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Abstract

Eukaryotic gene transcription is tightly regulated, such that a specific transcriptional program is turned on only when required in an organ/tissue/cell specific manner. The set of genes that is turned on in response to an environmental cue is determined by the activation of a selective group of transcription factors. Once activated, transcription factors bind specifically to DNA response elements in the regulatory region of the target genes and thereby activate transcription. This activation is mediated by the recruitment of other proteins to the promoter, the so-called co-activators. These proteins are recruited as part of multi-protein complexes via a direct or indirect interaction with chromatin bound transcription factors, resulting in the activation of transcription.

An emerging class of proteins involved in the regulation of transcription are bridging factors. These proteins do not harbour any known co-activator function or domain, but are recruited to the promoter via a direct interaction with transcription factors, and mediate an increase in transcriptional activity, presumably through the recruitment of other co-activators. Therefore they can be considered as co-activators. Proteins belonging to the class of LIM-domain proteins can function as co-activators via this mechanism. Our group could show that the nuclear isoform of the LIM-domain protein Trip6, nTrip6, is an important regulator of the transcription factors AP-1 and NF-κB. nTrip6 functions as a novel transcriptional co-activator for both transcription factors. Moreover, nTrip6 is essential for the repression of AP-1 and NF-κB by another transcription factor, the glucocorticoid receptor (GR). GR represses the transcription of AP-1 and NF-κB target genes without binding to DNA, but by being tethered to the promoter bound by AP-1 or NF-κB. nTrip6 is essential for this so-called trans-repression, by mediating the tethering of GR to the promoter. This negative crosstalk is reciprocal: AP-1 and NF-κB can trans-repress GR by a tethering mechanism.

In this work I showed that nTrip6 act as a selective co-activator for a subset of AP-1 dimers. Moreover, the interaction of AP-1 dimers with nTrip6 determines the ability of GR to trans-repress these AP-1 dimers, since GR is tethered to the promoter via its interaction with nTrip6. Similarly, nTrip6 acts as a co-activator for GR, and mediates the tethering of AP-1 and NF-κB to promoter-bound GR, as a first step in the trans-repression of GR by AP-1 and NF-κB. The co-activator function of nTrip6 necessitates its dimerisation through two dimerisation domains in its N-terminus. This dimerisation enables the recruitment of other proteins, presumably co-activators, to the promoter. These properties of nTrip6 are most likely common to other LIM-domain proteins. The ability of transcription factors to interact with several LIM-domain proteins enables fine tuning of the transcriptional response, by the recruitment of different co-activator complexes, and by the possibility of repressing crosstalks with other transcription factors.

Abstract

Die transkriptionelle Koaktivatorfunktion des LIM-Domänenproteins nTrip6

Die Transkription eukaryotischer Gene ist streng reguliert. Ein spezifisches transkriptionelles Programm wird in einem Organ/Gewebe/Zell-spezifischen Kontext nur ausgeführt wenn es benötigt wird. Die Gene, welche als Antwort auf einen Umweltreiz angeschaltet werden, werden durch die Aktivierung einer selektiven Gruppe von Transkriptionsfaktoren aktiviert. Nach ihrer Aktivierung binden Transkriptionsfaktoren an spezielle DNA-Erkennungssequenzen in der regulativen Region ihrer Zielgene und leiten die Transkription dieser ein. Diese Aktivierung wird durch die Rekrutierung von anderen Proteinen, den so genannten Ko-Aktivatoren, an den Promotor vermittelt. Diese Proteine werden als Teil von Multi-Protein Komplexen mittels einer direkten oder indirekten Interaktion mit dem chromatin-gebundenen Transkriptionsfaktor rekrutiert, welches zur Aktivierung der Transkription führt.

Eine neu entstandene Klasse von Proteinen, welche in der Regulation der Transkription eine Rolle spielen, sind Brückenfaktoren. Diese Proteine enthalten keine bekannten Ko-Aktivator Funktionen oder Domänen, werden allerdings mittels einer direkten Interaktion mit den Transkriptionsfaktoren an den Promotor rekrutiert, und führen dabei zu einer Steigerung in der transkriptionellen Aktivität, vermutlich durch die Rekrutierung zusätzlicher Ko-Aktivatoren. Proteine, welche zur Klasse der LIM-Domänen Proteine gehören, können mittels dieses Mechanismus als Ko-Aktivatoren fungieren. Unsere Gruppe konnte zeigen, dass die nukleäre Isoform des LIM-Domänen Proteins Trip6, nTrip6, ein wichtiger Regulator der Transkriptionsfaktoren AP-1 und NF-κB ist. nTrip6 ist ein neuer transkriptioneller Ko-Aktivator für diese beiden Transkriptionsfaktoren. Des weiteren ist nTrip6 essentiell für die Repression von AP-1 und NF-κB durch einen weiteren Transkriptionsfaktor, den Glukokortikoidrezeptor (GR). Der GR reprimiert die Transkription von AP-1 und NF-κB Zielgene ohne direkte Bindung an DNA, sondern mittels direkter Anbindung an die Promotor-gebundenen Transkriptionsfaktoren. nTrip6 ist essentiell für diese so genannte „Trans-Repression“, in dem es die Anbindung des GR an den Promotor vermittelt. Dieser negative „Crosstalk“ ist reziprok: AP-1 und NF-κB können ebenfalls den GR mittels Anbindung trans-reprimieren.

In dieser Arbeit konnte ich zeigen, dass nTrip6 ein selektiver Ko-Aktivator für AP-1 ist. Des weiteren bestimmt die Interaktion der AP-1 Dimere mit nTrip6 die Fähigkeit des GR zur Trans-Repression, da der GR mittels Interaktion mit nTrip6 an den Promotor herangeführt wird. Ferner fungiert nTrip6 als Ko-Aktivator für den GR, und vermittelt die Rekrutierung von AP-1 und NF-κB an den Promotor-gebundenen GR, wobei es sich um den ersten Schritt in der „Trans-Repression“ von GR durch AP-1 und NF-κB handelt. Um als Ko-Aktivator fungieren zu können, muss nTrip6 mittels zweier Domänen in seinem N-Terminus dimerisieren. Diese Dimerisierung ermöglicht die Rekrutierung weiterer Proteine, vermutlich Ko-Aktivatoren, an den Promotor. Diese Eigenschaften von nTrip6 sind vermutlich auch bei anderen LIM-Domänen Proteine zu finden. Die Fähigkeit von Transkriptionsfaktoren mit verschiedenen LIM-Proteinen zu interagieren ermöglicht eine Feinabstimmung der transkriptionellen Regulation durch Rekrutierung verschiedener Ko-Aktivator-Komplexe oder durch den Crosstalk mit anderen Transkriptionsfaktoren.

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1. Introduction:

3. Results:

µ	micro
AF1	Activation function 1
AF2	Activation function 2
AP-1	Activator protein 1
APS	Amoniumpersulfate
AR	Androgen receptor
ATP	Adenosine tri-phosphate
BiFC	Bimolecular fluorescence complementation
BSA	Bovine serum albumine
CBP	CREB-binding protein
CFP	Cyan fluorescent protein
CMV	cytomegalovirus immediate early promoter
collI	collagenase I
CRE	CREB responsive element
CREB	cAMP response element–binding protein
DBD	DNA binding domain
DCS	Donor calf serum
DD1	Dimerisation Domain 1
DD2	Dimerisation Domain 2
ddNTP	di-Desoxynucleotide-tri-phosphate
Dex	Dexamethasone
DMSO	Dimethyl sulfoxide
D-MEM	Dulbeco-modified Eagly medium
DNA	Desoxyribonucleic acid
DNA-FiSH	DNA fluorescent in situ hybridisation
DRIP	vitamin D receptor-interacting protein
DTT	Dithiothreitol
E.coli	Escherichia coli
Erk	extracellular signal-regulated kinase
EtOH	Ethanol
FCS	Fetal calf serum
FHL2	four -and-a-half-LIM only protein 2
g	g-force
GC	Glucocorticoids
GFP	Green fluorescent protein
GLyGly	Glycylglycin
GR	Glucocorticoid receptor
GRIP1	Glucocorticoid receptor interacting protein 1
GST	Glutathion-S-Transferase
HA	Hemagglutinin

HAT	Histone acetyltransferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hic-5/ ARA55	Androgen receptor activator
HLH	Helix-loop-Helix
HRP	Horse radish peroxidase
HTH	Helix-turn-Helix
IFN-γ	Interferone gamma
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JNK	Jun-N-terminal kinase
kDa	kilo Dalton
LB	Luria Broth
LBD	Ligand binding domain
LHX	LIM homeodomain-protein
LIM-D1	LIM-domain protein 1
LIMK	LIM-kinases
LMO	LIM-domain only protein
LPP	Lipoma preferred partner
Luc	Luciferase
M	Molar
MALDI-TOF	Matrix-assisted laser desorption/ionization – Time of flight
MKP-1	Mitogen-activated protein kinase phosphatase
mM	milli-Molar
MMTV	Mouse mammary tumor virus
mRNA	messanger RNA
MyoD	Myogenic determination gene
NcoA-1	Nuclear receptor co-activator
NES	Nuclear export signal
NF-κB	Nuclear factor κB
nGRE	negative GRE
NLS	Nuclear localisation signal
NP-40	Nonidet P-40
Oct	Octamer-motif-binding factor
OD	Optical density
P/CAF	p300/CBP-associated factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl-Fluorid
PVA	Poly vinyl alcohol
RNA	Ribonucleic acid

RT-PCR	real time PCR
SDS	Sodium dodecylsulfate
siRNA	Short interference RNA
SOC	Super Optimal broth with Catabolite repression
SRC	Steroid receptor co-activator
SWI/SNF	SWItch/Sucrose NonFermentable
TBP	TATA-box binding protein
TBS	Tris buffered saline
TCF	T-cell factor
TEMED	N,N,N´,N´-tetramethylenethylendiamine
TIF2	Transcriptional mediators/intermediary factor 2
TPA	Phorbol ester 12-O-tetradecanoate-13-acetate
TR	Thyroid receptor
TRAP	Thyroid receptor-associated protein
TRE	TPA responsive element
Trip6	Thyroid hormone receptor interacting protein 6
Tris	Tris(hydroxymethyl)-aminomethane
uPA	urokinase plasminogen activator
UV	Ultra Violet light
VC	C-terminal part of Venus
VN	N-terminal part of Venus
YC	C-terminal part of YFP
YFP	Yellow Fluorescent Protein
YN	N-terminal part of YFP

1. Introduction

Eukaryotic gene transcription is tightly regulated, such that a specific transcriptional program is turned on only when required in an organ/tissue/cell specific manner. The set of genes that is turned on in response to an environmental cue is determined by the activation of a selective group of transcription factors. Depending on the transcription factor, several mechanisms can be used for their activation, ranging from transcriptional induction, to posttranslational modifications or translocation of the cytosolic transcription factor to the nucleus. Once activated, transcription factors bind, via a DNA binding domain (DBD), specifically to DNA response elements in the regulatory region of the target genes and thereby activate transcription via a trans-activation domain.

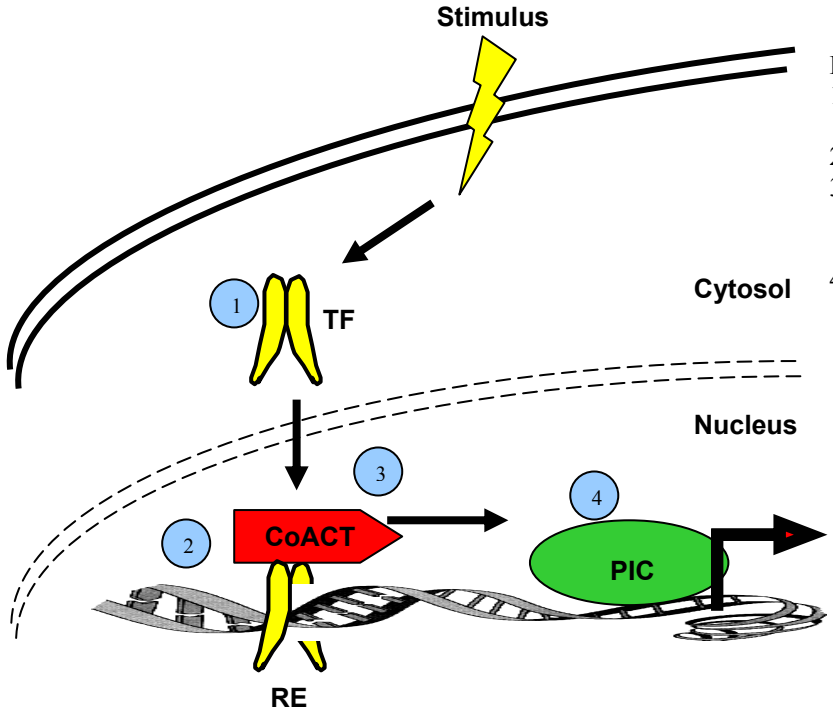

Fig 1. Signal transduction
1) Activation of the transcription factor(s)
2) DNA-binding
3) Recruitment of co-activators/co-activator complexes
4) Pre-initiation complex assembly and activation of transcription

This activation is mediated by the recruitment of other proteins to the promoter, the so-called co-activators. These proteins are recruited as part of multi-protein complexes via a direct or indirect interaction with chromatin bound transcription factors, resulting in the activation of transcription. Co-activators can be grouped into different classes depending on their functions and properties (Marmorstein and Trievel, 2008; McKenna and O'Malley, 2002; Naar et al., 2001; O'Malley et al., 2008; Peterson and Laniel, 2004; Smith and Denu, 2008).

1) Histone tail modifiers:

   Post-translational modifications of histones, like acetylation, methylation, phosphorylation, ubiquitination or/and sumoylation is an essential co-activation mechanism. The best-characterized modification is acetylation, catalyzed by histone acetyltransferases (HATs) like CREB binding protein (CBP) or p300. CBP/p300 provides a bridge directly interacting both with a wide variety of transcription factors,

such as C/EBPb, Ets, AP-1, NF-AT and GATA family members, and with components of the basal transcriptional machinery, including TBP, TFIIB, TFIIE and TFIIF. (Liu et al., 2004). Another class of proteins with an intrinsic HAT function are members of the p160/SRC/NCoA co-activator family: NCoA-1 (Nuclear receptor co-activator 1), also called SRC-1, NCoA-2 also called SRC-2, TIF2 or GRIP1 and NCoA-3, also called p/CIP, RAC3, ACTR, AIB1 or TRAM-1. These proteins were first identified as co-activators for nuclear receptors, but they are also capable of influencing the transcriptional regulation of other transcription factors, like STAT and NF-κB. The function of NCoA proteins is the recruitment of other co-activators, which harbour histone acetyl-transferase activity (HAT), like p300, CBP and p/CAF and co-factors with histone methyl-transferase activity (HMT), like CARM1 and PRMT1. In addition, NCoA-1 and NCoA-3 posses a weak intrinsic HAT activity at the C-terminus (Lodrini et al., 2008).

Histone methylation can be grouped into two families, lysine methylation and arginine methylation, which are catalyzed by lysine methyltransferases (HKMTs) and protein arginine methyltransferases (PRMTs), respectively. Nearly all HKMTs have the evolutionally conserved SET (SU(VAR)3-9, enhancer-of-Zeste, Trihorax) domain that is responsible for lysine methylation activity. Members of the protein arginine methyl transferase (PRMT) catalyzes the generation of di-methyl arginine residues on substrate proteins using S-adenosyl methionine (SAM) as the methyl donor. As an example, CARM1 methylates histone H3 and p300/CBP, thereby altering chromatin architecture and impacting transcriptional initiation. (Kleinschmidt et al., 2008; Purandare et al., 2008). The histone tail modifiers change the global charge of the histones resulting in a change of chromatin structure. Furthermore, these modifications also serve as docking sites for other proteins, e.g. other co-activators like chromatin remodellers.

2) Chromatin-remodellers:

The human SWI/SNF complex is a large multi-subunit complex of approximately 2MDa in size. The exact subunit composition of the SWI/SNF complex varies, although it contains at least nine or more subunits in humans. In humans there are two major subfamilies of the SWI/SNF complex: BAF (BRG1 or BRM-associated factor) and PBAF (polybromo-associated factor). These two groups are similar in their composition, sharing eight subunits (BRM or BRG1, BAF170, BAF155, BAF60a, BAF57, BAF53, actin, and BAF47). However, BAF contains BAF250, while PBAF contains BAF180 (also known as polybromo) and BAF200. All SWI/SNF complexes contain an ATPase subunit, which is either BRM (brahma) or BRG1 (brahma-related gene 1). These ATPase subunits are mutually exclusive. The ATPase subunit of SWI/SNF contains a bromodomain. A bromodomain is a protein domain that recognizes acetylated lysine residues such as those on the N-terminal tails of histones.

This recognition is often a prerequisite for protein-histone association and chromatin remodelling (Halliday et al., 2008). Via chromodomains these complexes are able to identify methylated histone tail. After binding to their specific docking site chromatin remodellers act by sliding the nucleosomes positioning relative to the DNA. Through this sliding the complex enables other proteins, e.g. co-activators, to be recruited to the promoter and to initiate transcription.

3) Mediator complex:

The human Mediator complex is a multi-subunit co-activator that regulates RNA polymerase II-dependent transcription (Conaway et al., 2005a; Conaway et al., 2005b). The mediator complex plays multiple roles in regulating the pre-initiation complex (PIC), which minimally consists of TFIIA, IIB, IID, IIE, IIF, IIH, and RNA polymerase II (Pol II). The Mediator complex interacts directly with Pol II and is involved in the recruitment of this complex to gene promoters. It also stimulates the TFIIH-dependent phosphorylation of the Pol II C-terminal domain, which is critical for transcription initiation. Furthermore, the mediator complex is a general target of DNA-binding transcription factors. Thus, the mediator complex is believed to function in part by communicating regulatory signals from promoter and enhancer-bound transcription factors directly to the PIC. Furthermore, this co-factor is able to interact and recruit other co-activators required for initiating transcription, like proteins harbouring a HAT-domain. One simple way in which Mediator activity is controlled is through subunit exchange: addition or subtraction of subunits to alter its biochemical function. For instance, a cdk8 sub-complex (consisting of cdk8, cyclin C, Med12 and Med13) can reversibly associate with the 'core' Mediator to inhibit its co-activator function. (Meyer et al., 2008).

Another class of proteins involved in the regulation and activation of transcription are bridging factors. These proteins do not harbour any known co-activator function like HAT or HMT, but are recruited to the promoter via a direct interaction with transcription factors, and mediate an increase in transcriptional activity, presumably through the recruitment of other co-activators. Therefore they can be considered as co-activators. Proteins belonging to the class of LIM-domain proteins can function as co-activators via this mechanism.

1.1. The family of LIM-domain proteins

The LIM-domain was first identified and described in three homeo-domain proteins, Lin1-l, Isl-1 and Mec-3. LIM-domains are cystein-and histidin rich regions. Each LIM-domain consists of 2 zinc-fingers, which harbour Zn^{2+}-ions, resulting in the specific structure of the zinc-finger (Dawid et al., 1998).

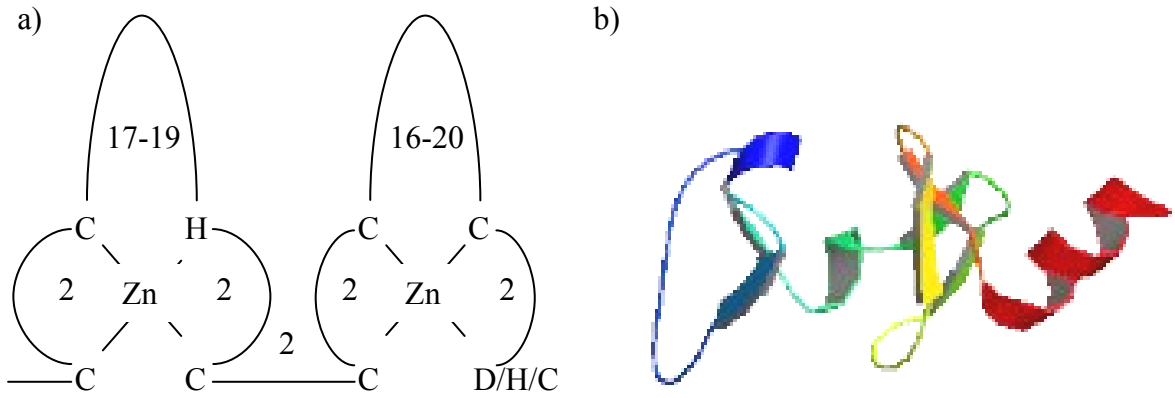

Fig 2. Structure of the LIM-domains
a) double zinc-finger motif of a LIM-domain
b) 3D-reconstruction of a crystallised LIM-domain

Based on sequence homology studies LIM-domain proteins were separated into three different classes:

Class I LIM-domain proteins contains LHX (LIM homeodomain)-proteins, LMO´s (LIM only proteins) and LIM-kinases (LIMK). Their LIM domains appear in tandem repeats and these proteins are localised mainly within the nucleus. Their function is diverse; LHX-proteins function as transcription factors by interacting with DNA directly through their homeo-domain, whereas LMO-proteins act as transcriptional co-activators. LIM-kinases are found in the cytosol as well as in the nucleus, where they are involved in actin polymerisation and are part of the Ras signalling pathway.

Class II LIM-domain containing proteins show a high sequence similarity. They are mainly composed of LIM-domains. Members of this class are CRIP and CRP. They are localised in the cytosol and function as bridging factors to associate proteins into signalling complexes. Interactions between CRP and Zyxin, a member of class III, are reported (Schmeichel and Beckerle, 1997, 1998).

Class III forms a very heterogeneous group of proteins mainly localised in the cytosol. Typical members of this class are proteins belonging to the Zyxin- and Paxillin-families. Members of the Zyxin-family, comprising Ajuba, LIM-D1, LPP and Trip6, are associated to focal adhesion sites. They are found in multi-protein complexes which are associated to cell matrix attachments or cell-cell contacts. They are involved in signal transduction pathways by activating signalling proteins (Turner, 2000), or by functioning as bridging factors for proteins involved in signalling (Matsuya et al., 1998). Moreover, members of this family have been reported to act as transcriptional co-activators for distinct classes of transcription factors. One of the best studied class III LIM domain protein involved in transcription regulation is the androgen receptor (AR) co-activator 55 (ARA55)/Hic-5 (Guerrero-Santoro et al., 2004). Other family members like Paxillin are involved in trans-activation of androgen receptor as well (Kasai et al., 2003). Lipoma preferred binding partner, LPP, functions as a co-activator for the ETS domain transcription factor PEA3 (Guo et al., 2006). Another class III LIM-

domain protein, LIM-D1, functions as an AP-1 co-activator (Feng et al., 2007). Finally, nTrip6 the nuclear isoform of Trip6, acts as a co-activator for AP-1 and NF-κB (Kassel et al, 2004).

1.1.1. The LIM-domain protein Trip6

Trip6 was first described and characterised in 1995 as thyroid hormone receptor interacting protein 6 (Trip6) (Lee et al., 1995), and its function and role was not identified at that time. The protein Trip6 comprises 476 amino acids encoded within 9 exons (Fig. 3).

