01
1417408_at	coagulation factor III	F3	2,09	1,38	1,99
1449168_a_at	A kinase (PRKA) anchor protein 2	Akap2	2,07	1,28	3,01
1449353_at	wild-type p53-induced gene 1	Wig1	2,06	1,34	3,01
1448623_at	RIKEN cDNA 2310075C12 gene	2310075C12Rik	2,03	1,34	0,00
1429487_at	protein phosphatase 1, regulatory (inhibitor) subunit 12A	Ppp1r12a	1,97	1,27	3,01
1418888_a_at	selenoprotein X 1	Sepx1	1,94	1,32	0,00
1436985_at	zinc finger protein 644	Zfp644	1,93	1,25	4,71
1437358_at	CDNA, clone:Y1G0111C17, strand:plus	---	1,92	1,35	3,01
1424608_a_at	similar to novel protein	LOC432458	1,84	1,26	3,75
1452113_a_at	RAB23, member RAS oncogene family	Rab23	1,83	1,27	1,14
1454876_at	RAB23, member RAS oncogene family	Rab23	1,82	1,38	3,01
1424700_at	transmembrane protein 38B	Tmem38b	1,81	1,20	3,75
1427742_a_at	Kruppel-like factor 6	Klf6	1,78	1,21	1,99
1417124_at	destrin	Dstn	1,78	1,35	0,00
1437735_at	protein phosphatase 1, regulatory (inhibitor) subunit 12A	Ppp1r12a	1,75	1,34	5,43
1416452_at	ornithine aminotransferase	Oat	1,72	1,30	0,00
1417472_at	myosin, heavy polypeptide 9, non-muscle	Myh9	1,72	1,31	4,71
1424002_at	phosducin-like 3	Pdcl3	1,71	1,29	0,00
1429019_s_at	paraoxonase 2	Pon2	1,70	1,19	1,14
1426717_at	non imprinted in Prader-Willi/Angelman syndrome 2 homolog (human)	Nipa2	1,63	1,27	0,00
1448810_at	glucosamine	Gne	1,53	1,13	1,14
1437211_x_at	ELOVL family member 5, elongation of long chain fatty acids (yeast)	Elovl5	1,47	1,15	3,01
1415906_at	thymosin, beta 4, X chromosome	Tmsb4x	1,44	1,19	0,00

Group 5 **Group 5:33 genes, 40 probes**

Probe.Set.ID	Gene.Title	Gene.Symbol	+CytoD	+CytoD+LatB	q-value(%)
1418255_s_at	serum response factor	Srf	7,90	4,43	0,00
1452519_a_at	zinc finger protein 36	Zfp36	6,22	4,14	0,00
1416250_at	B-cell translocation gene 2, anti-proliferative	Btg2	5,98	3,99	3,01
1438133_a_at	cysteine rich protein 61	Cyr61	5,83	4,05	1,14
1416442_at	immediate early response 2	Ier2	4,39	3,35	0,00
1416039_x_at	cysteine rich protein 61	Cyr61	4,20	3,25	0,00
1428319_at	PDZ and LIM domain 7	Pdlim7	3,48	2,32	3,01
1426791_at	RUN and SH3 domain containing 2	Rusc2	3,31	2,14	1,14
1415899_at	Jun-B oncogene	Junb	2,93	2,03	0,00
1444107_at	RIKEN cDNA 9430029N19 gene	9430029N19Rik	2,77	1,99	4,71
1416431_at	tubulin, beta 6	Tubb6	2,69	2,20	0,00
1417262_at	prostaglandin-endoperoxide synthase 2	Ptgs2	2,65	1,99	3,01
1424880_at	tribbles homolog 1 (Drosophila)	Trib1	2,42	1,82	3,75
1426377_at	zinc finger protein 281	Zfp281	2,42	1,73	1,14
1448467_a_at	tangerin	LOC114601	2,30	1,75	4,71
1424927_at	GLI pathogenesis-related 1 (glioma)	Glipr1	2,24	1,67	3,01
1452697_at	CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) phosphatase, subunit 1	Ctdp1	2,23	1,85	3,01
1426599_a_at	solute carrier family 2 (facilitated glucose transporter), member 1	Slc2a1	2,18	1,70	1,14
1416804_at	tangerin	LOC114601	2,15	1,60	2,74
1450981_at	calponin 2	Cnn2	2,14	1,60	1,14
1426792_s_at	RUN and SH3 domain containing 2	Rusc2	2,12	1,80	2,74
1426964_at	RIKEN cDNA 3110003A17 gene	3110003A17Rik	2,01	1,57	0,00
1417509_at	ring finger protein (C3HC4 type) 19	Rnf19	1,98	1,56	2,74
1434773_a_at	solute carrier family 2 (facilitated glucose transporter), member 1	Slc2a1	1,98	1,59	3,75
1434181_at	pleckstrin homology domain containing, family C (with FERM domain) member 1	Plekhc1	1,94	1,53	3,01
1416083_at	zinc finger, A20 domain containing 2	Za20d2	1,92	1,63	0,00
1424723_s_at	cleavage stimulation factor, 3' pre-RNA, subunit 3	Cstf3	1,85	1,56	3,01
1433883_at	tropomyosin 4	Tpm4	1,80	1,51	0,00
1417508_at	ring finger protein (C3HC4 type) 19	Rnf19	1,76	1,58	3,75
1423282_at	phosphatidylinositol transfer protein, alpha	Pitpna	1,74	1,40	0,00
1434343_at	RIKEN cDNA 5730403M16 gene	5730403M16Rik	1,70	1,38	3,75
1448129_at	actin related protein 2/3 complex, subunit 5	Arpc5	1,70	1,39	3,01
1423185_a_at	ubiquitin-associated protein 1	Ubap1	1,67	1,42	0,00
1419198_at	chromobox homolog 8 (Drosophila Pc class)	Cbx8	1,66	1,35	5,43
1458365_at	SCY1-like 1 binding protein 1	Scyl1bp1	1,57	1,29	5,43
1450893_a_at	ubiquitin-associated protein 1	Ubap1	1,55	1,29	3,01
1420056_s_at	phosphatidylserine receptor	Ptdsr	1,54	1,27	1,14
1454109_a_at	phosphatidylserine receptor	Ptdsr	1,52	1,27	3,75
1431299_a_at	RIKEN cDNA 2310014H01 gene	2310014H01Rik	1,51	1,27	3,01
AFFX-b-ActinMur/M1	actin, beta, cytoplasmic	Actb	1,31	1,19	3,01

Group 6 **Group 6:2 genes**

Probe.Set.ID	Gene.Title	Gene.Symbol	+CytoD	+CytoD+LatB	q-value(%)
1453851_a_at	growth arrest and DNA-damage-inducible 45 gamma	Gadd45g	1,83	0,63	3,01
1438658_a_at	endothelial differentiation, sphingolipid G-protein-coupled receptor, 3	Edg3	1,13	0,82	3,01