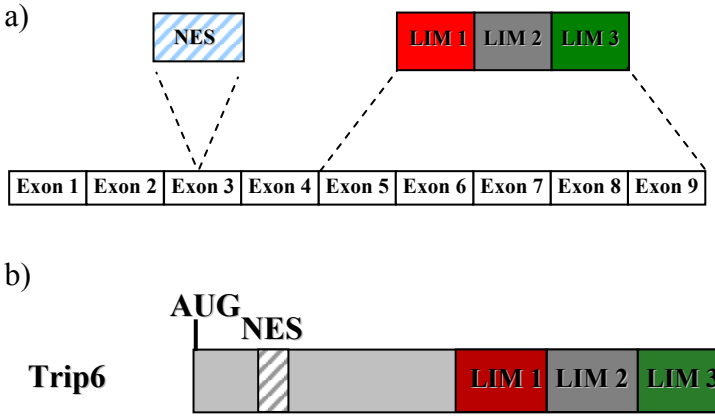

Fig.3: Structure of Trip6
a) Trip6 is encoded within 9 exons; The nuclear export signal (NES) is encoded within Exon 3, Exons 5-9 encode the 3 LIM domains
b) Schematic drawing of the Trip6 protein

Trip6 is mainly localised in the cytosol, where it co-localises with the actin filament (Cuppen et al., 2000) and is enriched at focal adhesions and at cell-cell contact sites, where it influences cell adhesion and migration (Bai et al., 2007; Lai et al., 2005; Lai et al., 2007; Xu et al., 2004; Yi et al., 2002). The three C-terminal LIM-domains of Trip6 are protein-protein interaction surfaces and not used to bind to DNA. They are highly conserved among the family members and all members of the Zyxin family contain three LIM-domains at the C-terminus (Wang and Gilmore, 2001).

Trip6 localisation in the cytosol was attributed to the presence of a functional nuclear export signal (NES) within its N-terminus, encoded by amino acids 100 to 107 (Wang and Gilmore, 2001). When this part of the protein was deleted, Trip6 accumulated within the nucleus (Wang and Gilmore, 2001). The NES, which is conserved among the different family members (Wang and Gilmore, 2001), is encoded within exon 3 and it is a leucine rich region (^{100}LSSTLAEL^{107}). Leucine-rich areas are known interaction surfaces for Crm-1. This protein is responsible for the nuclear export of proteins containing a NES-sequence. When Crm-1 is inhibited by Leptomycin B, Trip6 accumulates in the nucleus (Wang and Gilmore, 2001).

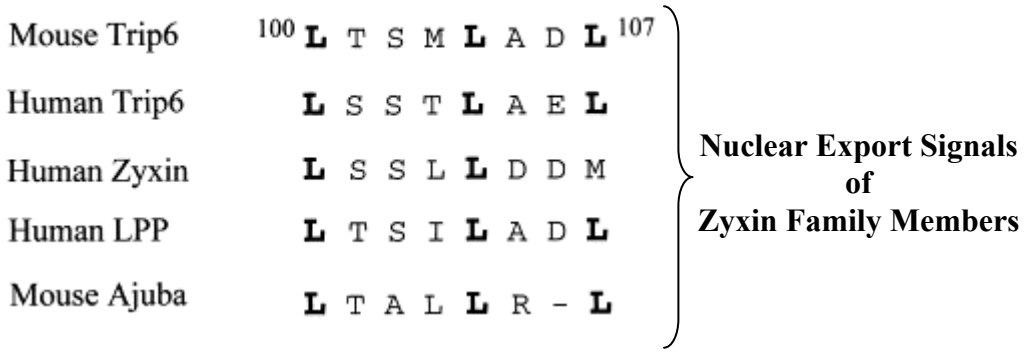

Fig.4: The NES is highly conserved among the Zyxin family members
from Wang et al., 2000

A possible nuclear function of Trip6 has been attributed to its putative capacity to translocate to the nucleus (Wang and Gilmore, 2001). However, this hypothesis has been dismissed, and the nuclear function of Trip6 was attributed to a shorter, exclusively nuclear isoform of Trip6, nTrip6 (Kassel et al., 2004).

1.1.2. The nuclear isoform of the LIM-domain protein Trip6, nTrip6

Our group identified a nuclear isoform of Trip6, nTrip6. This protein is shorter in size, lacks the NES, and is therefore exclusively present in the nucleus, and contains all three LIM-domains (Kassel et al., 2004) (Fig. 5).

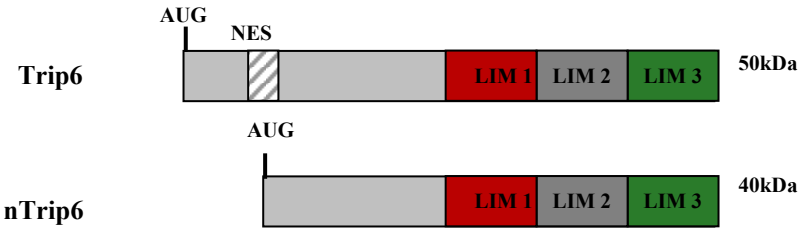

Fig.5: Schematic comparison of the different Trip6 isoforms

nTrip6 is generated via alternative translation initiation at an alternative AUG start codon, located in exon 3, directly within the NES (Winter, 2007). Translation from this secondary AUG results in a truncated and non-functional NES, and nTrip6 is thus localized in the nucleus.

1.1.3. nTrip6 functions as a co-activator for NF-kB RelA/p65 and for AP-1 c-Jun:c-Fos

Our group could show that nTrip6 functions as a novel transcriptional co-activator for the transcription factors AP-1 and NF-κB (Kassel et al., 2004). Recruited to AP-1 or NF-κB

bound to target promoters, nTrip6 acts as or binds additional co-activator(s). The interaction between the transcription factors and nTrip6 is mediated via its LIM-domains. Through a direct protein-protein interaction with LIM-domain 1 p65 as well as Fos are interacting with nTrip6 resulting in its recruitment to the promoter. Reducing the level of nTrip6 by siRNA decreased and over-expression increased the trans-activation by both AP-1 and NF-κB, suggesting that endogenous levels of nTrip6 can be limiting for AP-1 and NF-κB activity.

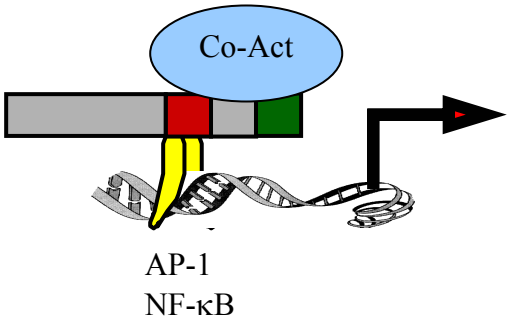

Fig.6: nTrip6 functions as a co-activator for AP-1 and NF-κB

nTrip6 is not only functioning as a co-activator for AP-1 and NF-κB, it is also mediating the repression of both transcription factor families by the glucocorticoid receptor (GR) to the (Kassel et al., 2004). We do not yet know the detailed mechanism of transcriptional regulation by nTrip6. Apart from the three LIM domains in the C-terminal half, nTrip6 carries no other known functional domain, motif, or catalytic activity that could mediate its co-activator function. We favour, therefore, the hypothesis that nTrip6 recruits additional co-activators by protein–protein interaction via its LIM-domains.

1.2.The transcription factor family AP-1

The Activator Protein-1 (AP-1) family of transcription factors was one of the first identified mammalian transcription factors. AP-1 consists of homo-and hetero-dimers, composed of the Jun (c-Jun, JunB, JunD), Fos (c-Fos, FosB, Fra1, Fra2) and CREB/ATF2 (ATF2, ATF3) subfamilies (Angel and Karin, 1991). AP-1 dimers bind to the consensus TPA responsive element (TRE) or to the related CREB responsive element (CRE). The AP-1 transcription factors belong to the class of basic zipper (bZip) sequence specific DNA-binding proteins. Via their leucine zipper motif the homo- or heterodimers are formed and bind DNA by their basic motif. The formation of each dimer depends on their relative dimerisation affinities, and on the abundance of each of the Jun, Fos and ATF proteins available in the cell at a given time. Fos members of the family cannot form stable homodimers but heterodimerise with Jun members, e.g. c-Jun:c-Fos. ATF family members can dimerise with c-Jun as well as with ATF/CREB family members (Benbrook and Jones, 1994), (Chatton et al., 1994), (Hai and Curran, 1991).

The different AP-1 hetero-dimers have different affinities to responsive elements. Jun hetero-dimerising with Fos recognises a heptameric responsive element, the phorbol ester 12-O-tetradecanoate-13-acetate (TPA) response element, TRE (TGAGTCA), which is for example present in the promoter of the collagenase I (MMPI) gene, whereas this heterodimer only weakly binds to the octameric cyclic-AMP response element, CRE (TTACCTCA), present in the *c-jun* promoter. In contrast, the other prototypical AP-1 heterodimer, c-Jun:ATF2, has a weak affinity towards the responsive elements recognised by c-Jun:c-Fos, but has a very high affinity to the octameric CRE (Benbrook and Jones, 1990; Hai and Curran, 1991). These binding sites have been identified in the regulatory regions of a wide range of genes, including transcription factors such as c-Jun and ATF3, matrix-degrading enzymes like collagenase I and urokinase plasminogen activator (uPA), cytokines like IFN-γ or adhesion molecules such as E-selectin (Angel et al., 1988a; Angel et al., 1988b; Angel et al., 1987; Brostjan et al., 1997; Cippitelli et al., 1995; De Cesare et al., 1995; Liang et al., 1996).
The ability of the AP-1 transcription factor to control many different biological processes stems primarily from its structural and regulatory complexity. For example, studies on combinatorial variants of AP-1 complexes, using Jun mutants that preferentially heterodimerise with either c-Fos or ATF2, revealed opposing roles of c-Jun:c-Fos and c-Jun:ATF2 dimers in transformation (van Dam et al., 1998). Jun-dependent cell transformation can be resolved into at least two distinct and independent processes: anchorage independence triggered by c-Jun:c-Fos and growth factor independence triggered by c-Jun:ATF2. These differences in the roles of c-Jun:c-Fos and c-Jun:ATF make these dimers particularly interesting to study.

1.2.1. The regulation of c-Jun:c-Fos vs. c-Jun:ATF2

The expression of AP-1 target genes is primarily regulated by the relative expression of the different dimerisation partners at a given time point. Additionally, the activation status of the different dimers determines the program of target genes expressed. Each AP-1 subunit is downstream of one or several signalling pathways. For instance, c-Jun:c-Fos dimers are mainly activated by the Ras-Raf-MEK-Erk MAP-kinase pathway, whereas c-Jun as well as ATF2 are activated by the stress activated MAP-kinases JNK and p38 (Karin, 1996; Whitmarsh and Davis, 1996).

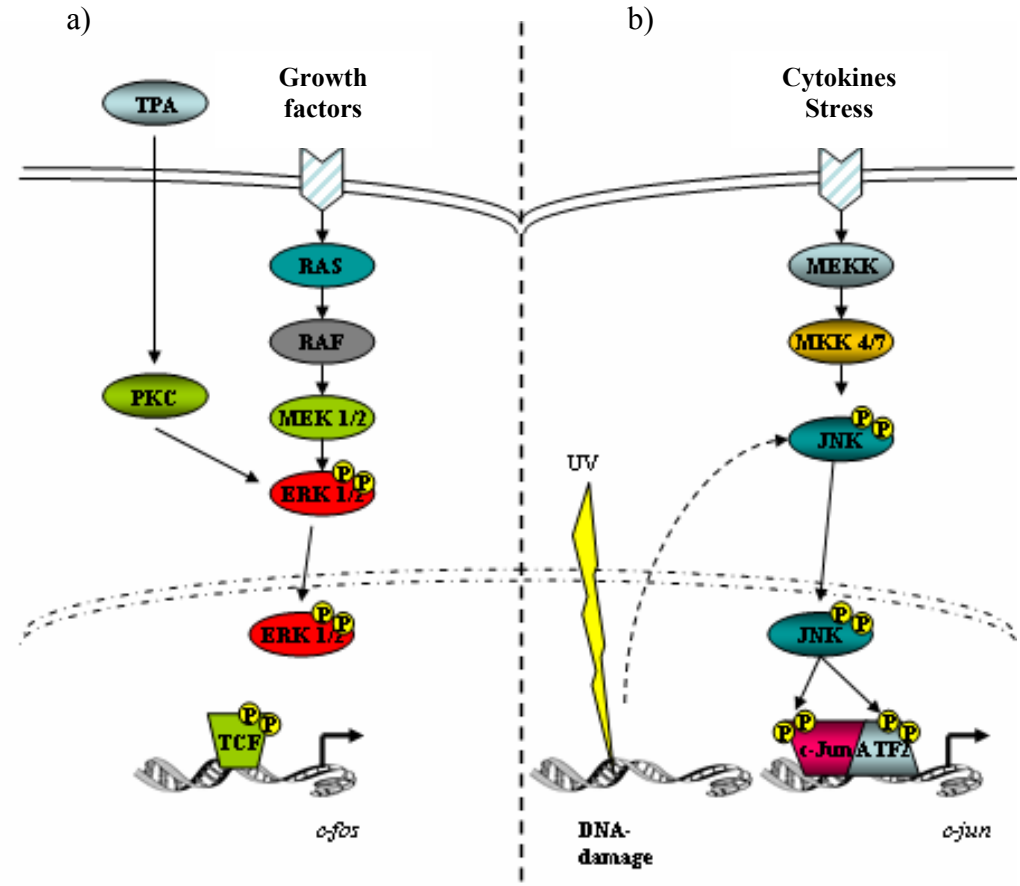

Fig.7. Different signalling pathways lead to the activation of different AP-1 dimers

a) The induction of *c-fos* expression is mediated through the activation of the ERK signalling pathway. Growth factors do stimulate the activation of Ras. Subsequently, the serine/threonine protein kinase Raf-1 binds to Ras and becomes activated, leading to phosphorylation and activation of the dualspecificity ERK-kinase, MEK. MEK activates the MAPKs ERK1 and ERK2, which translocate into the nucleus upon activation. In the nucleus, ERKs phosphorylate the transcription factor TCF, which is bound to the serum response element (SRE) of the *c-fos* promoter. Phosphorylation of TCF stimulates its trans-activation function which leads to rapid activation of *c-fos* transcription. An alternative way to activate *c-fos* expression is through TPA. TPA activates PKC which, through a crosstalk with ERK, stimulates the phosphorylation of TCF which regulates the expression of *c-fos*. (Adapted from Edmunds and Mahadevan, 2004).

b) Regulation of c-Jun and ATF2 through activation of JNK signalling pathway. Cytokines or stress stimulate the activation of MEKK1 resulting in the activation of MKK4/7. Both protein kinases are able to activate JNK by dual phosphorylation. Activated JNK can phosphorylate AP-1 family members like c-Jun and ATF-2. The phosphorylation of AP-1 stimulates its trans-activation function resulting in activation of the transcription, like *c-jun*. UV–induced DNA damage mediates the activation of JNK and subsequent phosphorylation of AP-1. (Adapted from Edmunds and Mahadevan, 2004).

Thus, different AP-1 dimers are activated by different signalling pathways, and activate different target genes. However, little is known about their specific regulation when bound to their target promoters. For example, several AP-1 co-activators are known (Bannister et al., 1995; Halliday et al., 2008; Naar et al., 2001), but whether there are co-activators specific for certain AP-1 dimers is not known. Our group showed that nTrip6 acts as an AP-1 co-activator (see above). Given that nTrip6 interacts with c-Fos and is recruited in a c-Fos dependent manner to TRE-containing target genes (Kassel et al., 2004), one could raise the question of the specificity of nTrip6 co-activator function for different AP-1 dimers.

1.2.2. Repression of AP-1 by the Glucocorticoid receptor

Little is known about the mechanisms negatively regulating the response to specific AP-1 dimers. A physiologically and therapeutically relevant negative control of the AP-1 response is exerted by Glucocorticoids (GCs). However, little is known about the dimer specificity of this repression. GCs act through the glucocorticoid receptor (GR), which regulates transcription by several mechanisms (Kassel and Herrlich, 2007). GR is a ligand-activated transcription factor and induces the expression of numerous target genes (Schoneveld et al., 2004). Liganded GR also interferes with several signal transduction pathways, including the Erk (Kassel et al., 2001), JNK (Caelles et al., 1997) and p38 pathways (Imasato et al., 2002). Finally, GR directly represses the activity of several other transcription factors, including AP-1 (Jonat et al., 1990; Schule et al., 1990; Yang-Yen et al., 1990), at the promoter of their target genes. This process is referred to as trans-repression (Kassel and Herrlich, 2007). Both AP-1 transcriptional activity at promoters and its trans-repression by GR requires the presence of the bridging factor nTrip6 (Kassel et al., 2004). Which of these different mechanisms are relevant for GR-mediated repression of the different AP-1 dimers remains unknown.

1.3. Glucocorticoids

The glucocorticoids as well as the mineralo-corticoids and the sex specific hormones estrogen, progesterone and testosterone belong to the group of steroid hormones. These molecules have a lipid-like structure, and the cholesterol is the common subunit. Cortisol (Fig. 8), the natural glucocorticoid in human is synthesised in the adrenal cortex and its secretion is regulated by the hypothalamic-hypophysis system. The secretion is circadialy regulated, but it can also be released upon stress.

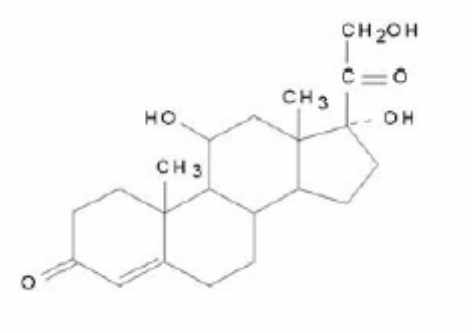

Fig.8: The glucocorticoid cortisol

The major impact of glucocorticoids on the organism is on glucose and protein-synthesis (Kasambalides and Lanks, 1983, 1985; Norton and Munck, 1980). In glucose-synthesis, corticoids lead to the production of glucose out of amino-acids within the liver (gluco-neogenesis), but at the same time, they hamper the peripheral sugar degradation, resulting in

an increase of blood-sugar concentration. The protein biosynthesis is hampered by corticoids. They interfere with the protein biosynthesis in muscles, bones and lymphatic organs.
Because of the interference with the protein-biosynthesis within the lymphatic system, Glucocorticoids block antibody production. They therefore have an "immuno-suppressive effect". This is used during organ transplantations to suppress the acceptors immune-system to tolerate the new organ and avoid rejection. Furthermore, a slowed immune response of the lymphocytes has an anti-inflammatory impact. This should hamper an overreaction by the organism towards an infection or inflammation.
Because glucocorticoids are able to interfere with the immune system, they are widely used as anti-inflammatory and immuno-suppressive therapeutics. They are used during acute allergic reactions, asthma, rheumatic arthritis and chronical inflammations. But there are side effects as well, especially during long term exposure to corticoids: diabetes, osteoporosis, adrenal-cortical obesity, and many more (Barnes, 1998; Barnes and Karin, 1997; Cato and Wade, 1996).

1.3.1. The Glucocorticoid receptor (GR)

Steroid hormones have a lipophilic character and are therefore able to diffuse passively through the cellular membrane. Within the cytosol, they bind to a specific steroid hormone receptor belonging to the superfamily of nuclear receptors. These receptors including the glucocorticoid receptor (GR) are ligand dependent transcription factors (Beato, 1989; Evans, 1988; Green and Chambon, 1988; Green et al., 1988; Webster et al., 1988). All family members share a common structure, illustrated for the human glucocorticoid receptor (Fig. 9).

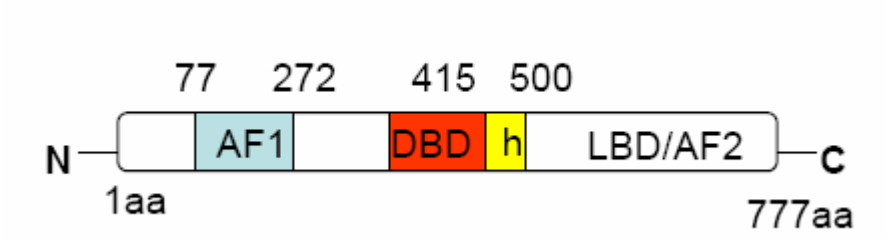

Fig.9: Schematic drawing of the domain structure of steroid receptor explained by the glucocorticoid receptor

N = amino-terminus; AF1 = activator function 1; DBD = DNA binding domain; h = hinge region; LBD/AF2 = ligand binding domain & activator domain 2; C = carboxy terminus; numbers indicate amino acid positions

The N-terminus harbours the activation function 1 (AF1). The central DNA-binding domain (DBD) is highly conserved among the steroid receptors. It is composed of two zinc finger motifs. Both contain a Zn^{2+}-ion linked to the cysteins necessary for the proper formation of the zinc finger (Freedman et al., 1988). Each zinc finger has a specific function: the first is responsible for binding to DNA, to the glucocorticoid response element (GRE), whereas the second zinc finger mediates the homo-dimer formation of the GR (Umesono and Evans,

1989). The hinge region (h) connects the DNA-binding domain with the ligand binding domain (LBD). The hinge region is essential for the translocation of the receptor upon ligand binding into the nucleus (Picard and Yamamoto, 1987). The ligand binding domain is located at the C-terminus of the protein and forms a "binding-pocket" comprised of 12 amphipatical α-helices and one β-sheet. Upon ligand binding to the "binding pocket", the pocket is closed by reorientation of α-helices 10, 11 and 12, resulting in a conformational change leading to its activation. Due to this conformational change the GR is capable of interacting with co-activator complexes. At the c-terminal end the second activation domain, AF2, as well as the trans-activation domain τ2, are located.

In the absence of hormone, GR resides in the cytoplasm as a multiprotein complex composed of chaperone proteins hsp90 and hsp70, immunophilin p59, and phosphoprotein p23 (Pratt and Toft, 1997). Glucocorticoids diffuse readily across cell membranes and bind to GR in the cytoplasm. Binding of hormone to the GR induces the release of hsp90, resulting in a conformational change that unmasks the nuclear localization signal. The receptor then translocates to the nucleus, where it can act through several modes of action. The glucocorticoid receptor regulates the transcription of genes by three basic modes of action: (*1*) binding to glucocorticoid response elements (GREs) in target genes to activate gene transcription, (*2*) inhibition of target gene transcription through direct DNA binding at negative GRE (nGREs), and (*3*) gene regulation by physical interaction with other transcription factors (Necela and Cidlowski, 2004). This crosstalk can be either positive, enhancing the transcription, or negative, repressing the transcription, depending on the transcription factor. The negative crosstalk is referred to as trans-repression.

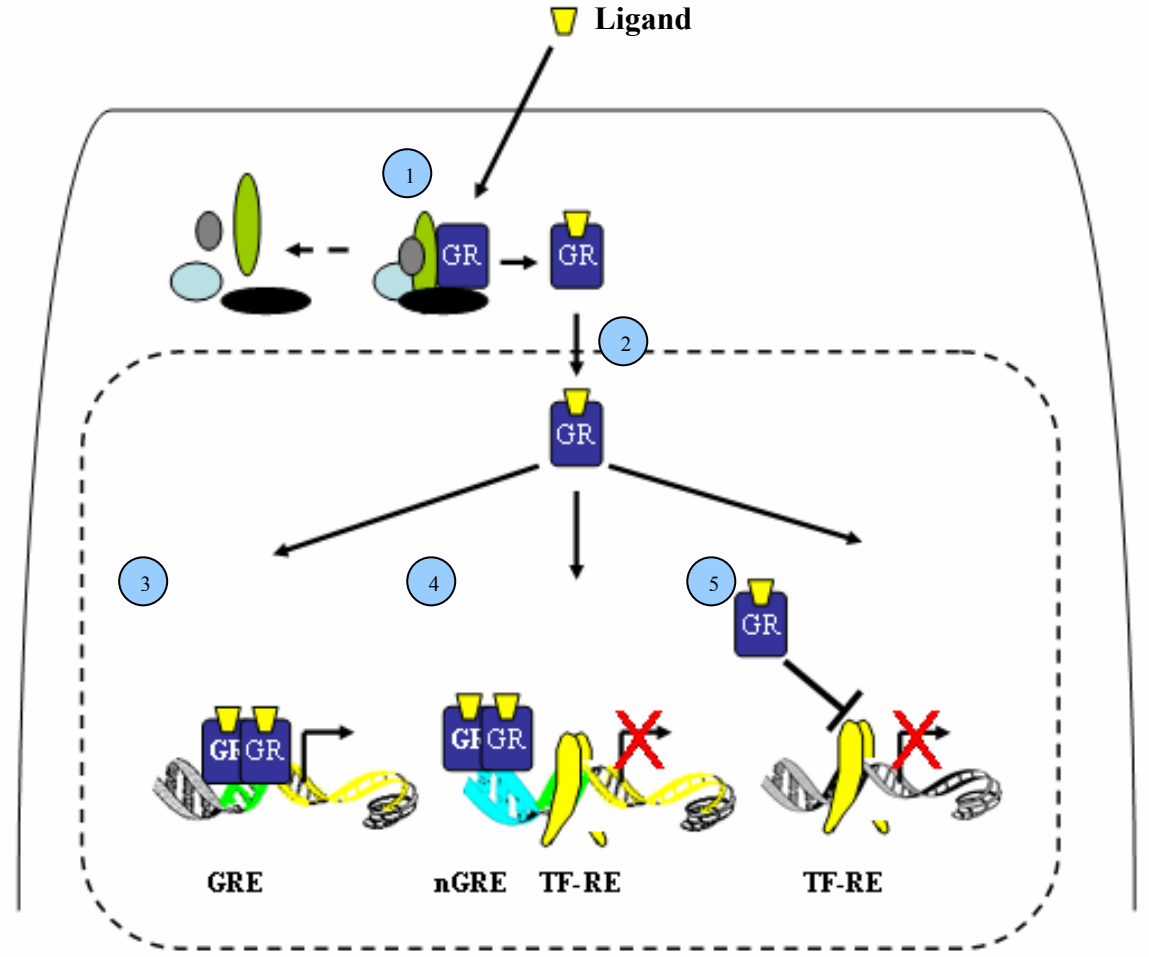

Fig.10: Different modes of glucocorticoid action
1) the glucocorticoid receptor is retained in the cytosol by interaction with chaperones and kept in an inactive state
2) upon ligand binding the receptor undergoes a conformational change and is released from the chaperone complex and translocated into the nucleus where it can function differentially
3) GR functions as a transcription factor and binds to its responsive elements of its target genes (e.g. iκB-α)
4) through binding to nGREs the GR is capable of competing with other TFs for binding to their responsive elements resulting in trans-repression
5) the GR is repressing the transcriptional activity of other TFs upon tethering to their promoter

1.3.2. DNA dependent mechanisms-"trans-activation"

The glucocorticoid receptor functions as a transcription factor and is able to bind directly DNA through its zinc-finger motif within the DBD. After activation by ligand-binding, it translocates into the nucleus and forms homo-dimers. Upon dimer formation it binds to glucocorticoid response elements (GRE) within the 5´ upstream region if its target genes and initiates the transcription (Fig. 10. 3)). GREs are composed of a palindromic sequence (consensus: AGAACANNNTGTTC; (Beato, 1989)). GR target genes are, for example, the tyrosin-aminotransferase, which is involved in gluco-neogenesis, MKP-1 (Kassel et al., 2001; Lasa et al., 2002), or osteocalcin (Morrison and Eisman, 1993).

However, the dimer can also bind to negatively regulating nGREs in close proximity to the response elements of other transcription factors (Fig. 10. 4)), resulting in a block of transcription (Dostert and Heinzel, 2004). This mechanism of action was proposed to account for repression of the proopiomelanocortin (Drouin et al., 1993) and prolactin (Sakai et al., 1988) genes.

1.3.3. DNA-independent mechanisms-"trans-repression"

The immunosuppressive function of glucocorticoids is achieved by repression of the expression of pro-inflammatory genes, encoding for instance cytokines, chemokines or cell adhesion proteins. Transcription-factors regulating these genes are for example the transcription factors of the AP-1 and NF-κB families. No nGRE could be detected in the majority of inflammatory genes whose transcription is repressed by GR and transcriptional interference was discovered to mostly result from protein-protein interactions between the GR and other transcription factors such as AP-1 (Jonat et al., 1990);(Schule et al., 1990; Yang-Yen et al., 1990), CREB/ATF (Akerblom et al., 1988; Cippitelli et al., 1995), NF-κB (Brostjan et al., 1997; McKay and Cidlowski, 1999; Reichardt et al., 2001). In contrast to the trans-activation function of GR, where GR forms homo-dimers, only monomers are required for trans-repression (Heck et al., 1994) (Fig. 10. 5)).
The first step in trans-repression is the tethering of activated GR to the promoter-bound transcription factors. Our group could show the involvement of nTrip6 in the trans-repression of AP-1 and NF-κB by GR (Kassel et al., 2004). The glucocorticoid receptor interacts with nTrip6 via its LIM-domains. Given that nTrip6 is recruited as a co-activator to AP-1 and NF-κB bound promoters, its ability to interact with GR mediates the tethering of GR to the promoter, and is thus essential for trans-repression (Kassel et al., 2004). Thus, nTrip6 has a dual function in the regulation of AP-1 and NF-κB. On the one hand it is an essential co-activator for these transcription factors, on the other hand it mediates their repression by GR (Fig. 11).

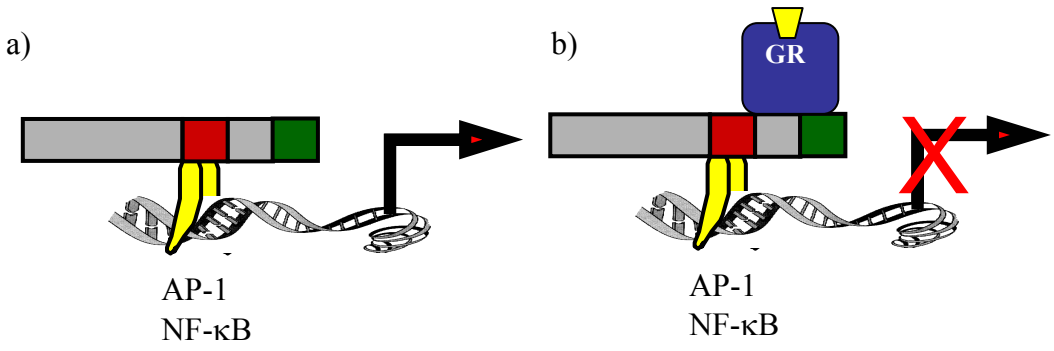

Fig.11: The dual function of nTrip6:
a) involvement in the positive transcriptional regulation as a co-activator for AP-1
b) mediating the repression of AP-1 by tethering the GR to promoter bound AP-1

1.3.4. Reverse crosstalk/ repression of GR by other transcription factors

Trans-repression is very often reciprocal. Transcription factors trans-repressed by GR are often able to trans-repress GR as well. Therefore, GR-mediated transcription is repressed by other transcription factors at promoters containing only GREs, but no binding site for the repressing transcription factors, e.g. AP-1 (Lucibello et al., 1990; Schule et al., 1990). They were able to show, that the glucocorticoid receptor and transcription factor AP-1 can reciprocally repress one another's transcriptional activation by a novel mechanism that is independent of DNA binding. Overexpression of c-Jun prevents the glucocorticoid-induced activation of genes carrying a functional glucocorticoid response element (GRE). Mutant analysis revealed that the ligand binding and DNA binding domains of GR and the region including the leucine zipper of AP-1 are required for repression. These data indicate that members of two distinct classes of transcription factors can oppose one another's activity through a mechanism likely involving protein-protein interactions.
Another family of transcription factors able to influence the transcriptional activity of GR is NF-κB (Ray and Prefontaine, 1994; Scheinman et al., 1995).
Although the repressing transcription factor does not bind directly DNA at the promoter of the GR target gene, it is tethered by protein-protein interaction to the promoter bound glucocorticoid receptor (Kassel and Herrlich, 2007). Considering the role of nTrip6 as a tethering factor for GR in the trans-repression of AP-1 and NF-κB, it might exert a similar function in the trans-repression of GR by AP-1 and NF-κB.

1.4. Aims of the project

Several questions remain open as to the regulation of transcription by nTrip6. The first part of my thesis will deal with the question if nTrip6 is generally required for the regulation of all AP-1 dimers, both in terms of its co-activator function and of its role in the trans-repression by GR or is it selective for certain AP-1 dimers?
At the start of my PhD work, nTrip6 had already been shown to interact selectively with certain AP-1 family members *in vitro*, and to increase the transcriptional activity of selective AP-1 dimers. My task in this project, involving several people in the laboratory, was more specifically to investigate the promoter occupancy, and represents the first part of this manuscript (this part of the work has been published in Diefenbacher et al., 2008).
The second part of this work concerns the putative function of nTrip6 as a co-activator for the glucocorticoid receptor, and whether it is involved in the trans-repression of GR by AP-1 and NF-κB. A manuscript is in preparation concerning this part of the work.
And finally, the third part of my work was to identify the mechanism by which nTrip6 exerts its co-activator function for AP-1.

2. Material and Methods

2.1. MATERIALS

2.1.1. Chemicals

All the chemicals were of the highest quality and were purchased from:

Acrylamid/N,N´-Methylenbisacrylamid (30:0,8)	Roth, Karlsruhe
Adenosintriphosphat (ATP)	Sigma, Deisenhofen
Agarose	Biozym Diagnostik, Hameln
Ampicillin	Roche Diagnostics, Mannheim
Bacto-Trypton	Biozym Diagnostik, Hameln
Blasticidin	Merck, Darmstadt
Bromphenol blue	Serva, Heidelberg
Chloroform	Roth, Karlsruhe
DMEM (synthetic culturmedium)	Gibco BRL, Eggenstein
D-Glucose	Biozym Diagnostik, Hameln
Dithiothreitol (DTT)	Roche Diagnostics, Mannheim
EDTA	Roth, Karlsruhe
Ethanol	Roth, Karlsruhe
Ethidiumbromide	Sigma, Deisenhofen
Formalin	Merck, Darmstadt
Fetal calf serum (FCS)	Bio Whittaker, Verviers
Glycerin	Roth, Karlsruhe
Glycerol	Sigma, Deisenhofen
Glycylglycin (GLyGly)	Sigma, Deisenhofen
Yeast-Extract	Biozym Diagnostik, Hameln
HEPES	Roth, Karlsruhe
Kanamycin	Biozym Diagnostik, Hameln
Luciferin	Biosynth AG, Staad (Schweiz)
Lysisbuffer (5 x passive lysis buffer*)*	Promega, Madison
β-Mercaptoethanol	Serva, Heidelberg
Sodium acetate	Roth,Karlsruhe
Sodium dodecylsulfate (SDS)	Roth, Karlsruhe
Mounting medium, Immu-mount	Shandon, Pittsburgh, USA
N,N,N´,N´-tetramethylenethylendiamine (TEMED)	BioRad, München
Nonidet P-40 (NP-40)	Sigma, Taufkirchen
Nucleotide triphosphate	Sigma, Deisenhofen
PBS	Gibco BRL, Eggenstein
Phenylmethylsulfonyl-Fluorid (PMSF)	Sigma, Deisenhofen
2-Propanol	Roth, Karlsruhe
Bovine serum albumine (BSA)	Sigma, Deisenhofen
RNasin	Gibco BRL (Eggenstein)
TPA	
Tris-HCl	Serva, Heidelberg
Tris base	Roth, Karlsruhe
Triton X-100	Sigma, Taufkirchen
Trizol	Invitrogen, Karlsruhe
Trypsine	Difco, Detroit

All other chemicals, unless otherwise stated, were purchased from Carl Rotch GmbH&Co, Karlsruhe, Germany; Merck, Darmstadt, Germany or Sigma, Deisenhofen, Germany.

2.1.2. Hardware and consumables

Bacteria-Petri dishes	Greiner, Nürtingen
8 well Chamber slides	Nunc, Rochester, USA
Cell Incubator	Hereus, Stuttgart
Electrophoresis-Chambers	BioRad, München
Developer for X-Ray-films	Kodak, New Haven
Dialysis Cassettes	Thermo Scientific, Germany
ECL Hyperfilm	Amersham, Freiburg, Germany
Eppendorf-reaction tubes, 1,5 ml	Eppendorf, Hamburg
ELISA plate reader	
Gel drier	BioRad, München
Hyperfilm MP®	Amersham Pharmacia Biotech
Image Reader FLA-3000	Fujifilm, Nakanuma, Japan
Immobilon-P (PVDF membrane)	Millipore; Bedford, USA
Heraeus Centrifuge 400R	
with inserts #3324, #8177, #8179	Heraeus instruments
Coolling Centrifuge J2-HS with inserts JS-13.1 & JA-1	Beckmann, Stuttgart
Luminometer	Perkin Elmer,
Perkin Elmer Cetus 9600 Thermocycler	Perkin Elmer Cetus, Norwalk
Phosphoimagerplate	Fujifilm, Nakanuma, Japan
Spectralphotometer (Spekol UV/VIS)	Zeiss, Jena
Stereomicroscope	Zeiss, Jena
Table-Centrifuge Typ 5410	Eppendorf, Hamburg
Tissue culture plastics	Greiner, Nürtingen
Microscope LSM-Meta 510	Zeiss, Jena
Microtome	Leica
MP Hyperfilm	Amersham, Freiburg, Germany

2.1.3 Kits

Easy Pure DNA purification Kit	Biozym Diagnostik GmbH, Oldendorf, Germany
Qiagen Plasmid Maxi Kit	Qiagen, Hilden, Germany
Zero® Blunt® TOPO PCR Kit	Invitrogen, Karlsruhe, Germany
pcDNA 3.1+TOPO direct.clon. Kit	Invitrogen, Karlsruhe, Germany

2.1.4. Bacterial strains

E.coli DH5α:

Bacterial strain for plasmid expression

Genotype: *sup*E44Δ*lac*U169 (Φ*lac*ZΔM15) *hsd*R7 *rec*A1 *gyr*A96 *thi*-1 *rel*A1

2.1.4.1. bacterial growth media

Normal growth medium (Luria Broth, LB) :

Yeast extract	10g/L
NaCl	5g/L
Tryptone	5g/L
pH 7.5	

Super Optimal broth with Catabolite repression (SOC):

Tryptone	2%
Yeast extract	0.5%
NaCl	10mM
KCl	2.5mM
$MgSO_4$	10mM
$MgCl_2$	10mM
Glucose	20mM
pH 7.4	

$MgSO_4$, $MgCl_2$ and KCL were sterile filtered and added after autoclavation.

2.1.5. Tissue culture

2.1.5.1. Cell lines

Hela:
Human Negroid cervix epithelial carcinoma
ECACC No. 930210013

Cos 7:
Monkey African green kidney, SV-40 transformed
ECACC No. 87021302

NIH3T3:
Murine embryonal fibroblast (*Swiss mouse embryo*).
ECACC No. 93061524

NIH3T3 clone 2U

Stable NIH-3T3 clones bearing an array of amplified uPA-Luc gene unit were obtained by Blasticidin selection after co-transfection of equimolar amounts of -1977/-1858uPA-TATA-Luc and pΔBN-AR1, which promotes an amplification of co-transfected plasmids (Shimizu et al, 2003). Copy number was estimated by real time PCR analysis, using primers amplifying both the Chinese hamster DHFR genomic region contained in pΔBN-AR1 and the endogenous mouse counterpart, as described (Bosisio 2006).

NIH3T3 clone 7m

Stable NIH-3T3 clones bearing an array of amplified MMTV-Luc gene unit generated like described above.

NIH3T3 clone 12c

Stable NIH-3T3 clones bearing an array of amplified Collagenase I minimal promoter-Luc gene unit generated like described above.

2.1.5.2. Tissue culture conditions

All cell lines were cultured at 6% CO_2, 95% humidity, 37°C in an incubator. Manipulation of cells was performed under a sterile hood. Media, Buffers and glassware were sterilised before work (120°C, 1.4 bar, 20min).

2.1.5.3. Cell line culture media

	Growth-medium 10%DCS-DMEM	Starvation-medium 0,5%DCS-DMEM	Growth-medium 10%FCS-DMEM	Starvation-medium 0,5%DCS-DMEM
Hela	+	+	-	-
Cos-7	-	-	+	+
NIH3T3	+	+	-	-
2U	+	+	-	-
7m	+	+	-	-
12c	+	+	-	-

2.1.6. Enzymes

Restriction-enzymes and buffers were used from the following companies:

NEB	Frankfurt am Main, Germany
Promega	Mannheim, Germany
Fermentas	St. Leon-Rot, Germany

2.1.7. Plasmids

2.1.7.1. Mammalian expression vectors

pcDNA 3.1(+)
Empty vector, basic mammalian expression cloning vector.

pcDNA 3.1(+) HA
Contains the cDNA encoding for 8 amino acids of the haemaggluthinin protein, which was used to tag proteins (provided by Marga Litfin, ITG, FZKA Karlsruhe).

pcDNA 3.1(+) HA-Trip6$^{190-476}$
Contains the cDNA of Trip6$^{190-476}$ fused to a HA-tag. The start-codon is missing within the clone and was introduced via the marker. The construct was cloned via EcoRI/XoI (provided by Christine Heilbock, ITG, FZKA Karlsruhe).

pcDNA 3.1(+) HA-Trip6$^{190-476}$ΔDD2
Contains the cDNA of Trip6$^{190-476}$ with a deletion of the second dimerisation-domain fused to a HA-marker. The start-codon is missing within the clone and was introduced via the marker. The deletion was introduced by a fusion PCR using the primer pairs Trip6$^{190-476}$-forward KpnI:ΔDD2-reverse and ΔDD2:Trip6$^{190-476}$-reverse XbaI. The construct was cloned via KpnI/XbaI.

pcDNA 3.1(+) HA -LIM
Contains the cDNA encoding only for the LIM-domains of Trip6, fused to a HA-marker. The start-codon is missing within the clone and was introduced via the marker. The sequence was amplified by PCR using the primer pairs LIM-forward KpnI and Trip6$^{190-476}$-reverse XbaI. The construct was cloned via KpnI/XbaI.

pGAL4$_{DBD}$
Contains the cDNA for the DNA-Binding-Domain of the GAL4-yeast transcription factor under the control of a CMV-promoter (provided by Marga Litfin, ITG, FZKA Karlsruhe).

pGAL4$_{DBD}$-Trip6
Contains the cDNA of Trip6 fused to the DNA-Binding-Domain of the GAL4-yeast transcription factor under the control of a CMV-promoter (provided by Marga Litfin, ITG, FZKA Karlsruhe).

pGAL4$_{DBD}$- -LIM
Contains the cDNA of Trip6 LIM-domains alone, fused to the DNA-Binding-Domain of the GAL4-yeast transcription factor under the control of a CMV-promoter (provided by Marga Litfin, ITG, FZKA Karlsruhe).

pCGcJun~cFos
Expression of cJun~cFos fusion under the control on CMV promoter.
Described in Bakiri et al., 2002

pCGcJun~ATF2
Expression of cJun~ATF2 fusion under the control on CMV promoter.
Described in Bakiri et al., 2002

2.1.7.2. Expression vectors for fluorescent protein fusions

pcDNA3.1(+)-HA-CFP
Contains the cDNA for the cyan fluorescent protein (CFP) under the control of a CMV-promoter, which is fused to a HA tag. Generated via PCR using pcDNA3.1-CFP (Invitrogen, Karlsruhe) as a template using the primer pair Cherry-forward NotI and Cherry-reverse XbaI and cloned into pcDNA 3.1(+)-HA via NotI/XbaI.

pcDNA3.1(+)-HA-YFP
Contains the cDNA for the yellow fluorescent protein (YFP) under the control of a CMV-promoter, which is fused to a HA tag. Generated via PCR using pcDNA3.1-YFP (Invitrogen, Karlsruhe) as a template using the primer pair Cherry-forward NotI and Cherry-reverse XbaI and cloned into pcDNA 3.1(+)-HA via NotI/XbaI.

pcDNA3.1(+)-HA-mCherry
Contains the cDNA for the cherry-red fluorescent protein (mCherry, provided by Roger Y. Tsien, UCSD, La Jolla CA, USA) under the control of a CMV-promoter, which is fused to a HA tag. Generated via PCR using the primer pair Cherry-forward NotI and Cherry-reverse XbaI and cloned into pcDNA 3.1(+)-HA via NotI/XbaI.

pcDNA-3.1(+)-mCherry
Contains the cDNA for the cherry-red fluorescent protein (mCherry, provided by Roger Y. Tsien, UCSD, La Jolla CA, USA)) under the control of a CMV-promoter. Generated via PCR using the primer pair Cherry-forward NheI and Cherry-reverse KpnI and cloned into pcDNA 3.1(+)-HA via NheI/KpnI.

pcDNA-3.1(+)-mOrange
Contains the cDNA for the orange fluorescent protein (mOrange, provided by Roger Y. Tsien, UCSD, La Jolla CA, USA)) under the control of a CMV-promoter. Generated via PCR using the primer pair Cherry-forward NheI and Cherry-reverse KpnI and cloned into pcDNA 3.1(+)-HA via NheI/KpnI.

pcDNA-3.1(+)-mCherry-NLS-DD1
Contains the cDNA of Trip6 dimerisation domain I fused to a nuclear localisation signal (NLS) of the SV-40 virus and the sequence of the cherry-red fluorescent protein (mCherry) under the control of a CMV-promoter. Generated via Oligocloning using primer pair DD1 for BamHI and DD1 rev XbaI and cloned into pcDNA 3.1(+) via BamHI/XbaI.

pcDNA-3.1(+)-mCherry-NLS-DD1-Srambled
Contains the scrambled cDNA of Trip6 dimerisation domain I fused to a NLS and the sequence of the cherry-red fluorescent protein (mCherry) under the control of a CMV-promoter. Generated via Oligocloning using primer pair DD1-SCR for BamHI and DD1-SCR rev XbaI and cloned into pcDNA 3.1(+) via BamHI/XbaI.

pcDNA-3.1(+)-mCherry-NLS-DD2
Contains the cDNA of Trip6 dimerisation domain II fused to a NLS and the sequence of the cherry-red fluorescent protein (mCherry) under the control of a CMV-promoter. Generated via Oligocloning using primer pair DD2 for BamHI and DD2 rev XbaI and cloned into pcDNA 3.1(+) via BamHI/XbaI.

pcDNA-3.1(+)-mCherry-NLS-DD2Scrambled
Contains the scrambled cDNA of Trip6 dimerisation domain II fused to a NLS and the sequence of the cherry-red fluorescent protein (mCherry) under the control of a CMV-promoter. Generated via Oligocloning using primer pair DD2-SCR for BamHI and DD2-SCR rev XbaI and cloned into pcDNA 3.1(+) via BamHI/XbaI.

pcDNA3.1(+)-HA-Trip6$^{190\text{-}476}$-CFP
Contains the cDNA of Trip6$^{190\text{-}476}$ fused to CFP. The construct was generated via PCR using the primer pair Trip6$^{190\text{-}476}$ forward KpnI and Trip6$^{190\text{-}476}$ reverse NotI and cloned into pcDNA3.1(+)-HA-CFP via KpnI/NotI.

pcDNA3.1(+)-HA-Trip6$^{190-476}$-YFP
Contains the cDNA of Trip6$^{190-476}$ fused to YFP. The construct was generated via PCR using the primer pair Trip6$^{190-476}$ forward KpnI and Trip6$^{190-476}$ reverse NotI and cloned into pcDNA3.1(+)-HA-YFP via KpnI/NotI.

pCMV-GFP-GR
Expression of Glucocorticoid receptor fused to GFP under the control of CMV promoter (provided by Andrew Cato, ITG, FZKA Karlsruhe).

pCMV-GFP-POLII
Expression of RNA-Polymerase-II fused to GFP under the control of a CMV promoter. (provided by Kimihiko Sugaya, National Institute of Radiological Sciences Chiba, Japan).

pCMV-cFos-Venus
Expression of cFos fused to Venus under the control of a CMV promoter. (provided by Chang-Deng Hu, Purdue University School of Pharmacy, USA).

pcDNA3.1(+) HA-GR-mCherry
Contains the cDNA of the Glucocorticoid-receptor (GR) fused to mCherry. The construct was generated via PCR using the primer pair GR-VN forward KpnI and GR-VN reverse NotI and cloned into pcDNA3.1(+)-mCherry via KpnI/NotI.

2.1.7.3. Expression vectors encoding for Bimolecular fluorescent complementation assay

pBiFC-YC
Composed of the pCMV-HA vector (Clontech); the coding sequence of aminoacids 155-238 of the C-terminal part of the Yellow Fluorescent Protein (YFP) is cloned directly after the multiple cloning side (provided by Tom Kerppola, University of Michigan).

pBiFC-YN
Composed of the pFLAG-CMV vector (Sigma); the coding sequence of aminoacids 1-155 of the C-terminal part of the Yellow Fluorescent Protein (YFP) is cloned directly after the multiple cloning side (provided by Tom Kerppola, University of Michigan).

pBiFC–Trip6$^{190-476}$-YC
Containing the cDNA of Trip6$^{190-476}$. It was amplified from a pcDNA 3.1(+)-HA-Trip6$^{190-476}$ vector via PCR using the primer pair Trip6$^{190-476}$-YC for EcoRI and Trip6$^{190-476}$-YC rev XhoI and cloned using EcoRI/XhoI.

pBiFC–Trip6$^{190-476}$-LIM1m-YC

Containing the cDNA of Trip6$^{190-476}$ with a mutation within the two zinc-fingers of LIM-domain 1 (replacement of cysteins 279,282,307,310 to alanine). It was amplified from a pcDNA 3.1(+)-HA-Trip6$^{190-476}$ vector via PCR using the primer pair Trip6$^{190-476}$-YC for EcoRI and Trip6$^{190-476}$-YC rev XhoI and cloned using EcoRI/XhoI.

pBiFC– Trip6$^{190-476}$-LIM3m-YC

Containing the cDNA of Trip6$^{190-476}$ with a mutation within the two zinc-fingers of LIM-domain 3 (replacement of cysteins 399, 402, 432, 435 to alanine). It was amplified from a pcDNA 3.1(+)-HA-Trip6$^{190-476}$ vector via PCR using the primer pair Trip6$^{190-476}$-YC for EcoRI and Trip6$^{190-476}$-YC rev XhoI and cloned using EcoRI/XhoI.

pBiFC–LIM-YC

Containing the cDNA of Trip6$^{227-476}$. It was amplified from a pcDNA 3.1(+)-HA-Trip6$^{190-476}$ vector via PCR using the primer pair LIM-YC for EcoRI and LIM-YC rev XhoI and cloned using EcoRI/XhoI.

pBiFC–Trip6$^{190-476}$-YN

Containing the cDNA of Trip6$^{190-476}$. It was amplified from a pcDNA 3.1(+)-HA-Trip6$^{190-476}$ vector via PCR using the primer pair Trip6$^{190-476}$-YN for HindIII and Trip6$^{190-476}$-YN rev KpnI and cloned using HindIII/KpnI.

pBiFC–cJun~cFos-YC

Containing the cDNA of the fusionprotein cJun~cFos. It was amplified via PCR from pCGcJun~cFos vector and cloned via EcoRI/XhoI (provided by Sylwia Sekula, ITG, FZKA Karlsruhe).

pBiFC–cJun~ATF2-YC

Containing the cDNA of the fusionprotein cJun~ATF2. It was amplified via PCR from pCGcJun~ATF2 vector and cloned via EcoRI/XhoI (provided by Sylwia Sekula, ITG, FZKA Karlsruhe).

pcDNA3.1(+)-HA-VN

Composed of the pcDNA-3.1(+)-HA vector in fusion with the coding sequence of aminoacids 1-172 of the N-terminal part of the Venus Fluorescent Protein (VN-CT), amplified by PCR using the primer pair VN-CT for NotI and VN-CT rev XbaI and cloned via NotI/XbaI.

pcDNA3.1(+)-HA-VC

Composed of the pcDNA-3.1(+)-HA vector in fusion with the coding sequence of aminoacids 155-238 of the C-terminal part of the Venus Fluorescent Protein (VC-CT), amplified by PCR using the primer pair VC-CT for NotI and VC-CT rev XbaI and cloned via NotI/XbaI.

pcDNA3.1(+)-HA-Trip6$^{190-476}$-VN

Containing the cDNA of Trip6$^{190-476}$. The construct was generated via PCR using the primer pair Trip6$^{190-476}$ forward KpnI and Trip6$^{190-476}$ reverse NotI and cloned into pcDNA3.1(+)-HA-VN via KpnI/NotI.

pcDNA3.1(+)-HA-Trip6$^{190-476}$-VC

Containing the cDNA of Trip6$^{190-476}$. The construct was generated via PCR using the primer pair Trip6$^{190-476}$ forward KpnI and Trip6$^{190-476}$ reverse NotI and cloned into pcDNA3.1(+)-HA-VC via KpnI/NotI.

pcDNA3.1(+)-HA-LIM-VC

Containing the cDNA of Trip6$^{227-476}$. The construct was generated via PCR using the primer pair LIM forward KpnI and Trip6$^{190-476}$ reverse NotI and cloned into pcDNA3.1(+)-HA-VC via KpnI/NotI.

pcDNA3.1(+)-HA-Trip6$^{190-476}$ΔLIM-VC

Containing the cDNA of Trip6$^{190-476}$ΔLIM. The construct was generated via PCR using the primer pair Trip6$^{190-476}$ forward KpnI and Trip6$^{190-476}$ΔLIM reverse NotI and cloned into pcDNA3.1(+)-HA-VC via KpnI/NotI.

pcDNA3.1(+)-HA-GR-VN

Containing the cDNA of the Glucocorticoid-receptor (GR). The construct was generated via PCR using the primer pair GR-VN forward KpnI and GR-VN reverse NotI and cloned into pcDNA3.1(+)-HA-VN via KpnI/NotI.

pcDNA3.1(+)-HA-cFos-VC

Containing the cDNA of cFos. The construct was generated via PCR using the primer pair c-Fos-VC forward KpnI and c-Fos-VC reverse NotI and cloned into pcDNA3.1(+)-HA-VC via KpnI/NotI.

pcDNA3.1(+)-VC

Composed of the pcDNA-3.1(+) vector in fusion with the coding sequence of the 155 aminoacids of the N-terminal part of the Venus Fluorescent Protein (VC-CT), which is cloned directly after the multiple cloning side using the primer pair VC-NT-for NheI and VC-NT-rev KpnI via NheI/KpnI.

pcDNA3.1(+)-VN

Composed of the pcDNA-3.1(+) vector in fusion with the coding sequence of the 175 aminoacids of the N-terminal part of the Venus Fluorescent Protein (VN-CT), which is cloned directly after the multiple cloning side using the primer pair VN-NT-for NheI and VN-NT-rev KpnI via NheI/KpnI.

pcDNA3.1(+)-VC-Trip6

Containing the cDNA of Trip6. It was amplified from a pcDNA 3.1(+)-HA-Trip6 vector via PCR using the primer pair Trip6 forward KpnI and Trip6 reverse NotI and cloned using KpnI/NotI.

pcDNA3.1(+)-VN-Trip6

Containing the cDNA of Trip6. It was amplified from a pcDNA 3.1(+)-HA-Trip6 vector via PCR using the primer pair Trip6 forward KpnI and Trip6 reverse NotI and cloned using KpnI/NotI.

2.1.7.4. Expression vectors to generate recombinant proteins

pGEX-4T-1

Basic cloning vector for the expression of recombinant proteins fused a GST-protein (glutathion-s-tranferase) (Promega, Mannheim) and expression in bacteria.

pGex-4T1-Trip6$^{190\text{-}476}$

contains the cDNA of Trip6$^{190\text{-}478}$, which is fused to GST, and ligated via EcoI/XhoI into pGEX-4T-3.

2.1.8. Antibodies

	Isotype	Concentration	Company
Human Trip6 BL443	Rabbit	1/100//1/1000 IF WB	Bethyl
Vinculin sc-5573	Mouse	1/250	Santa Cruz
E-Cadherin #610181	Mouse	1/100	BD Bioscience
anti-Flag M2 Clone F1804	Mouse	1/100	Sigma
anti-HA Clone 3F10	Rat	1/100//1/1000 IF WB	Roche
anti –GST Clone GST-2	Mouse	1/500	Sigma
anti -rat-Alexa 488	Goat	1/1000	Invitrogen
anti -rat-Alexa 546	Goat	1/1000	Invitrogen
anti -rabbit-Alexa 488	Goat	1/1000	Invitrogen
anti -rabbit-Alexa 546	Goat	1/1000	Invitrogen
anti -mouse-Alexa 488	Goat	1/1000	Invitrogen
anti -mouse-Alexa 546	Goat	1/1000	Invitrogen
Phalloidin Rhodamine		1/1000	Molecular Probes
DRAQ5		1/1000	Biostatus Limited
anti -goat HRP	Rabbit	1/1000	Dako
anti -mouse HRP	Goat	1/1000	Dako

2.1.9. Primer used for amplification and cloning into expression vectors

Insert	Sequence
Trip6-forward KpnI	GGTACCTCGGGGCCCACCTGGCTG
Trip6-reverse NotI	GCGGCCGCCTCAGCAGTCAGTGGTGACGGT
Trip6-reverse XbaI	TCTAGATCAGCAGTCAGTGGTGACGGT
nTrip6-forward KpnI	GGTACCCTGAATGGGGGTCGGGGTC
nTrip6-reverse NotI	ATTGCGGCCGCGGCAGCAGTCAGTGGTGACGGTGGC
$Trip6^{190-476}$-forward KpnI	GGTACCCAGGCCTCTGGGCCCCTC
$Trip6^{190-476}$-reverse NotI	GCGGCCGCCTCAGCAGTCAGTGGTGACGGT
LIM-forward KpnI	GGTACCGGAGAAGATGTGGTTGGGGATGGGGCTGG
Trip6-reverse NotI	GCGGCCGCCTCAGCAGTCAGTGGTGACGGT
CFP/YFP-forward NheI	GCGGCTAGCATGGTGAGCAAGGGCGAGGAG
CFP/YFP-reverse NotI	GCGGGTACCCTCGTACAGCTCGTCCATGCCGAGAGTG
Cherry/Orange/Tomato-forward NheI	GCGCTAGCATGGTGAGCAAGGGCGAGGAGCTG
Cherry/Orange/Tomato-reverse KpnI	GCGGTACCACCGGATCCTCCACCTCCGCCTGAGCCCTTGTACAGCTCGTCC
Cherry/Orange/Tomato-forward NotI	GCGGCCGCGGCTCAGGCGGAGGTGGAGGATCCGGTGTGAGCAAGGG
Cherry/Orange/Tomato-reverse XbaI	GCTCTAGATAACTTGTACAGCTCG
VN-NT-forward NheI	GCGCTAGCATGGTGAGCAAGGGCGAGGAGCTG
VN-NT-reverse KpnI	GCGGTACCACCGGATCCTCCACCTCCGCCTGAGCCCTCGATGTTGTGGCGG
VC-NT-forward NheI	GCGCTAGCATGGACAAGCAGAAGAACGGCATCAAGGCC
VC-NT-reverse KpnI	GCGGTACCACCGGATCCTCCACCTCCGCCTGAGCCCTTGTACAGCTCGTCC
VN-CT-forward NotI	GCGGCCGCGGCTCAGGCGGAGGTGGAGGATCCGGTGTGAGCAAGGG
VN-CT-reverse XbaI	GCTCTAGACTACTCGATGTTGTGGCGG
VC-CT-forward NotI	GCGGCCGCGGCTCAGGCGGAGGTGGAGGATCCGGTGACAAGCAGAAGAACG
VC-CT-reverse XbaI	GCTCTAGATAACTTGTACAGCTCG
NES-for KpnI	GGTACCCCTACAAAAGAAGCTAGAAGAACTAGAACTAGAA
NES-rev BamHI	GGATGTTTTCTTCGATCTTCTTGATCTTGATCTTGGATCC
NLS-for KpnI	GGTACCCCCAAAGAAGAAGCGAAAGGTA
NLS-rev BamHI	GGGTTTCTTCTTCGCTTTCCATGGATCC
GR-VN-for KpnI	GCGGTACCATGGACTCCAAAGAATCATTAAC
GR-VN-rev XbaI	AAAGCGGCCGCGCTTTTGATGAAACAGAAGTTTTTTG
$Trip6^{190-476}$-YC-for EcoRI	CGAATTCGGCAGGCCTCTGGGCCCCTCCCG
$Trip6^{190-476}$-YC-rev XhoI	ACCTCGAGAGCAGTCAGTGGTGACGGTGGC
LIM-YC-for EcoRI	CGAATTCGGGGCCAGTGTGGTGGCTGCGGA
LIM-YC-rev-XhoI	ACCTCGAGAGCAGTCAGTGGTGACGGTGGC
$Trip6^{190-476}$-YN-for HindIII	AAGCTTCAGGCCTCTGGGCCCCTCCCG
$Trip6^{190-476}$-YN-rev KpnI	TGGTACCGAGCAGTCAGTGGTGACGGTGGC
$Trip6^{190-476}$ΔLIM-reverse NotI	CTCGAGAAAAGTACTCCCCGCTGGGCGG
c-Fos-for VC KpnI	GCGGTACCTTCTCGGGTTTCAACGCCG
c-Fos-for VC NotI	GCGCGGCCGCCAGGGCCAGCAGCGTGGG

2.2. Methods

2.2.1. DNA-Methods

2.2.1.1. DNA digestion via restriction-endonucleases

2-3 units of restriction-endonuclease were used per µg DNA. The total volume of the digest was 10 times the volume of the enzyme/glycerol-solution. Additionally a 10-times concentrated buffer-solution was added to the DNA-enzyme mix in order to achieve the required milieu for the enzyme. The DNA-enzyme-buffer mix was incubated, if not recommended differently by the supplier, for at least 2h at 37°C.

2.2.1.2. Ligation of DNA-fragments

Ligation reactions were performed using T4 DNA ligase according to manufacturer's instructions (Fermentas).

2.2.1.3. Polymerase chain reaction (PCR)

By using two sequence-specific oligonucleotides DNA-fragments were amplified by using the thermo-stable Pfu-DNA-polymerase. The reaction was carried out in a Perkin-Elmer-Thermo-Cycler (Norwalk, USA).

PCR Mixture:

10xPCR buffer	5µl
DMSO	5µl
0.1µg template DNA	1µl
forward primer 10mM	1µl
reverse primer 10mM	1µl
10mM dATP	1µl
10mM dCTP	1µl
10mM dGTP	1µl
10mM dTTP	1µl
Pfu-DNA polymerase	0.25µl
H_2O	
	add 50µl

PCR-Program

1cycle:		
	5min	94°C
35cycles:		
	1min	95°C
	1min	55°
	xmin	72°C (depending on size of fragment)
1cycle:		
	hold at	4°C

For screening a large number of bacterial clones, the PCR was modified to perform a "colony-PCR". Here, a bacterial clone was picked with a small pipette-tip a directly passed over into the PCR-reaction mixture and then the tip was further passed into LB or SOC containing selection-markers (e.g. Ampicilin or Kanamycin).
Instead of the Pfu-DNA-polymerase the faster GoTaq-polymerase from Promega was used due to its capability to amplify 1kb/minute.

PCR Mixture:		PCR-Program		
10xPCR buffer	5µl	1cycle:		
bacterial colony	0µl		5min	94°C
forward primer 10mM	0.5µl	35cycles:		
reverse primer 10mM	0.5µl		1min	95°C
10mM dATP	0.5µl		1min	55°
10mM dCTP	0.5µl		xmin	72°C (depending on size of fragment)
10mM dGTP	0.5µl	1cycle:		
10mM dTTP	0.5µl		hold at	4°C
Taq-DNA polymerase	0.25µl			
H_2O	_____			
	add 25µl			

The PCR-reactions were then loaded on a 1%-2% agarose-gel, according to expected fragment size, to analyse the fragment length.

2.2.1.4. Electrophoresis via agarose-gels

Depending on fragment size, agarose-gels were cast at a concentration between 0,8-2%. Between 50ml and 400ml of gel solution depending on chamber size was used and covered with a layer of buffer (between 100ml and 1000ml). The agarose was dissolved in 1xTAE (40mM Trips pH 8,3; 40mM NaAzetate; 2mM EDTA pH8,0) buffer and melted in a microwave oven. Afterwards the glass-beaker was cooled down by running water and ethidium bromide was added to the gel-solution. Finally it was poured into the chambers equipped with a comb and cooled down to room temperature. The solid gel was covered with 1x TAE-buffer, the comb was removed and the samples (10mM EDTA; 10% Glycerol; 0,1% SDS; 0,02% Bromphenol blue) were loaded. The separation was achieved by applying voltage between 80-120V. DNA could be visualized by UV-light (302nm) and was photographed for documentation.

2.2.1.5. Isolation of DNA-fragments out of agarose gels

Fragments corresponding to DNA of interest were cut out of the agarose-gel at the UV-table with a scalpel and transferred onto parafilm for "freeze-squeeze" or into an Eppendorf-reaction tube for kit-based elution (Promega) according to manufacturers' protocol.

"freeze-squeeze":

Excised DNA-fragments were transferred to -20°C till they were completely frozen. Afterwards the frozen gel pieces were packed into parafilm and squeezed by hand. The extracted solution containing the DNA was collected in a Eppendorf-reaction tube. The DNA was precipitated in 100%EtOH, 1/40 of total volume 3M NaAc followed by a centrifugation step, 12k rpm, 4°C, 15min. The pellet was then washed in 70% EtOH followed by a centrifugation step, 12k rpm, 4°C, 15min. then the DNA was dried at room temperature till no EtOH was left and dissolved in 50µl TE-buffer (10mM Tris-HCL; 1mM EDTA pH 8,0).

2.2.1.6. "Mini-Preparation"/small-scale preparation of plasmid-DNA

This technique was applied to screen bacteria-clones for DNA-content after transformation of a ligated vector. The bacteria-clone was put into 2ml LB containing an antibiotic selection marker (e.g. Ampicilin, Kanamycin,…) and was cultivated over night in a shacking incubator at 37°C. Next day 1.5ml of this culture was transferred into an Eppendorf reaction tube and centrifuged (3500rpm, 4°C, 3min). The supernatant was removed and the pellet was resuspended in 200µl ice-cold TELT-buffer (50 mM Tris-HCl pH 8,0, 62,5 mM EDTA, 2,5 M LiCl, 0,4% Triton X-100) followed by administering 20µl Lysozyme (10 mg/ml in TE-Buffer (10 mM Tris-HCl, 1 mM EDTA pH 8,0)). This mix was then incubated for 3min on a heating block at 96°C, transferred onto ice for 5min, and centrifuged (12000 rpm, 4°C, 15min). After centrifugation the supernatant, which is containing the DNA, was mixed with 100µl Isopropanol, vortexed and centrifuged again (12000rpm, 4°C, 15min). The DNA pellet was washed with 1ml 70% EtOH and dried at room temperature. Afterwards it was dissolved in 50µl 1xTE-buffer (10mM Tris-HCL; 1mM EDTA pH 8,0).

2.2.1.7. ”Maxi-Preparation”/large-scale preparation of plasmid-DNA

LB-medium containing an antibiotic selection marker was inoculated with a bacteria-clone selected via “mini-preparation” or with bacteria directly transformed with DNA of interest and transferred to a shacking incubator till next day at 37°C, shaking. The preparation was performed according to manufacturers protocol (Qiagen).
The precipitated DNA was washed again with 70%EtOH. The DNA pellet was dissolved in 100-400µl 1xTE-buffer and quantified.

2.2.1.8. Estimation of DNA-concentration

DNA concentration within aqueous solutions was estimated by spectra photometry. An OD of 1 measured at 260nm corresponds to 50µg/ml double-stranded DNA or 40µg/ml single-stranded RNA. To estimate the purity of the sample, OD at 280nm was measured. At this wavelength phenol, ethanol and proteins lead to a shift in the spectra. The ration OD_{260}/OD_{280} should be ideally ~1,8 for DNA and ~2,0 for RNA.

2.2.2. Generating chemically competent bacteria

A single colony of *E. coli* DH5α was incubated in 5ml LB medium overnight at 37°C with shaking (200 rpm). The 4 ml were used to inoculate 400 ml of fresh LB medium and allowed to grow to an OD_{600} of 0.4. After chilling on ice for 10 min the cells were centrifuged at 3600g for 10 min at 40C. The pellet was re-suspended in 20 ml of ice cold 0.1M $CaCl_2$ and allowed to stand on ice for 10 min. Then bacteria were centrifuged once more and again re-suspended in $CaCl_2$. This procedure was repeated once more. Finally the pellet was re-suspended in 2 ml of ice-cold $CaCl_2$ with 10% glycerol. After 5 min incubation on ice, the bacteria were aliquoted and frozen at -80°C.

2.2.3. Transformation of chemically competent *E.coli* bacteria

2.2.3.1. Transformation of ligated vector-DNA or vector DNA

Chemical transformation was used for propagation of plasmids and DNA ligation products. 1µl plasmid DNA or 2-7µl of a ligation mix was added to 100µl ice-thawed chemically competent *E.coli*. After mixing and incubation on ice for 15 min bacteria were heat-shocked at 42°C for 30 sec and incubated on ice for another 2 min. The transformed bacteria were mixed with 0,5ml of SOC medium and incubated at 37°C with shaking for 1h. Finally the bacteria were plated onto the LB agar plates supplemented with appropriate antibiotics for

selection and allowed to grow for 16-24h at 37°C or directly transferred into 100ml LB containing the appropriate antibiotics and allowed to grow for 12-24h at 37°C.

2.2.3.2. TOPO-Cloning

Topo-cloning is the direct ligation of PCR products in vectors, and was carried out according to manufacturers manual.

2.2.4. Protein-methods

2.2.4.1. Measurement of protein content according to Bradford

5µl of protein containing solution was mixed with 200µl Bradford-solution and transferred into a 96well flat-bottom plate. This well was afterwards placed in an ELISA-plate reader and protein content was estimated via extinction at 660nm. The total protein concentration in the samples was interpolated from a bovine serum albumine (BSA) standard curve.

2.2.4.2. Separation of proteins via a polyacrylamide gel electrophoresis (SDS-Page)

Protein samples (2x sample buffer: 125mM Tris-HCL pH 6,8 ;4% SDS; 20% Glycerol; 0,01% Bromphenolblue; 2% 2-Mercapthoethanol) were electrophoretically separated on the basis of the size using the method from Laemmli (1970). The resolving gels containing between 8-12% acryl amide depending on experiment and 5% stacking gel (6-12% Acrylamid; 0,16-0,32% N,N´-Methylenbisacrylamide; 375mM Tris-HCL pH 8,8; 0,1% APS; 0,1% TEMED) were cast according to Sambrook et al. (1989). Samples were run into the stacking gel (running buffer: 25mM Tris; 192mM Glycin; 0,1% SDS) at 60-80V and then run at 100-140V in the separating gel (4% Acrylamid; 0,1% N,N´-Methylenbisacrylamide; 125mM Tris-HCL pH 6,8; 0,1% APS; 0,1% TEMED) with a mini gel system (Hoefer, San Francisco, USA).

2.2.4.3. Coomassie-brilliant-blue stain of proteins

To stain and visualize proteins separated by SDS-page the gel was incubated with a Coomassie-brilliant-blue staining solution (0,25% Coomassie-brilliant-blue R250; 50% Methanol; 10% Acetic acid) for 1h at room temperature followed by a destaining step in destain solution I (50% Methanol; 10% Acetic acid) 1-2h followed by destain solution II (5% Methanol; 7% Acetic acid) over night. Destaining was carried out till protein bands appeared. The gel was then dried via a gel-drier.

2.2.4.4. Western blotting

Proteins in SDS-PAGE gels were transferred (20mM Tris; 192mM Glycine; 10% Methanol) to methanol soaked Immobilon-P membranes in a semi-dry blotter at 1mA/cm^2 for 1h. After the transfer membranes were incubated in blocking buffer (5% skimmed milk powder; TBS) room temperature for 1h to reduce unspecific binding. Primary antibodies were diluted in blocking buffer at concentration recommended by the supplier (generally 1:500-1:3000). Membranes were incubated in the primary antibody-containing buffer for 1-2h at room temperature or overnight at 4°C. The membranes were then washed three times with TBST buffer ((TBS: 20mM Tris; 150mM NaCl; pH 7,6)+ 0,05% TWEEN 20) for 5 minutes each. With appropriate horse radish peroxidase (HRP) conjugated 2^{dary} antibody diluted in blocking buffer membranes were incubated for additional 1h at room temperature. Membranes were washed again with TBST buffer for three times. Detection of specific protein signals were achieved using enhanced chemiluminescence (ECL) Western blotting detection reagents and ECL Hyperfilm following manufacturer's instructions.

2.2.4.5. Production of Glutathion-S-Transferase-fusion proteins

By fusing eukaryotic proteins to the protein Glutathion-S-Transferase (GST) from *Schisostoma japonicum* they can be produced in high amounts and soluble states. The cDNA of the protein of interest was fused by cloning into the vector pGEX-4T-1 directly after the 3´end of the GST coding sequence. The affinity of GST to glutathione allows to purify the fusion protein under mild conditions via affinity-chromatography by using immobilized glutathione.

80ml of a stationary pre-culture of a transformed bacteria-strain was diluted 1:10 in LB containing an appropriate antibiotic and incubated at 37°C till an OD_{600} of 0,6 was reached. The protein expression was induced by adding IPTG and by addition of 10µM $ZnSO_4$ Zinc Finger-containing proteins were stabilised. Protein production was carried out for 4h at 30°C. Afterwards the bacteria were transferred onto ice for 10 min then pelleted via centrifugation (3000 rpm, 4°C, 15 min). The supernatant was discarded and the pellet resuspended in 8ml ice-cold fusionproteinbuffer.

All following steps were carried out on ice:

The bacteria and the bacteria-DNA were mechanically sheared by sonifying the solution. The insoluble bacterial components were then separated from the soluble fraction containing the proteins by ultracentrifugation (25000g, 4°C, 45 min). Glutathione immobilized on agarose beads was stored in 20% Ethanol. 1ml of this 1:1 solution mix was washed twice shortly before use in PBS. The supernatant of the ultracentrifuged bacteria was poured onto the beads and incubated for 45min at 4°C under constant rotation. The beads were washed 3 times with fusion protein buffer. Thereafter the bead-protein mix was transferred into a column and the fusion protein was eluted fractionwise from the agarose-beads by adding 5mM Glutathion

(dissolved in 50mM Tris-HCL) and collected in Eppendorf-reaction tubes. The protein of each fraction was measured via Bradford, and the fusion proteins were detected by SDS-page followed by a Coomassie-brilliant-blue staining of the acrylamide-gel.
The fusion proteins were dialysed against PBS over night at 4°C using Dialyses-hoses and stored at -80°C until usage.

2.2.4.6. *in vitro* protein-protein interaction studies – Spot®-Technique

This system is based on the specific synthesis of peptides in a single position (spot) on a cellulose-membrane. Depending on the format of this membrane several different peptides could be *spotted* parallel. Peptide synthesis on the membrane was performed fully automatic by a robot under the supervision and control of Claudia Ester (ITG, FZKA, Germany). After synthesis of the selected peptide-sequences they were subjected to interaction studies by incubation of the membrane with proteins of interest.

After finishing the production process, the peptides immobilized on the membrane were used as baits to investigate their interaction with recombinant expressed GST-fusion-proteins.
In a first step, the membrane was activated by placing it in 100% methanol for 10min and rinsed 3 times in TBS for 5min. Afterwards the membrane was transferred into a blocking buffer-solution for 3h to reduce unspecific binding of primary antibody followed by washing the membrane in TBS for 5min. That was followed by incubation with a recombinant expressed fusion protein (10µg/ml in blocking buffer), which should be observed for interaction with Trip6 protein sequence. Incubation was carried out by adding GST-Trip6$^{190-476}$ over night at 4°C. Next day the membrane was washed 3 times with TBS to get rid of unbound protein and incubated for 3h with an α-GST-antibody (diluted 1:1000 in blocking buffer) at room temperature followed by washing steps, 3 times TBS-T, 5min, and incubated with a appropriate secondary antibody coupled to Horse-reddish peroxidase (HRP; diluted 1:5000 in blocking buffer) for 1½ h. After washing the membrane 3 times with TBS-T, proteins interacting with the spotted peptide-sequences were highlighted via a enhanced chemiluminescence ECL Western blotting detection reagents and ECL Hyperfilm following manufacturer's instructions.

2.2.5. DNA-Pulldown

This pulldown-assay using a biotinylated DNA-template as bait allows to investigate the composition of protein-complexes recruited to a known DNA template. For my studies, I used a template generated by PCR containing a TRE (TPA-responsible element), a TATA-box and a part of the luciferase gene coding sequence. This construct mimics a promoter area with all

necessary binding sites to allow AP-1 to bind and to recruit co activator complexes and the basal transcription factor machinery.

2.2.5.1. Preparation of nuclear extracts

$2x10^6$ logarithmically growing HeLa-cells were seeded in a 15cm dish in 10% DCS containing D-MEM and incubated over night at 37°C. Next day the medium was changed and cells were starved with 0.5% DCS D-MEM for 24-48h. Normal medium contains growth factors which lead to the activation of AP-1. Therefore the starvation leads to the reduction of already present AP-1. Only after stimulation at a given time point with TPA AP-1 is activated and leads to the activation of all other factors necessary for activating transcription of AP-1 target genes. After that starvation time cells were stimulated with the phorbol ester TPA for 4h at 37°C to activate AP-1. Cells were washed 3 times with ice cold PBS-/-,scraped in 1ml PBS-/- with a rubber-blade cell scraper, and pelleted by centrifugation (1200rpm, 3min, 4°C). The supernatant was discarded and cells were washed in 5 times pellet-volume of hypotonic lysis buffer (10mM HEPES KOH pH 7,9; 1,5mM $MgCl_2$; 10mM KCl; add PIC; 0,5mM DTT) followed by resuspension in 3 times cell pellet volume of hypotonic lysis buffer, and allowed to swell on ice for 10min. Swollen cells were then disrupted on ice in a douncer (10-15 strokes). The cell lysate was then centrifuged to pellet the nuclei (4000rpm, 4°C, 15 min). The nuclei pellet was resuspended in ½ nuclei pellet volume of low salt extraction buffer (20mM HEPES KOH pH 7,9; 25% glycerol; 1,5mM $MgCl_2$; 20mM KCl; 0,2mM EDTA; add PIC; 0,5mM DTT). Then ½ nuclei pellet volume of high salt buffer (20mM HEPES KOH pH 7,9; 25% glycerol; 1,5mM $MgCl_2$; 1,2M KCl; 0,2mM EDTA; add PIC; 0,5mM DTT) was added dropwise, and nuclear proteins were extracted by incubation at 4°C for 30min under constant rotation. The extracted nuclei were ultracentrifuged (15000rpm, 4°C, 30min). The supernatant containing nuclear proteins was then dialysed in dialysis-cassettes (Thermo scientific) against DNA-pulldown buffer (DPD; 20mM HEPES KOH pH 7,9; 10% glycerol; 50mM NaCl; 5mM $MgCl_2$; 0,5mM EDTA; 0,1% NP-40; add PIC; 0,5mM DTT) over night at 4°C. Next day the dialysed protein solution was collected and protein content was measured using Bradford and stored until usage at -80°C.

2.2.5.2. Preparation of biotinylated DNA template

The biotinylated DNA fragment was generated by PCR using the Collagenase-I-promoter-Luc vector. The forward primer of the PCR to generate the DNA-template was ordered biotinylated at its 5´end. Due to this, the bait can be coupled to streptavidin.

Template-preparation via PCR:

PCR Mixture:		PCR-Program		
5xPCR buffer	4µl	1cycle:		
DMSO	1µl		5min	94°C
forward primer biotin 10mM	1,5µl	40 cycles:		
reverse primer 10mM	1µl		1min	95°C
10mM dNTP	0,45l		1min	55°
Taq-DNA polymerase	0.25µl		xmin	72°C (depending on size of fragment)
Template CollI-Luc	0,1µg	1cycle:		
H_2O	_____		hold at 4°C	
	add 25µl			

To purify the PCR by electrophoresis a sufficient amount of the 180bp bait fragment, 40-50 PCR reactions were pooled and loaded on a 2% agarose-gel. The bait fragment was purified by the "freeze-squeeze" method. The biotinylated DNA-template was dissolved in 50µl 1xTE-buffer, and concentration was estimated by comparing with a DNA of known concentration followed by a semi-quantitative gel electrophoresis.

2.2.5.3. DNA pulldown

50µg nuclear extracts were mixed together with 100ng of biotinylated DNA template, 100ng of salmon sperm DNA, to block unspecific binding to DNA, in DPD containing 0,5mM DTT in a total volume of 100µl. The reaction was incubated for 20min at room temperature under constant rotation. The biotinylated DNA bait and the bound proteins were captured by magnetic beads coupled to streptavidin (Dynabeads) blocked by incubation with digested BSA to reduce unspecific binding of proteins.

BSA-digest:
0,5g of BSA were dissolved in 10ml 50mM NH_4CO_3 and denatured for 10min at 95°C. Then 10mg/ml trypsin was added to the solution and incubated at 37°C over night to digest the BSA. To check if the digest was complete, 1mg of digested BSA was loaded on a 10% acrylamide-gel and after electrophoresis stained with coomassie-brilliant blue. Undigested BSA would lead to a signal at ~70kDa, digested BSA can not be visualized with Coomassie-brilliant-blue.

Afterwards 10µl of pre-blocked streptavidin-Dynabeads were added to the mix and incubated for 30min at 4°C under mild rotation to allow the magnetic beads the capture of the biotinylated DNA and associated protein complexes. The magnetic beads were washed using a magnet and washing was performed by soaking for 5 times in 0,5ml of DPD at 4°C. The

pulled down protein complexes were harvested by adding 30µl 2x sample buffer and boiling for 5 min at 95°C, separated from the beads by centrifugation, and separated by SDS-PAGE on a 8% or 12% acrylamide gel to resolve high-molecular and low-molecular proteins, respectively.
To visualize the separated proteins, a modified silver staining protocol was used, which allows a characterization of the proteins by MALDI-TOF.

Modified Silverstain-protocol:
The acrylamide-gels were first fixed twice by transferring in 10% acetic acid, 40% ethanol for 15min at room temperature. Then the gels were sensitized by incubating with 250ml sensitizing solution (75ml Ethanol; 10ml Na-thiosulphate (5%); 17g Na-acetate (250ml ddH_2O)) for 30min at room temperature. After sensitizing, the gels were washed 3 times for 5 min each in 250ml ddH_2O and the silver solution (25ml silver nitrate (2,5%; 250ml ddH_2O))was added for 20min. After washing the gels twice in ddH_2O for 1min each the staining was developed by adding a developer solution (6,25g Na-carbonate; 100µl formaldehyde (add250ml ddH_2O)) for 4-5min at room temperature. The developing reaction was stopped by exchanging the solution with a stop solution (3,65g EDTA in 250ml ddH_2O). Finally the gels were washed 3 times with ddH_2O for 5min each and gels could then be scanned, bands excised and stored at 4°C.

2.2.6. Tissue culture methods

2.2.6.1. Passaging cells

After removing the old tissue-culture media Trypsin-containing solution (2ml, 0,25% trypsin) was added to the cells and transferred to a incubator for 5min. Thereafter 10ml fresh medium was applied to the detached cells to stop trypsin-dependent digest and mixture was resuspended to separate cells. This mixture was administered to new Petri dishes according to the planned dilution.

2.2.6.2. Seeding cells

After removing the old tissue-culture media Trypsin-containing solution (2ml, 0,25% trypsin) was added to the cells and transferred to a incubator for 5min. Thereafter 10ml fresh medium was applied to the detached cells to stop trypsin-dependent digest and mixture was resuspended to separate cells.
Thereupon cells were collected within a falcon-tube (15ml), centrifuged (1500 rpm, 5min), and the supernatant was removed. The cell-pellet was resuspended in 10ml fresh medium.
To count cells per ml, 10µl were transferred to a Neubauer counting chamber. Cells were counted by using a brightfield-microscope.

2.2.6.3. Freezing and thawing of cells

For freezing, logarithmically growing cells were trypsinised as described above and collected by centrifugation. Cells were resuspended in freezing medium (DMEM, 40%FSC, 10%DMSO) and transferred into cryostatic tubes. After incubation on ice for 30 minutes, cells were slowly frozen at -80°C and then transferred to liquid nitrogen. For re-propagation cells were thawed quickly at 37°C and transferred to fresh medium. The next day medium was replaced with fresh cell culture medium.

2.2.6.4. Transfecting cells

Cells were seeded 24h before transfection into dishes or plates with a cell number according to the surface.

	Surface in cm^2	HeLa	Cos-7	NIH3T3
10cm	55	$2x10^6$	-	-
6well	9,4	$5x10^5$	$1x10^5$	-
12well	3,8	$3x10^5$	-	-
24well	1,9	$1x10^5$	$5x10^4$	-
96well	0,4	$1x10^4$	$5x10^3$	-
8well chamber slide	0,95	$1,5x10^4$	$5x10^3$	$1,5x10^4$

Transfection cells using Lipofectamin2000 (Invitrogen):
According to 8-well-chamber slide

In a 1.5ml Eppendorf-reactiontube 25µl D-MEM without serum were provided and the calculated amount of DNA was added (max. 200ng).
After the preparation of all samples Lipofectamin2000 was added into a separated vessel (15ml falcon-tube or 1,5ml Eppendorf reactiontube depending on total number of transfections), mixed with D-MEM (25µl/well) and incubated 3min at room temperature. The amount of Lipofectamin2000 to use is dependent on the amount of DNA and cell line. Next step was the mixture of DNA-D-MEM with Lipofectamin2000-D-MEM. 25µl of the transfectionagent D-MEM mix was administered to the DNA mix and inverted several times to mix properly. This mixture was then incubated 15min at room temperature.
In the mean time the growing medium was changed and fresh prewarmed medium was applied onto the cells.
After incubation the DNA-transfection reagent-mixture was applied onto the cells and transferred again to the incubator.
After 5h incubation the medium was changed again and replaced by fresh growing medium.

Transfection cells using FuGENE6 (Roche):
According to 8-well-chamber slide

In a 1.5ml Eppendorf-reactiontube 12,5µl D-MEM without serum were provided and the calculated amount of DNA was added (max. 200ng).
After the preparation of all samples FuGENE6 was added into a separated vessel (15ml falcon-tube or 1,5ml Eppendorf reactiontube depending on total number of transfections), mixed with D-MEM (12,5µl/well). The amount of FuGENE6 to use is dependent on amount of DNA and cell line. Next step was the mixture of DNA-D-MEM with FuGENE6-D-MEM. 12,5µl of the transfectionagent D-MEM mix was administered to the DNA mix and inverted several times to mix properly. This mixture was then incubated 30-45min at room temperature.
In the mean time the growing medium was changed and fresh pre-warmed medium was applied onto the cells.
After incubation the DNA-transfectionagent-mixture was applied onto the cells and transferred again to the incubator.

2.2.6.5 Inducing cells

UV irradiation: the medium was collected; cells were washed with PBS -/- and irradiated with 40J/m2 UV-C light; the collected medium was poured back on cells.
Dexamethasone (DEX): after starvation of cells DEX (10^{-6}M) was added to the medium. Stock of DEX (10^{-2}M) is stores at -20°C in EtOH.
Phorbol ester (TPA): after starvation TPA was added to medium to final concentration 50ng/ml. Stock of TPA (200µg/ml) is stored at -20°C.

2.2.6.6. DNA-fluorescent in situ hybridisation (DNA-FiSH)

Fixation and denaturing:
Cells were seeded and cultured on glass coverslips (1,5cm ∅, Roth, Germany), then fixed by incubating with 8% paraformaldehyde for 30 min at room temperature. Cells were then washed 3 times in PBS(-/-) for 10 min each, and permebealized for 10 min in 0.5%Triton x-100 in PBS(-/-) and washed again in PBS(-/-). Cells were then incubated with 50µg/ml RNAse for 60 min and then washed 3x for 10 min with PBS(-/-). Denaturing of DNA was achieved by incubating the coverslips at 95°C for 5min in presence of 50% formamid/2xSSC, then for 5min on ice in 50%formamide/2xSSC. The cells were then dehydration for 5min each in 70, 90 and 100% ethanol on ice.

Probe preparation and hybridisation:
As a probe a fragment of luciferase coding sequence was amplified by PCR:
A dUTP conjugated to Alexa 546 (Chromatide dUTP, Molecular Probes/Invitrogen) was incorporated into the PCR reaction to fluorescently label the PCR product. After gel-purification the probe was dissolved in 140µl ddH_2O, denatured by addition of 100µl formamide and heated for 10 minutes at 70°C. The probe was chilled in iced water and 160µl of 2,5x hybridisation solution (5xSSC,25%Dextran, 2,5mg/ml tRNA) was added. The fluorescent probe was stored until used at -20°C.

Primers:
Forward: TCTACTGGTCTGCCTAAAGG
Reverse: AAACATTCCAAAACCGTGAT

PCR Mixture:

10xPCR buffer	5µl
25mM $MgCl_2$	3µl
1ng/µl template DNA	1µl
forward primer 10mM	1µl
reverse primer 10mM	1µl
10mM dATP	1µl
10mM dCTP	1µl
10mM dGTP	1µl
2mM dTTP	3,75µl
1mM Chromatide dUTP	2,5µl
GoTaq®DNA polymerase	2,5U
H_2O	
	add 50µl

PCR-Program

1cycle:	
5min	94°C
5min	55°C
30cycles:	
2min	72°C
1min	94°C
1min	55°C
1cycle:	
5min	72°C
hold at	4°C

Hybridisation:

20µl of hybridisation solution containing the fluorescent probe was deposited on a glass slide and the cover slips with the cells were inverted onto the solution and incubated in the dark over night at 37°C. On next day, cells were washed with 2xSSC,0,05%Triton-X100 for 10minutes, 2xSSC for 10 minutes, 4xSSC for 5minutes and rinsed with PBS(-/-). Finally the cover slips were mounted on glass slides using an aqueous mounting medium (DAKO).

2.2.6.7. Reporter gene assays

2.2.6.7.1. Collagenase-I-Luciferase reporter assay

$1x10^5$ logarithmically growing HeLa-cells were seeded in a 24well plate and grown overnight. The next day cells were transfected with Lipofectamine 2000 reagent in ratio 3µl of

transfection reagent to 1µg of DNA. 100-400ng of plasmid DNA and 50 ng of reporter DNA (-517/+63Coll-Luc) were used for transfection. After transfection cells were incubated for 20h before starvation in 0.5% DCS containing DMEM medium. The cells were pre-starved for 8h and then cells were not induced or induced with TPA (50ng/ml) over night. Next day cells were subjected to measurement. They were washed twice with ice cold PBS-/- and lysed on ice in 25µl of 1x lysis buffer (Promega) and after successful lyses the luciferase activity was measured. Measurements were performed on Luminescence Counter.

2.2.6.7.2. Gal-Luciferase reporter assay

$1x10^5$ logarithmically growing HeLa-cells seeded in a 24well plate. Day after seeding cells were transfected with Lipofectamine 2000 reagent in ratio 3µl of transfection reagent to 1µg of DNA. 100-400ng of plasmid DNA and 100 ng of reporter DNA (GAL4UAS-Luc) were used for transfection. After transfection cells were incubated for 20h before starvation in 0.5%DCS containing DMEM medium over night. Next day cells were subjected to measurement. They were washed twice with ice cold PBS-/- and lysed on ice in 25µl of 1x lysis buffer (Promega) and after successful lyses the reporteractivity was measured. Measurements were performed on Luminescence Counter.

2.2.6.7.3. Array cell lines

$1,5x10^4$ logarithmically growing NIH3T3-2u, 7m or 12c-cells (or NIH3T3-parental cells as control) were seeded in a specific 96well plate, which is designed for luminometers. The cells can be directly lysed in these plates and subjected to measurement. Day after seeding cells were pre-starved for 8h and then cells were not induced or induced (clone 2u with TPA(50ng/ml) or UV-irradiated ($40J/m^2$); clone 7m with Dexamethasone (final concentration of 10^{-6}M); clone 12c with TPA (50ng/ml)) over night. Next day cells were subjected to measurement. They were washed twice with ice cold PBS-/- and lysed on ice in 25µl of 1x lysis buffer (Promega) and after successful lyses the reporteractivity was measured. Measurements were performed on Luminescence Counter.

2.2.6.7.4. Inhibiting AP-1 function by blocking peptides

$1x10^4$ logarithmically growing HeLa-cells seeded in a specific 96well plate, which is designed for luminometers. The cells can be directly lysed in these plates and subjected to measurement. Day after seeding cells were transfected with Lipofectamine 2000 reagent in ratio 3µl of transfection reagent to 1µg of DNA. 25-100ng of plasmid DNA encoding blocking peptides (pcDNA3.1(+)-mcherry-NLS-DD1 or mCherry-NLS-$DD1_{SCR}$) and 25 ng of reporter DNA (-517/+63Coll-Luc) were used for transfection. After transfection cells were incubated for 20h before starvation in 0.5%DCS containing DMEM medium. The cells were

pre-starved for 8h and then cells were not induced or induced with TPA (50ng/ml) over night. Next day cells were subjected to measurement. They were washed twice with ice cold PBS-/- and lysed on ice in 25µl of 1x lysis buffer (Promega) and after successful lyses the reporteractivity was measured. Measurements were performed on Luminescence Counter.

2.2.6.7.5. Reverse Crosstalk-inhibiting GR transcription factor activity through c-Fos

$1x10^5$ logarithmically growing Cos-7-cells seeded in a 24well plate. Day after seeding cells were transfected with FuGENE6 reagent in ratio 3µl of transfection reagent to 1µg of DNA. 100-400ng of plasmid DNA encoding for pcDNA-3.1(+)-HA-c-Fos, pCMV-p65-GFP and pCMV-p65-YFP, 20ng of pcDNA3.1(+)-HA-GR and 20 ng of reporter DNA (MMTV-Luc) were used for transfection. After transfection cells were incubated for 20h before starvation in 0.5%FCS containing DMEM medium. The cells were pre-starved for 8h and then cells were not induced or induced with DEX (10^{-6}M) over night. Next day cells were subjected to measurement. They were washed twice with ice cold PBS-/- and lysed on ice in 25µl of 1x lysis buffer (Promega) and after successful lyses the reporteractivity was measured. Measurements were performed on Luminescence Counter.

2.2.6.7.6. Luciferase activity measurement

The amount of expressed *Photinus pyralis*-luciferase (Firefly) was measured from the lysed cell extracts.
24h-48h post transfection cells were washed twice in ice-cold PBS-/- followed by adding lysis buffer in a volume appropriate to the well size (e.g. 25µl in 96well-plate, 100µl in 24well-plate) and lysis was carried out for 15min at 4°C. After lysis, 25 µl of lysates were transferred into a white 96well plate and subjected to luciferase measurement in an automated luminometer (Perkin Elmer). The reaction buffer and substrate were injected automatically into each well (70µl/well reaction buffer, 20µl/well substrate). The luminescence was measured for 2 seconds.

Luciferin stock solution:	1mM luciferin (0,28mg/ml) in Gly-Gly-buffer
Luciferin reactionmix:	1:5 dilution of stock in Gly-Gly-buffer
Gly-Gly-buffer:	25mM Gly-Gly; 15mM $MgSO_4$; 4mM EGTA; pH 7,8
Reaction-buffer:	1mM DTT; 2mM ATP in Gly-Gly-buffer
Lysis-buffer:	1:5 Dilution of 5x passive lysis buffer (Promega) in ssH_2O

2.2.6.8. *in vivo* protein-protein-interaction via bimolecular fluorescent complementation assay

A system to study protein-protein interaction in living cells is the bimolecular fluorescence complementation (BiFC) (Hu et al., 2002; Hu et al., 2006). This assay is based on the splitting of the yellow fluorescent protein (YFP) into two parts, YC (C-terminal part of YFP) and YN (N-terminal part of YFP). These two parts do not spontaneously re-associate and do not fluoresce. By fusing YC and YN to two interacting proteins, the two halves of YFP are brought together, leading to complementation of the fluophore. The advantage of the BiFC system compared to other protein-protein interaction systems is that the localisation of the interaction can be monitored by microscopy.

1. YFP-based BiFC (Hu et al., 2002)

Cells were seeded with a density of $1.5x10^4$ cells/well in NUNC-8-well chamber slides and grown overnight at 37°C. Then they were transfected with 150ng of vector-DNA fused to the N-terminal part of YFP (YN) alone or together with vector-DNA fused to the C-terminal part of YFP (YC). As a transfection control cells were co-transfected with pcDNA3.1+-mCherry or dsRed-mito (30ng/well). As a transfection reagent I used Lipofectamin2000 (Invitrogen) with a DNA:Lipofectamin2000 ratio of 1:3. After 5h the medium was changed. Next day cells were transferred into a 30°C incubator for two hours to allow the maturation of the complemented YFP-protein after interaction. Imaging was performed using a Zeiss LSM 510 Meta in confocal multitracking mode.

2. Venus-based BiFC (Hu et al., 2006)

Cells were seeded with a density of $1.5x10^4$ in NUNC-8-well chamber slides and grown overnight at 37°C. Then they were transfected with 750ng of vector-DNA fused to the N-terminal part of Venus (VN) alone or together with vector-DNA fused to the C-terminal part of Venus (VC). As a transfection control cells were co-transfected with pcDNA3.1+-mCherry (15ng/well). As a transfection reagent I used Lipofectamin2000 (Invitrogen) with a DNA:Lipofectamin2000 ratio of 1:3. After 5h the medium was changed. Next day cells were directly subjected to microscopy using a Zeiss LSM 510 Meta in confocal multitracking mode.

2.2.6.9. Fluorescence resonance energy transfer (FRET)

This protein-protein interaction assay is also termed Förster resonance energy transfer, named after the scientist Theodor Förster. FRET describes an energy transfer mechanism between two chromophores or fluorescent proteins. A donor chromophore in its excited state can transfer energy by a nonradiative mechanism to an acceptor chromophore in close proximity

(<10nm or <100Å). The energy transfer is analogous to a near field radio, because the radius of interaction is much smaller than the wavelength of light used. The excited atom emits a virtual photon which is accepted by the receiving atom. The recipient or acceptor chromophore is excited emits light with a shift in wavelength compared to the wavelength of the emitted light from the donor, reflecting the close proximity of the two interaction partners during a protein-protein interaction, (Piston and Kremers, 2007).

To study the dimerisation of nTrip6 in living cells, I used Trip6$^{190-476}$ fused to CFP as a FRET donor, and Trip6$^{190-476}$ fused to YFP as an acceptor. Cells were seeded with a density of 1.5x10^4 cells/well in NUNC-8-well chamber slides and grown overnight at 37°C. Then they were transfected with 100ng of Trip6$^{190-476}$ fused to CFP alone, with Trip6$^{190-476}$ fused to YFP alone or transfected together. As a transfection reagent I used Lipofectamin2000 (Invitrogen) with a DNA:Lipofectamin2000 ratio of 1:3. After 5h the medium was changed. Next day cells were directly subjected to microscopy using a Zeiss LSM 510 Meta in confocal multitracking mode (see Results section for details).

2.2.6.10. Immunofluorescence

1. Antibody staining of adherent cells

Cells seeded and cultured on glass-coverslips (1,5cm ∅, Roth, Germany) were fixed with 3,7% Formalin in PBS-/- directly added onto cells for 10 min at room temperature followed by permeabilisation in 0,5% Triton X-100 in PBS for 5min. Cells were then washed 3 times with PBS-/- for 10 min. To avoid unspecific binding, cells were blocked with 1%BSA/1%Goat-serum in PBS-/- for 30min. Antibodies diluted in blocking buffer (1g BSA/1ml goat serum in 100ml PBS-/-), were deposited on parafilm and the cells were incubated for 1h with the antibodies by inverting coverslips onto the antibody drop.
The coverslips were collected using forceps and transferred into a 24 well plate for washing steps. Cells were washed 3 times in PBS-/- for 5 min.
The appropriate secondary fluorescent labelled antibody was added for 30 min and then washed as described for the primary antibody.
Finally coverslips were mounted in polyvinylalcohol (PVA: 20% Polyvinyl alcohol (Vinol 205); 80ml PBS-/-) onto a glass slide. Images were taken with a Zeiss (Oberkochen, Germany) LSM510meta in confocal multitracking mode using a x63/ -oil lens.

2. Antibody-staining to study array-occupancy of NIH3T3 2U cells

This procedure is similar to antibody staining of adherent cells except fixation. Cells were fixed by adding 8% PFA (8 g Paraformaldehyd in 100 ml H_2O at 60°C and adding 1M NaOH until solution is clear and PFA-powder completely dissolved) directly onto cells still in culture medium (final concentration PFA: 4%) for20 min at room temperature.

3. Results

Trip6 is described as a cytosolic protein, enriched at focal adhesions and at cell-cell contacts (Wang et al., 1999; Wang and Gilmore, 2001; Yi and Beckerle, 1998). This is illustrated by the Trip6 immunofluorescence-staining (Fig. 12), where focal adhesions were stained with an anti-Vinculin antibody and cell-cell contacts by an anti-E-Cadherin.

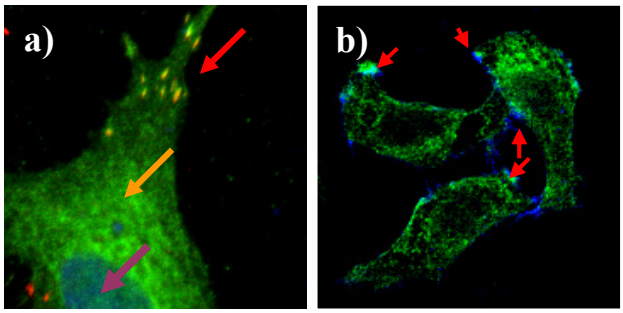

Fig.12: Trip6 colocalizes with Vinculin, a marker for focal adhesions and E-Cadherin, marker for cell cell contacts
HeLa were fixed and stained with a Trip6-specific antibody together with an antibody against Vinculin or E-Cadherin, and nuclei were counterstained using DRAQ5.
a) anti-Trip6 (green) and anti-Vinculin (red); DRAQ5 (nuclear marker, blue)
b) anti-Trip6 (green) and anti-E-Cadherin (blue)
An overlap between nTrip6 and DRAQ5 can be observed within the nucleus as well as an overlap between Trip6 and Vinculin at focal adhesion sites and Trip6 and E-Cadherin at cell-cell contac sites.

Our group identified nTrip6, a shorter isoform of Trip6, presumably lacking the nuclear export signal (NES), and thus exclusively present in the nucleus. At the time of this work, the exact nature of nTrip6 was not known. I therefore used Trip6$^{190\text{-}476}$ as a tool (Kassel et al., 2004). This protein is shorter in size, lacks the NES and is thus exclusively nuclear (Fig. 13), and is fully functional to regulate AP-1 activity (Kassel et al., 2004).

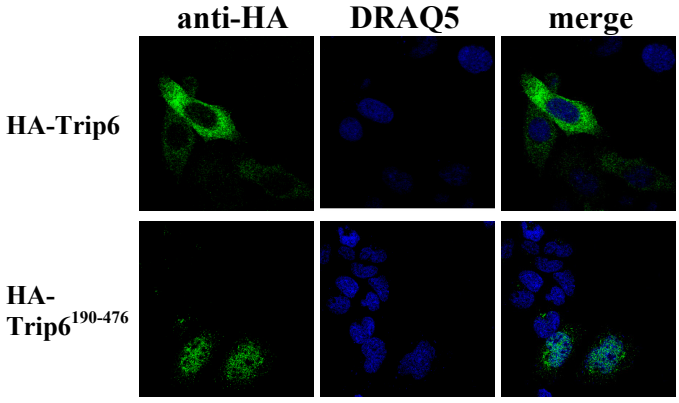

Fig.13: Subcellular localisation of Trip6 and nTrip6
HeLa-cells were transfected with expression vectors for Trip6 fused to HA (HA-Trip6) or with HA fused Trip6$^{190\text{-}476}$ (HA-Trip6$^{190\text{-}476}$). Cells were then stained with an HA specific antibody and the nuclei were counterstained with DRAQ5.
Trip6 is detectable in the cytosol, whereas Trip6$^{190\text{-}476}$ is localised exclusively in the nucleus.

An HA-tagged version of Trip6$^{190\text{-}476}$ is exclusively expressed in the nucleus, whereas HA-Trip6 is only cytosolic, as shown by immunofluorescence staining (Fig. 13).

3.1. nTrip6 is a specific co-activator for c-Fos containing AP-1 dimers

To investigate the co-activator function of nTrip6 for AP-1, I co-transfected HeLa cells with the AP-1-dependent -517/+63CollI-Luc reporter gene and increasing amounts of Trip6$^{190-476}$, and monitored the transcriptional activity after activation of AP-1 by treatment of the cells with the phorbol ester TPA.

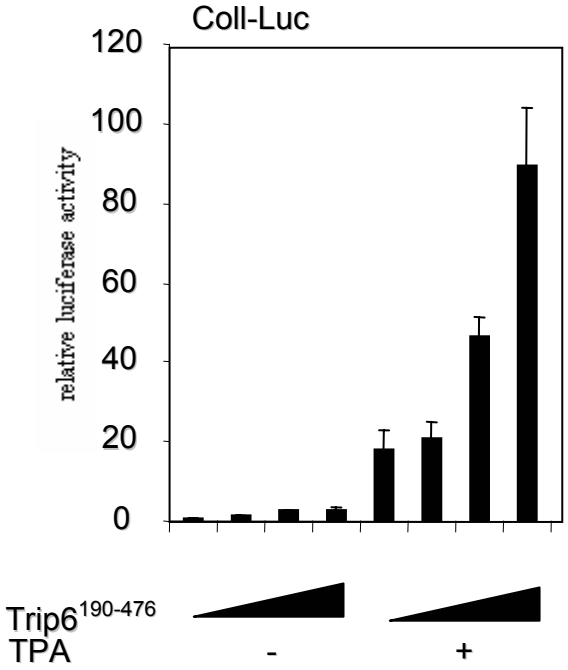

Fig.14: nTrip6 functions as a co-activator for AP-1
1,5x10^5 logarithmically growing HeLa-cells were transfected with the AP-1 regulated -517/+63CollI-Luc reporter gene together with increasing amounts of Trip6$^{190-476}$. 24h post transfection the cells were serum-starved with 0,5% DCS-DMEM and were induced overnight by treatment with TPA to induce AP-1. Next day cells were lysed and subjected to Luciferase reporter assay. Results are presented as fold induction, and are the mean ± SD of a representative experiment performed in triplicates.

When I co-transfected HeLa cells with increasing concentrations of Trip6$^{190-476}$, in untreated conditions the reporter showed only a weak expression (Fig. 14). This basal level indicates low amounts of AP-1 present under unstimulated conditions. Treating cells with TPA resulted in a 20 fold increase in reporter activity. This activation was further increased in a dose dependent manner by co-transfection of increasing concentrations of Trip6$^{190-476}$ (Fig. 14). This illustrates the already documented (Kassel et al., 2004) co-activator function of nTrip6 for AP-1.

3.1.1. nTrip6 interacts exclusively with AP-1 dimers containing Fos-family members

At the start of this work, unpublished results of our group showed that over-expression of nTrip6 was not able to increase the transcriptional activity of the c-Jun:ATF2 AP-1 dimer. AP-1 is a transcription factor family composed of dimers of members of the Jun, Fos and CREB/ATF family. Is nTrip6 a common co-activator for all family members or is it limited to a certain dimer composition? As a first step to address this question, I investigated the interaction of nTrip6 with two typical AP-1 dimers c-Jun:c-Fos and c-Jun:ATF2. Both dimers are activated by different pathways and bind to dimer specific responsive elements in the

vicinity of their target genes. c-Jun:c-Fos binds to the heptameric TPA responsive elements (TRE) whereas cJun:ATF2 recognizes an octameric CREB-responsive element (CRE). To investigate the interaction between nTrip6 and these dimers, I used single chain AP-1 dimers, c-Jun~c-Fos and c-Jun~ATF2, provided by Latifa Bakiri (Bakiri et al., 2002). In these constructs c-Jun is linked to c-Fos or ATF2 by a flexible linker. Due to this tethering c-Jun dimerisation capacity is limited to its fusion partner. This ensures that any measured interaction is really via the studied dimer, and not due to c-Jun dimerising with endogenous AP-1 family members.

A system to study protein-protein interaction in living cells is the bimolecular fluorescence complementation (BiFC) (Hu et al., 2002; Hu et al., 2006). This assay is based on the splitting of the yellow fluorescent protein (YFP) into two parts, YC (C-terminal part of YFP) and YN (N-terminal part of YFP). These two parts do not spontaneously re-associate and do not fluoresce. By fusing YC and YN to two interacting proteins, the two halves of YFP are brought together, leading to complementation of the fluophore. The advantage of the BiFC system compared to other protein-protein interaction systems is that the localisation of the interaction can be monitored by microscopy.

To study the interaction between nTrip6 and the different dimer pairs, I designed fusion proteins composed of Trip6$^{190-476}$ C-terminaly fused to YN and cJun~cFos as well as cJun~ATF2 fused to YC. To investigate if the expression and localisation of the fusion proteins was proper, I transfected HeLa-cells with these constructs and performed an antibody staining against the HA or the FLAG epitope also encoded in the construct.

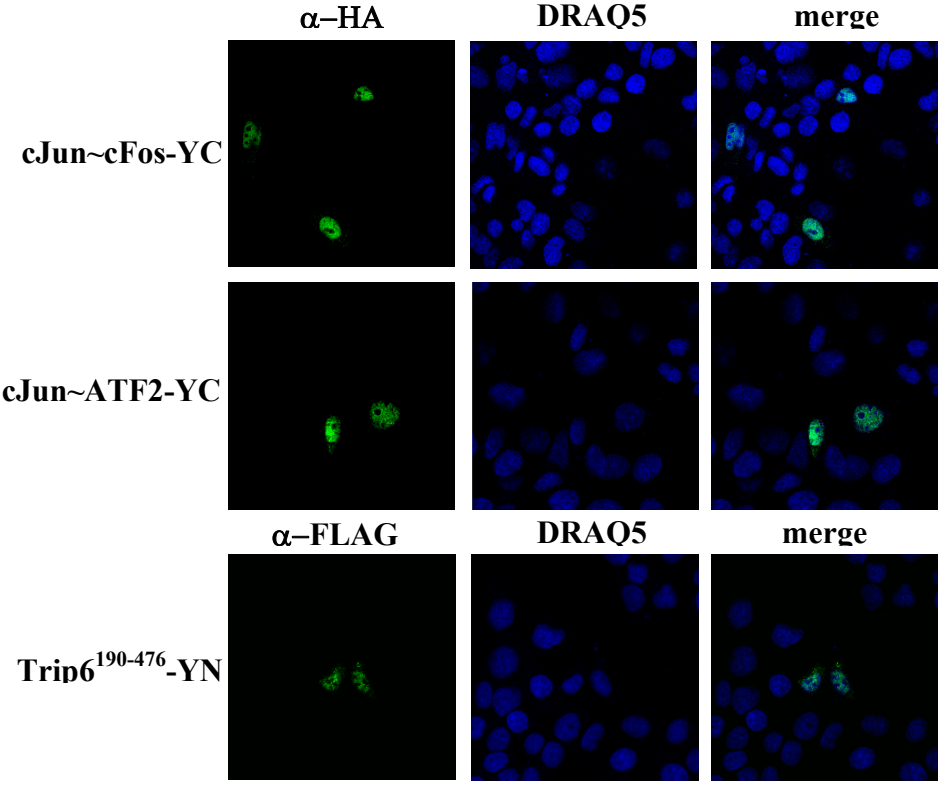

Fig. 15: BiFC-fusion of Trip6$^{190-476}$ and the single chain AP-1 dimers does not hamper proper localisation
HeLa cells were transfected with Trip6$^{190-476}$ fused to the N-terminal part of YFP (YN), with cJun~cFos or cJun~ATF2 fused to the C-terminal half of YFP (YC) and probed with an antibody against the HA- or FLAG-tag. The nuclei of live cells were stained with DRAQ5 and fluorescent images were acquired by confocal microscopy.

Immunoreactivity was detectable within the nucleus of transfected cells (Fig. 15), showing that the fusion of Trip6$^{190-476}$ to YN as well as the fusion of the single-chains c-Jun~c-Fos and cJun~ATF2 to YC did not hamper the localisation of the protein. I then used these constructs to investigate by BiFC the protein-protein interaction between nTrip6 and the two prototypical AP-1 dimers in living cells (Fig. 16).

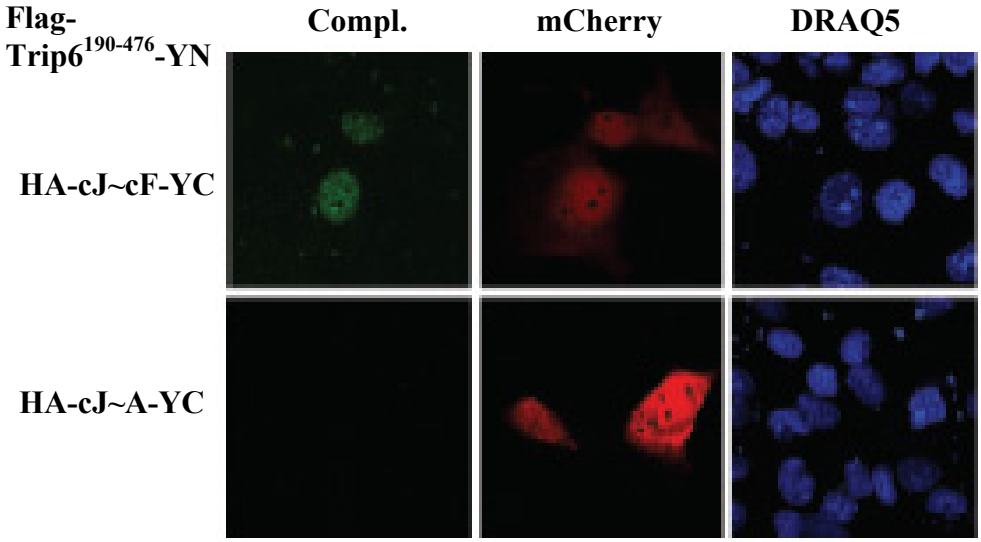

Fig. 16: nTrip6 selectively interacts with c-Fos containing AP-1 dimers
HeLa cells were transfected with Trip6$^{190-476}$ fused the N-terminal half of YFP (YN) together with either c-Jun~c-Fos YC or c-Jun~ATF2 fused to YC. As a transfection control cells were co-transfected with mCherry and nuclei were highlighted by DRAQ5. 16h post transfection cells were imaged using a laser scanning confocal microscope.

The complementation of YFP was observed in the nucleus of 70 to 80% of cells co-transfected with equal amounts of the single chain c-Jun~c-Fos fused to YC and Trip6$^{190-476}$ fused to YN. No complementation was detected between c-Jun~ATF2-YC and Trip6$^{190-476}$-YN, although the cells were efficiently transfected, as judged by the expression of the cherry-red fluorescent protein mCherry, which served as a transfection control (Fig. 16). This result shows that nTrip6 interacts with c-Jun~c-Fos, but not with c-Jun~ATF2 in living cells, confirming the results of in vitro experiments with individual AP-1 family members (Heilbock, 2004).

We therefore conclude that nTrip6 is selectively interacting with AP-1 dimers containing Fos-family members, and thus might be a selective co-activator for these AP-1 dimers.

3.1.2. Recruitment of nTrip6 to AP-1 dependent promoter in a dimer specific fashion

If nTrip6 is a selective co-activator for certain AP-1 dimers, it should be selectively recruited to promoters of target genes together with the AP-1 dimer.

To directly visualize nTrip6 recruitment, I used a reporter cell line containing an integrated array of multiple copies of the -1977/-1858uPA-Luc reporter gene generated in our lab. This part of the uPa gene contains both a CRE and a TRE, and can be activated by both, c-Jun:c-Fos and c-Jun:ATF2. The array was generated by transfecting the reporter plasmid together with the pΔBN-AR1 plasmid (Shimizu et al., 2003). pΔBN-AR1 contains a mammalian replication initiation origin and a matrix attachment region from the Chinese hamster dhfr gene: when integrated into the genome, it initiates events similar to gene amplification in cancer cells, leading to tandem repeats of up to 10 000 copies (Shimizu et al., 2003; Shimizu et al., 2001).

The high local concentration of binding sites on the promoter of the amplified gene unit enables the direct visualization of binding to the gene array of proteins tagged with a fluorescent marker, as previously shown for an NF-κB-dependent array cell line (Bosisio et al., 2006). Using this amplification method NIH-3T3 fibroblasts carrying several hundred integrated uPA-Luc plasmid copies in discrete loci were obtained (Fig. 17). One of the cell clones, clone 2U, was shown by real-time PCR to harbour an integrated gene-array of about 2000 gene units (data not shown).

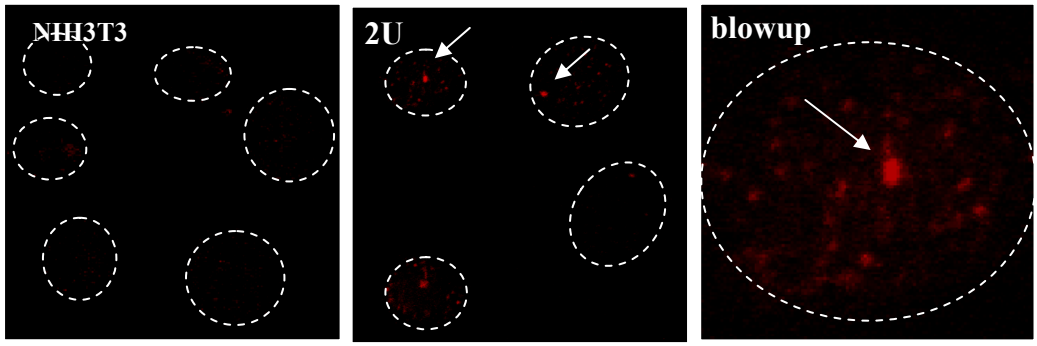

Fig. 17: DNA fluorescence in situ hybridisation (DNA-Fish) highlights the uPA-Luc gene-array
Clone 2U and parental NIH-3T3 fibroblasts were subjected to DNA *in situ* hybridization using a fluorescently labeled cDNA probe complementary to the luciferase coding sequence. A single gene array is visible in 100% of the 2U cells.

The presence of the uPA-Luc gene-array was confirmed by DNA fluorescent in situ hybridization (FiSH) using a fragment of the luciferase coding sequence as a probe. The staining was detectable as a single spot within the nucleus of the 2U cells, and was present in 100% of the cells, whereas only background staining was visible in the parental NIH-3T3 cells (Fig. 17).

To investigate if the gene array was functional, clone 2U cells were subjected to different stimuli: either a treatment with TPA to activate c-Jun:c-Fos (Angel et al., 1988b) or UV-irradiation to activate c-Jun:ATF2 (Adler et al., 1995; Adler et al., 1996), and the reporter gene activity was measured.

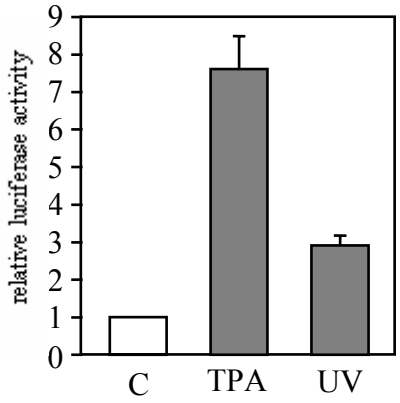

Fig. 18: The uPA-Luc gene array within clone 2U is functional
$1x10^4$ logarithmically growing 2U-cells were seeded in a 96 well plate. 24h post seeding cells were serum-starved with 0,5% DCS-DMEM and c-Jun:c-Fos was induced overnight by adding TPA, or c-Jun:ATF2 was induced by UV-irradiation. Next day cells were lysed and subjected to Luciferase reporter assay. Results are presented as fold induction, and are the mean ± SD of a representative experiment performed in triplicates.

Both TPA treatment and UV irradiation induced luciferase activity in clone 2U (Fig. 18), suggesting that both c-Jun:c-Fos and c-Jun:ATF2 are able to bind to their cognate response elements on the array and to activate transcription from the uPA-Luc gene array.

The next step to confirm the functionality of the array was to study the recruitment of the two AP-1 dimers to the array, and their ability to mediate the recruitment of the RNA polymerase II (Pol II) to the array (Fig. 19).

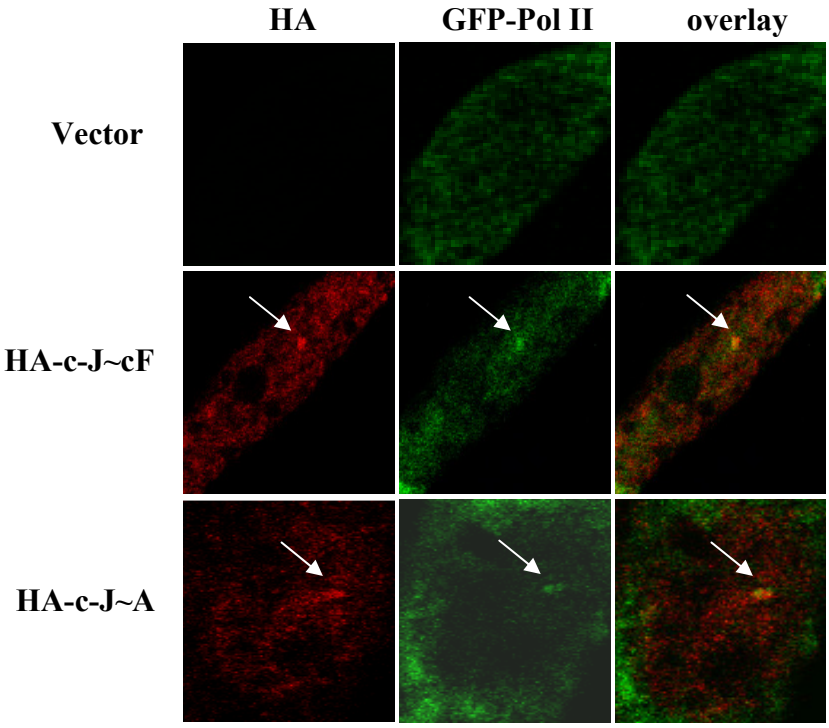

Fig. 19: AP-1 is functionally recruited to the uPA promoter-array
2U cells were transfected with GFP-Pol II, together with the empty vector (Vector), an expression vector for an HA-tagged c-Jun~c-Fos single chain (HA-c-J~c-F), or an expression vector for an HA-tagged c-Jun~ATF2 single chain (HA-c-J~A). The localization of the AP-1 single-chain proteins was determined by immunofluorescence using an anti-HA antibody. The enrichment of the AP-1 proteins and of GFP-Pol II was observed in 70–80% of the co-transfected cells.

Over-expression of an HA-tagged version of the c-Jun~c-Fos single chain construct led to its recruitment to the array, as shown by the enrichment to a single bright spot in the nucleus after immunostaining with an anti-HA antibody. Furthermore, c-Jun~c-Fos mediated the

recruitment of Pol II to the array, as shown by the co-localization of the HA staining with GFP-Pol II (Fig. 19, overlay). Similarly, co-transfected HA-c-Jun~ATF2 and GFP-Pol II were co-recruited to the array. These specific enrichments to the array were observed in 70 to 80% of the transfected cells. GFP-Pol II was not enriched to the array when transfected alone, as shown by the homogenous nuclear fluorescence (Fig. 19). These results confirm that the uPA reporter gene array is functional and can respond to c-Jun:c-Fos and c-Jun:ATF2.

The next question was: is nTrip6 recruited to the promoter together with c-Jun:c-Fos but not with c-Jun:ATF2?

To investigate the recruitment of nTrip6 to the promoter I fused Trip6$^{190-476}$ to the cyan fluorescent protein (CFP) or to YFP. I verified that the fusion to the fluorescent protein did not hamper the nuclear localisation by fluorescence microscopy (Fig. 20. a)). I also confirmed that the fusion to CFP or YFP did not interfere with the AP-1 co-activator function by performing a reporter gene assay using the AP-1 dependent -517/+63Coll-Luc reporter gene (Fig. 20. b)).

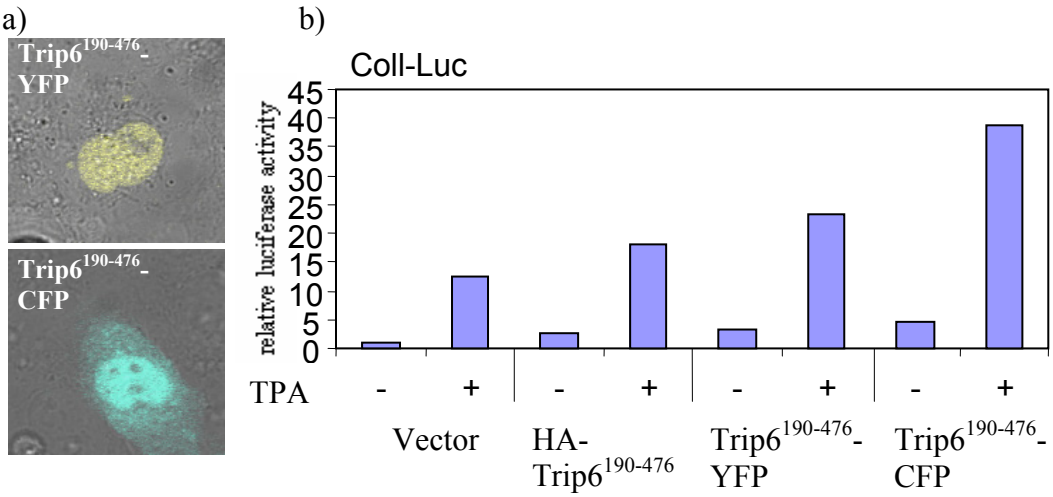

Fig. 20: Fusion of nTrip6 to fluorescent proteins does not hamper its co-activator function
1,5x10^5 logarithmically growing HeLa-cells were seeded in a 96 well plate and next day transfected with empty vector, Trip6$^{190-476}$ fused to a HA-tag or Trip6$^{190-476}$ fused to YFP or CFP. 24h post transfection cells were serum-starved with 0,5% DCS-DMEM and induced overnight by administration of TPA to induce AP-1. Next day cells were lysed and subjected to Luciferase reporter assay. Results are presented as fold induction.

In cells transfected with the -517/+63Coll-Luc reporter gene together with empty vector as a control treatment with TPA resulted in an increase in the reporter activity (Fig. 20. b)). When HA-tagged Trip6$^{190-476}$ was co-transfected, the transcriptional activity of AP-1 was further increased, showing the co-activator function of nTrip6. Transfection of the cells with the fluorescent protein tagged Trip6$^{190-476}$ led to the same extent to an increase in transcription activity (Fig. 20. b)). Thus, the fusion of CFP and YFP to Trip6$^{190-476}$ did not change its capacity to function as a co-activator for AP-1 and made them suitable tools to investigate the recruitment of nTrip6 to different AP-1 promoters.

I then studied the recruitment of nTrip6 to an AP-1 dependent promoter by co-transfection of the uPa-Luc array cell line clone 2U with Trip6$^{190-476}$-YFP together with the single chains c-Jun~c-Fos or c-Jun~ATF2 (Fig. 21).

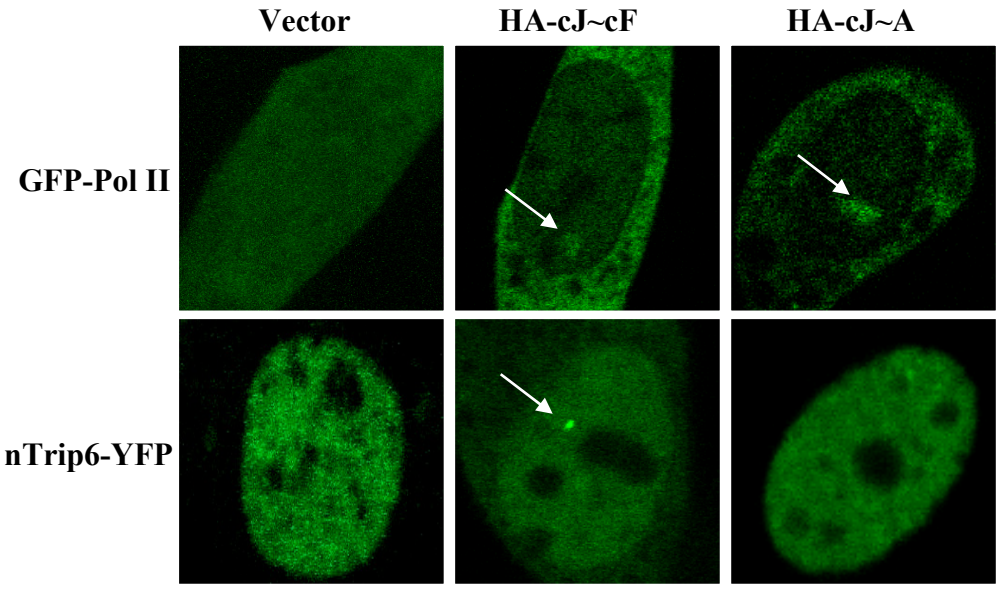

Fig. 21: nTrip6 is not tethered to c-Jun:ATF2 occupied promoter.
2U cells were transfected with empty vector, an expression vector for c-Jun~c-Fos single chain (cJ~cF), or an expression vector for c-Jun~ATF2 single chain (cJ~A), together with an expression vector for GFP-Pol II and an expression vector for Trip6$^{190–476}$ fused to YFP (YFP-Trip6$^{190–476}$). Images were acquired by confocal microscopy. Nuclei of representative cells are shown.

When transfected alone Trip6$^{190-476}$-YFP showed a homogenous distribution in the nucleus, without specific local enrichment. Co-transfection with c-Jun~c-Fos resulted in a recruitment of Trip6$^{190-476}$-YFP to the promoter array, whereas co-transfection with c-Jun~ATF2 did not (Fig. 21). GFP-Pol II was not enriched to the array when transfected alone, as shown by the homogenous nuclear fluorescence (Fig. 21). Upon co-transfection of the forced dimers Pol II was enriched at the gene array. These results confirm that the uPA reporter gene array is functional and can respond to c-Jun~c-Fos and c-Jun~ATF2.
To summarize, nTrip6 interacts with c-Jun:c-Fos but not with c-Jun:ATF2, and is tethered to c-Jun:c-Fos, but not to c-Jun:ATF2 bound promoters. Together with the results showing that nTrip6 increases the transcriptional activity of only Fos-containing AP-1 dimers (Diefenbacher et al., 2008; Sekula, 2006), these results demonstrate that nTrip6 is a selective co-activator for Fos-containing AP-1 dimers.

3.1.3. c-Jun:ATF2 is not trans-repressed by the glucocorticoid receptor

The next question addressed in this work concerns the specificity of nTrip6 action in the repression of the different AP-1 dimers by GR (Sekula, 2006). The first step in the trans-

repression of c-Jun:c-Fos by the GR is the tethering of the GR to the AP-1 bound promoter, via the interaction of GR with nTrip6. Since c-Jun:ATF2 does not mediate the recruitment of nTrip6 to the promoter, I hypothesise that GR cannot repress c-Jun:ATF2, because it cannot be tethered to the c-Jun:ATF2 bound promoter. To address this question, I studied the recruitment of GR to the uPa promoter in the array cell line, in the presence of c-Jun:c-Fos or c-Jun:ATF2.

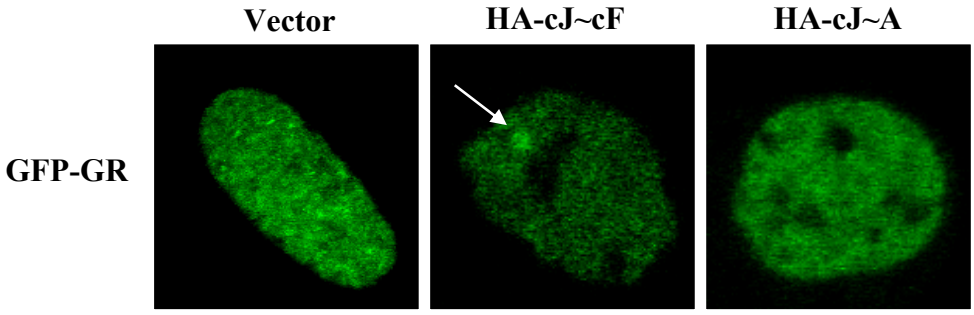

Fig. 22: GR is not tethered to c-Jun:ATF2 occupied promoter
2U cells were transfected with empty vector, an expression vector for c-Jun~c-Fos single chain (cJ~cF), or an expression vector for c-Jun~ATF2 single chain (cJ~A), together with an expression vector for GR fused to GFP. Cells were treated with dexamethasone for 30 min and imaged by confocal microscopy. Nuclei of representative cells are shown.

In cells co-transfected with GFP-GR and empty vector, treatment with the synthetic glucocorticoid dexamethasone resulted in a homogenous distribution of GP-GR within the nucleus (Fig. 22, left). This result confirms that GR does not directly bind to the uPa-promoter. When GFP-GR was transfected together with c-Jun~c-Fos, dexamethasone treatment resulted in the enrichment of GR in a single spot within the nucleus (Fig. 22, middle). This result illustrates the tethering of GR to c-Jun:c-Fos occupied promoters. In cells co-transfected with GFP-GR and c-Jun~ATF2, dexamethasone treatment did not result in a recruitment of GR to the array (Fig. 22, right). Thus, GR can not be recruited to c-Jun:ATF2 bound promoters.

These result led me to propose that the lack of repression of c-Jun:ATF2 by GR is due to the inability of this AP-1 dimer to interact with nTrip6, which is the platform for the tethering of GR to promoter bound c-Jun:c-Fos (Diefenbacher et al., 2008; Kassel et al., 2004).

3.2. Role of nTrip6 in the repression of GR by AP-1

Since nTrip6 behaves as a co-activator and is interacting with GR (Kassel et al., 2004) one could hypothesise that nTrip6 is also a co-activator for GR. Moreover, nTrip6 is essential for GR mediated trans-repression of c-Jun:c.Fos (Kassel et al., 2004). If nTrip6 is a co-activator for GR, is it also required for the repression of GR by AP-1 or NF-κB?

3.2.1. nTrip6 functions as a co-activator for GR

To investigate the possibility that nTrip6 is a co-activator for GR, I performed reporter gene assays using the mouse mammary tumor virus promoter long terminal repeat (MMTV) containing a glucocorticoid receptor responsive element (GRE) fused to luciferase.

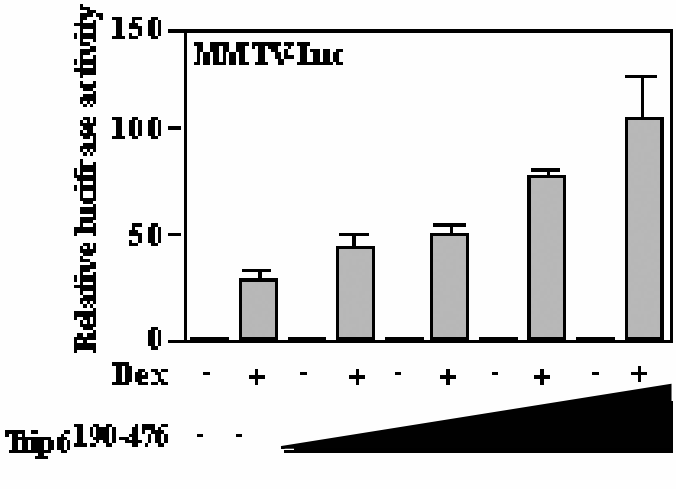

Fig. 23: nTrip6 increases GR-dependent transcription
$1{,}5x10^5$ logarithmically growing HeLa-cells were transfected with increasing amounts of $Trip6^{190-476}$ together with a GR dependent luciferase gene (MMTV-Luc). 24h post transfection the cells were serum-starved with 0,5% DCS-DMEM and treated with the synthetic glucocorticoid dexamethasone. Next day cells were lysed and subjected to Luciferase reporter assay. Results are presented as fold induction, and are the mean ± SD of a representative experiment performed in triplicates.

In HeLa cells transfected with vector control no GR transcriptional activity was observed in untreated conditions (Fig. 23). After treatment with dexamethasone, GR was activated resulting in an increase in transcriptional activity. This activity was further increased in a dose dependent manner upon co-transfection of increasing amounts of $Trip6^{190-476}$ (Fig. 23). Thus, as observed for AP-1 (Diefenbacher et al., 2008; Kassel et al., 2004), nTrip6 is able to increase the transcriptional activity of GR, suggesting that it might act as a co-activator for GR.

I then investigated the interaction between nTrip6 and GR in living cells using the advanced BiFC-system based on the Venus fluorescent protein (Hu et al., 2006), a GFP derivate. If nTrip6 is a co-activator for GR, it should be recruited to GR regulated promoters through its interaction with GR. The first step was thus to confirm that nTrip6 interacts with GR in living cells.

I designed fusion proteins composed of Trip6$^{190-476}$ C-terminaly fused to the C-terminal part of Venus (VC) and GR fused to the N-terminal part of Venus (VN) and verified that the fusion was not altering the localisation of the protein (Fig. 24).

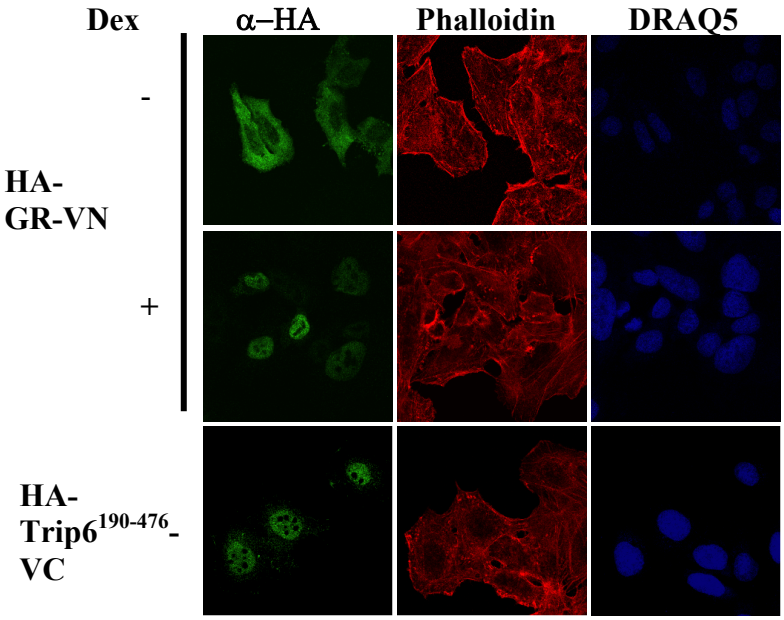

Fig. 24: BiFC-fusion of Trip6$^{190-476}$ and the GR does not hamper their localisation
HeLa cells were transfected with Trip6$^{190-476}$ fused to the C-terminal part of Venus (VC) or with GR fused to VN, treated with dexamethasone (10^{-6}M) for 2h, and probed with an antibody against the HA-epitope and Phalloidin coupled to rhodamine to highlight the cell shape. The nuclei of cells were stained with DRAQ5 and fluorescent images were acquired by confocal microscopy.

Trip6$^{190-476}$ fused to VC was detectable within the nucleus of transfected cells (Fig. 24, lower panel). In untreated cells GR-VN was localized in the cytosol (Fig. 24, upper panel), only upon adding dexamethasone it translocated to the nucleus (Fig. 24, middle panel), showing that the fusion did not hamper GR translocation. The fusion of Trip6$^{190-476}$ to the C-terminal part of Venus as well as the fusion of the glucocorticoid receptor to the N-terminal part of Venus did not hamper the localisation of the proteins. I then used the constructs to investigate GR interaction with nTrip6 in living cells (Fig. 25).

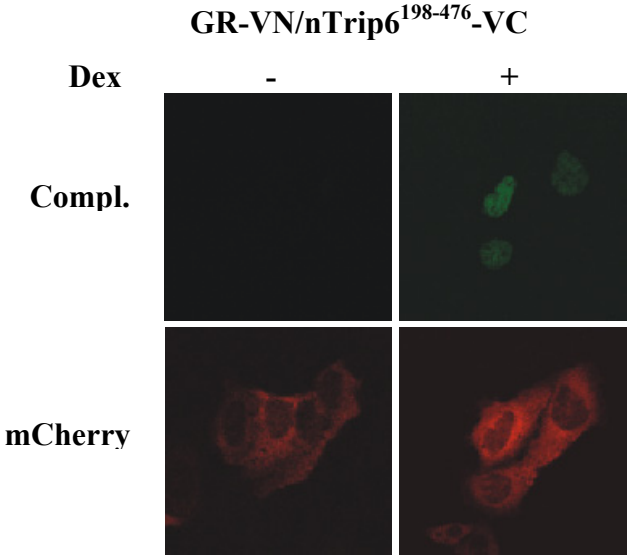

Fig. 25: GR interacts with nTrip6 in living cells only in the nucleus
HeLa cells were transfected with Trip6$^{190-476}$ fused the C-terminal half of Venus (VC) together with GR fused to the N-terminal part of Venus (VN), and with mCherry fused to a nuclear export signal (NES) as a transfection indicator. 24h post transfection cells were imaged before and after 2h of dexamethasone treatment. Fluorescent images were acquired by confocal microscopy.

In untreated cells co-transfected with GR-VN and Trip6$^{190-476}$-VC, no complementation of the Venus protein was detected although cells were efficiently transfected as indicated by the

expression of mCherry fused to a nuclear export signal (Fig. 25, left row). After treatment with dexamethasone, the complementation of Venus fluorescence was detectable. This complementation was exclusively observed in the nucleus of 70 to 80% of the transfected cells (Fig. 25, right row).

Previous work of our group based on *in vitro* tecniques showed that GR was interacting with nTrip6 via the LIM-domain 3. To verify these results in living cells I generated VC-fusions of Trip6$^{190\text{-}476}$ harbouring point mutations of the coordinating cysteins in the zinc fingers of the LIM-domains 1 and 3 (Trip6$^{190\text{-}476}$-LIM1m-VC, Trip6$^{190\text{-}476}$-LIM3m-VC).

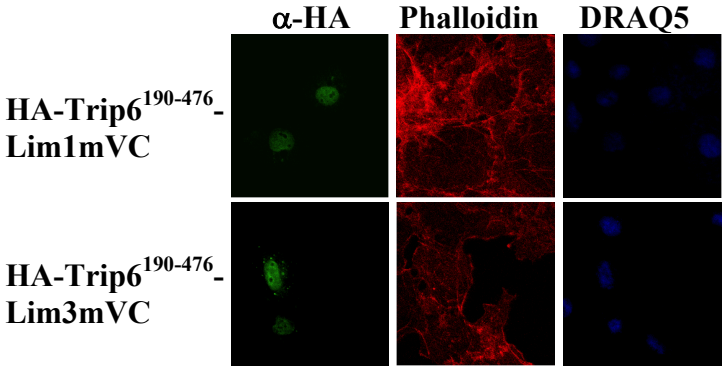

Fig. 26: BiFC-fusion of Trip6$^{190\text{-}476}$-LIM-mutants does not hamper their nuclear localisation
HeLa cells were transfected with Trip6$^{190\text{-}476}$ harboring mutations within the LIM-domains, LIM1m and LIM3m, fused to the C-terminal part of Venus (VC) and probed with an antibody against the HA-tag. The nuclei of cells were stained with DRAQ5 and fluorescent images were acquired by confocal microscopy.

In cells transfected with Trip6$^{190\text{-}476}$-LIM1m-VC or Trip6$^{190\text{-}476}$-LIM3m-VC, the fusion proteins were detectable by immunofluorescene in the nucleus as indicated by DRAQ5 staining (Fig. 26). Therefore the fusion of VC to the LIM-mutants did not hamper their proper localisation. I then used these constructs to study their interaction with GR in a BiFC experiment.

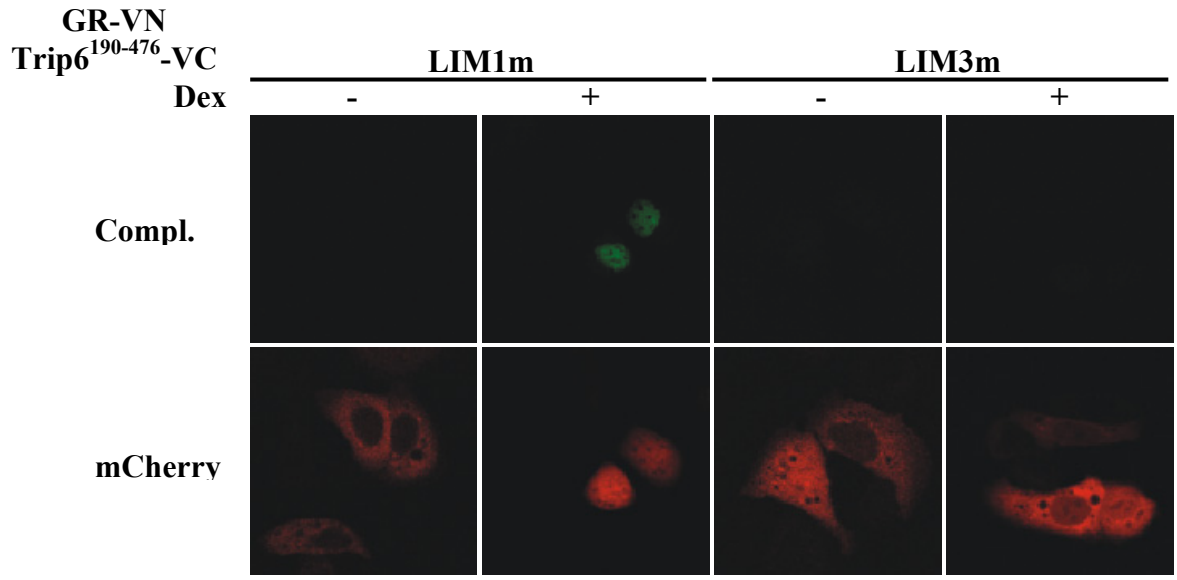

Fig. 27: The Glucocorticoid receptor interacts with nTrip6 via LIM-domain 3
HeLa cells were transfected with Trip6$^{190\text{-}476}$-LIM1m-VC or Trip6$^{190\text{-}476}$-LIM3m-VC together with GR fused to VN, and with mCherry-NES as a transfection indicator. 24h post transfection cells were imaged before and after 2h of dexamethasone treatment. Fluorescent images were acquired by confocal microscopy.

No complementation was detectable in unstimulated cells transfected with Trip6$^{190-476}$-LIM1m-VC or Trip6$^{190-476}$-LIM3m-VC together with GR (Fig. 27). After treatment with dexamethasone, the complementation of Venus was detectable in the nucleus of cells transfected with Trip6$^{190-476}$LIM1m-VC together with GR-VN (Fig. 27). No complementation was observed in cells transfected with Trip6$^{190-476}$LIM3m-VC together with GR-VN, although cells were efficiently transfected as illustrated by the expression of mCherry-NES (Fig. 27).

This result shows that the activated GR is interacting with nTrip6 and that the interaction is mediated via the LIM domain 3.

3.2.2. nTrip6 is recruited to GR-dependent promoters

Thus, nTrip6 interacts with GR and enhances its transcriptional activity. If nTrip6 functions as a co-activator for GR, it should be recruited to the promoter of GR target genes in a GR-dependent manner.
To visualize the recruitment of nTrip6 in a living cell to a GR-dependent promoter I used another array cell line established in our group. This cell line, the clone 7m, was generated as described above for the uPA array cell line, using the MMTV-Luc reporter gene as a template for amplification and insertion. The presence of the gene-array was also visualized by DNA-FiSH using a fragment of the luciferase coding sequence as a probe (Fig. 28).

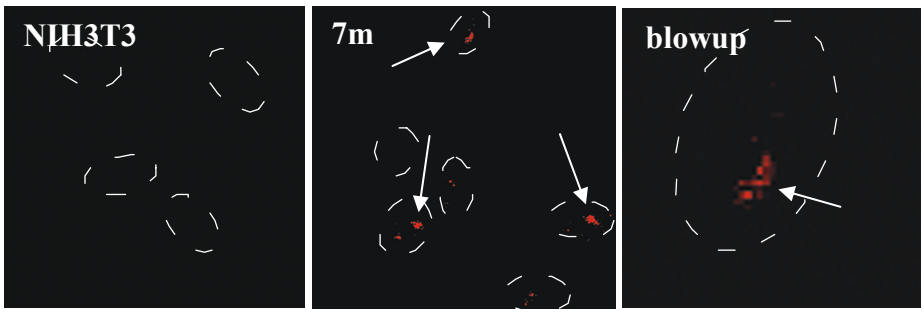

Fig. 28: DNA fluorescence in situ hybridisation (DNA-Fish) highlights the MMTV-Luc gene array
Clone 7m and parental NIH-3T3 fibroblasts were subjected to DNA *in situ* hybridization using a fluorescently labelled cDNA probe complementary to the luciferase coding sequence. A single gene array is visible in 100% of the 7m cells.

Using this probe, the array was detectable in a single spot within the nucleus of the 7m cells, and was present in 100% of the cells, whereas only background staining was visible in the parental NIH-3T3 cells (Fig. 28).
To investigate if the gene array was functional, cells were treated with dexamethasone to activate GR, and luciferase activity was measured (Fig. 29).

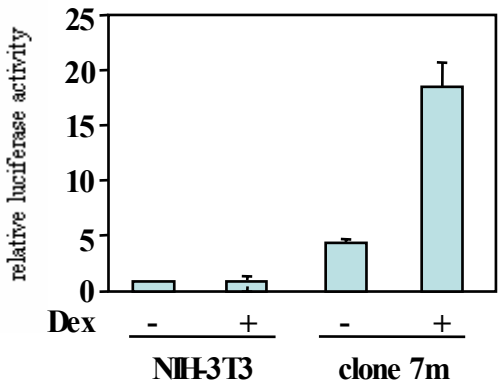

Fig. 29: The MMTV-Luc gene array in clone 7m is functional
$1x10^4$ logarithmically growing 7m-cells as well as parental cells were seeded in a 96 well plate. 24h post seeding cells were serum-starved with 0,5% DCS-DMEM and treated with the synthetic glucocorticoid dexamethasone. Luciferase activity was determined 16h later, and was plotted relative to the untreated NIH3T3 control cells. Results are presented as fold induction, and are the mean ± SD of a representative experiment performed in triplicates.

Treatment of the parental cell line NIH3T3 with dexamethasone did not result in any detectable luciferase activity, as expected. The 7m clone showed a significant basal luciferase activity, which was strongly increased upon treatment of cells with dexamethasone (Fig. 29). This result shows that the array is functional. I therefore used this cell line to investigate whether nTrip6 is recruited together with GR on the promoter of target genes upon activation. I transfected the clone 7m with GFP fused RNA-Poll II to use the recruitment of Pol II as an indicator of the transcriptional activity of the gene array, with GFP fused GR or with Trip6$^{190-476}$-CFP.

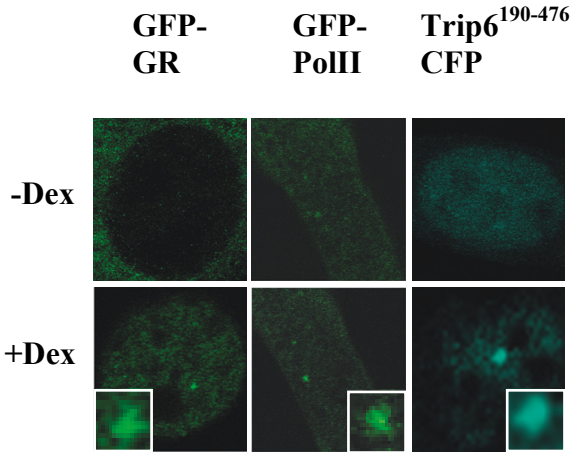

Fig. 30: nTrip6 is recruited to a GR dependent promoter.
7m cells were transfected with expression vectors for GFP-Pol II, GFP-fused GR or Trip6$^{190–476}$ fused to CFP (Trip6$^{190–476}$-CFP). Cells were imaged by confocal microscopy before and 2h after treatment with dexamethasone.

GFP-GR was located in the cytosol and upon dexamethasone treatment it translocated into the nucleus and was recruited to the array, as illustrated by the enrichment in a single locus within the nucleus (Fig. 30, left row). GFP-Pol II was not recruited to the array when GR was not active, but after treatment with the synthetic corticoid, RNA-Pol II was recruited to the array (Fig. 30, middle row). These enrichments were detectable in 70-80% of the transfected cells. These data further confirm that the array is functional and responds to glucocorticoids. Transfection of CFP-fused Trip6$^{190-476}$ resulted in a homogeneous distribution in the nucleus of untreated cells. Upon treatment with dexamethasone Trip6$^{190-476}$ was recruited to the array as indicated by an enrichment of Trip6$^{190-476}$-CFP in a single spot in the nucleus (Fig. 30, right row).

This result shows that nTrip6 is recruited to the promoter of GR target genes in a glucocorticoid dependent manner.
However, if nTrip6 acts as a co-activator for GR, it should be recruited to the promoter via its interaction with GR. I therefore studied whether nTrip6 interacts with GR at the promoter upon activation using the advanced BiFC system in the array cell line.

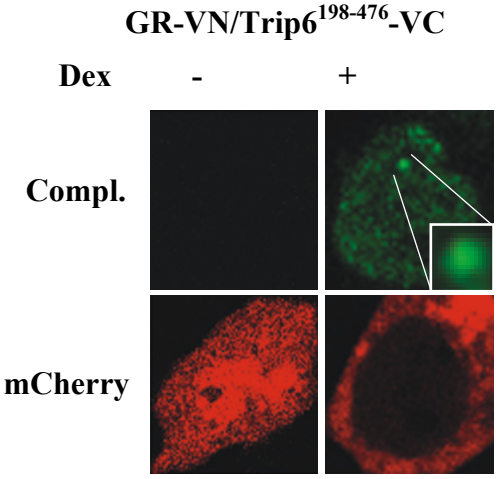

Fig. 31: nTrip6 interacts with GR on the promoter.
7m cells were transfected with an expression vector for GR fused to VN together with an expression vector for Trip6$^{190–476}$ fused to VC. As a transfection marker cells were co-transfected with mCherry fused to a nuclear export signal. 24h post transfection, cells were imaged by confocal microscopy before and 2h after treatment with dexamethasone.

I co-transfected 7m cells with GR-VN and Trip6$^{190-476}$-VC together with mCherry-NES as a transfection control. In the absence of dexamethasone no complementation of Venus was detectable in the nucleus of positively co-transfected cells, as expected (Fig. 31). Following treatment with glucocorticoids, complementation was observed in the nucleus, and this interaction between Trip6$^{190-476}$ and GR was enriched at the array (Fig. 31).
Thus, nTrip6 is recruited to activated GR-regulated promoters via its interaction with GR. This, together with the observation that nTrip6 increases GR transcriptional activity, demonstrates that nTrip6 functions as a co-activator for GR.

3.2.3. nTrip6 mediates the trans-repression of GR by AP-1

GR represses the transcriptional activity of AP-1 (Jonat et al., 1990). But on the other hand AP-1 is also able to interfere with the transcriptional activity of GR by trans-repressing GR (Schule et al., 1990).
Since only Fos family members containing AP-1 dimers are trans-repressed by GR (see above) one could hypothesise that Fos plays a major role in GR repression by AP-1. To address this question, I studied the effect of over-expressing only Fos on GR-mediated transcription using the MMTV-Luc reporter gene construct.

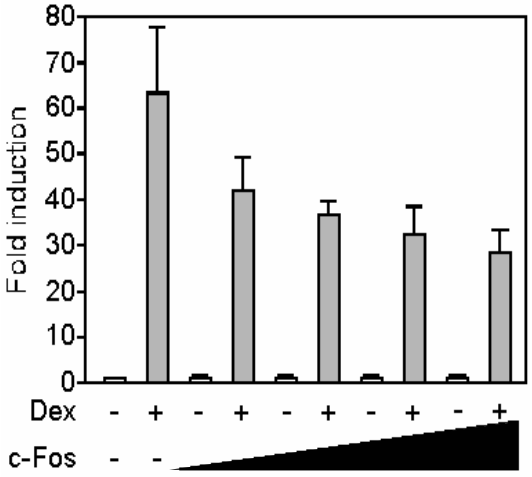

Fig. 32: Overexpression of c-Fos inhibits GR trans-activation
$1x10^5$ logarithmically growing Cos-7-cells were transfected with increasing amounts of c-Fos together with the MMTV-Luc, a reportergene regulated via GR. 24h post transfection the cells were serum-starved with 0,5% FCS-DMEM and treated with dexamethasone. Results are presented as fold induction, and are the mean ± SD of a representative experiment performed in triplicates.

Cells only transfected with GR showed no expression of the reporter in unstimulated conditions (Fig. 32). Treatment with dexamethasone resulted in a strong reporter gene activity. By co-transfection of increasing amounts of c-Fos the transcriptional activity of GR was repressed, in a dose dependent manner (Fig. 32). This results shows that, as hypothesised, c-Fos alone is sufficient to trans-repress GR.

In the repression of AP-1 by GR, nTrip6 interacting with c-Fos and GR, mediates the tethering of GR to the promoter-bound c-Fos. It therefore seemed logical to hypothesise that in the trans-repression of GR by AP-1, c-Fos is tethered to the promoter-bound GR via its interaction with nTrip6.
To test this hypothesis, I co-transfcetd the MMTV gene array cell line 7m with c-Fos fused to Venus, GR fused to mCherry together with Trip6$^{190-476}$ fused to CFP, and observed the recruitment of these proteins to the GR dependent promoter (Fig. 33).

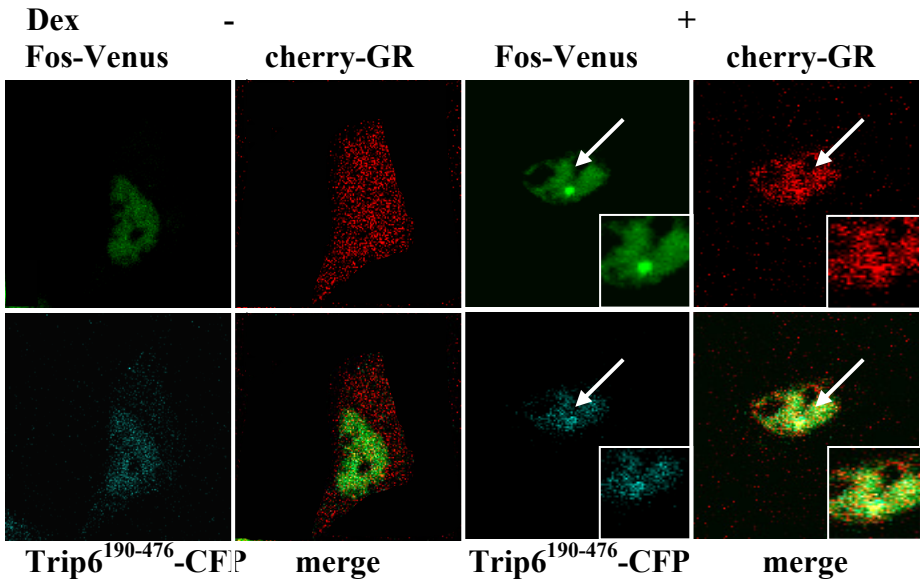

Fig. 33(page before): nTrip6, c-Fos and GR co-localise at GR-dependent promoters
7m cells were transfected with an expression vector for GR fused to mCherry together with an expression vector for Trip6$^{190–476}$ fused to CFP and an expression vector for c-Fos fused to Venus. 16h post transfection, cells were imaged by confocal microscopy before and 2h after treatment with dexamethasone.

In untreated co-transfected 7m cells GR was located in the cytosol, and c-Fos as well as Trip6$^{190-476}$ showed a homogeneous distribution within the nucleus, without any visible enrichment. After treatment with dexamethasone GR translocated to the nucleus and was recruited to the array. nTrip6 was recruited to this array in a GR dependent manner as already shown. And at the same time c-Fos was also recruited to the array at which both nTrip6 and GR were located (Fig. 33).

Thus, in repressing conditions, c-Fos interacting with nTrip6, is tethered to GR dependent promoters.

In conclusion, nTrip6 is a common co-activator for GR and c-Fos containing AP-1 dimers. Additionally, nTrip6 is involved in the reciprocal crosstalk between these two transcription factors, by mediating the tethering of the repressing transcription factors to the promoter-bound repressed transcription factor.

3.3. Mechanism of nTrip6 co-activator function

nTrip6 exerts a co-activator function for AP-1, NF-κB and GR (Diefenbacher et al., 2008; Kassel et al., 2004). However, nTrip6 does not harbour any known co-activator domain or function. The only known functional domains of nTrip6 are the three LIM-domains, which mediate protein-protein interactions. We thus hypothesised, that nTrip6 exerts its co-activator function through the recruitment of other co-activators to the promoter of the target genes, through an interaction with the LIM-domains.

3.3.1. The N-Terminus of nTrip6 is essential for its co-activator function

To investigate via which part nTrip6 exerts its co-activator like function, I first studied whether the LIM-domains were sufficient, in an AP-1-dependent reporter gene assay. I transfected HeLa cells with different nTrip6 mutants together with the AP-1 dependent -517/+63Coll-Luc reporter gene (Fig. 34). On the one hand cells were co-transfected with Trip6$^{190-476}$, on the other hand transfected with the LIM-domains alone, which are essential for the interaction with the transcription factors (Diefenbacher et al., 2008; Kassel et al., 2004).

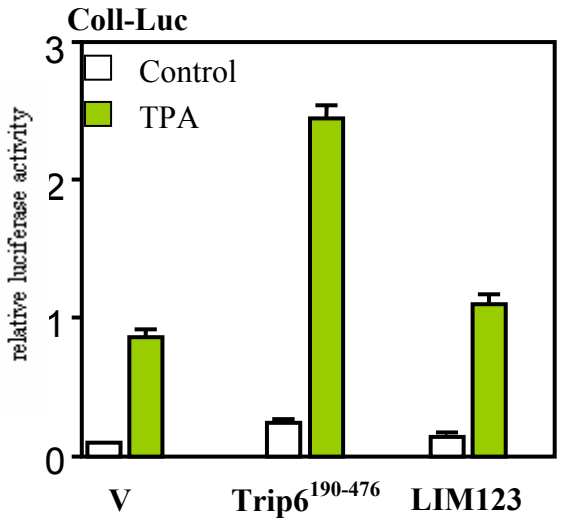

Fig. 34: The N-terminus of nTrip6 is essential for its co-activator function
1,5x10^5 logarithmically growing HeLa-cells were transfected with vector control (V), Trip6$^{190-476}$ or LIM-domains (LIM123) alone. 24h post transfection cells were serum-starved with 0,5% DCS-DMEM and treated witrh TPA to induce AP-1. Results are presented as fold induction, and are the mean ± SD of a representative experiment performed in triplicates.

In cells transfected with vector control alone (V), treatment with TPA, which activates AP-1, resulted in an increase in reporter activity. When I co-transfect Trip6$^{190-476}$, the transcriptional activity of AP-1 was further increased, illustrating the co-activator-like function of nTrip6.
This increase was not seen after co-transfection with the construct expressing the LIM-domains alone (Fig. 34). This result shows, that contrary to the hypothesis, the N-terminus of nTrip6 is essential for its co-activator function for AP-1. Furthermore, it suggests that the co-activators might be recruited through an interaction with nTrip6 N-terminus.

3.3.2. Co-activators are recruited via the LIM-Domains

To confirm this result, I directly studied the autonomous co-activator function of nTrip6. To directly measure the ability of nTrip6 or of the LIM-domains alone to recruit co-activators to a promoter, they where fused to the DNA-binding domain of the yeast transcription factor GAL4 (GAL_{DBD}), transforming Trip6 into a pseudo-transcription factor. As a reporter gene I used Luciferase under the control of the GAL4-UAS, a promoter-region recognized by GAL_{DBD}. In this assay, nTrip6 is directly recruited to the promoter, without the need of protein-protein interactions via its LIM-domains.

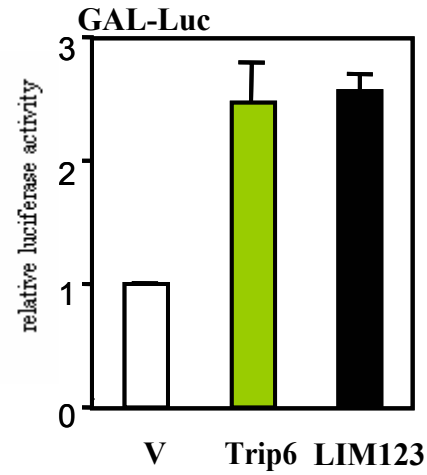

Fig. 35: The N-Terminus of nTrip6 is dispensable for its autonomous co activator function
$1{,}5*10^5$ logarithmically growing HeLa cells were seeded in a 24 well plate and transfected next day with vector cxontrol (V), $Trip6^{190\text{-}476}$ or LIM-domains (LIM123) fused to the Gal-DNA-binding -Domain (DBD) together with a GAL4-UAS driven luciferase reporter construct. Cells were harvested 16h later and luciferase activity was measured. Results are presented as fold induction, and are the mean ± SD of a representative experiment performed in triplicates.

In cells transfected with vector control alone (V), only a weak transcriptional activity was observed. When I co-transfect $Trip6^{190\text{-}476}$fused to $GAL4_{DBD,}$ the transcriptional activity was increased, showing the autonomous co-activator function of nTrip6. To my surprise, the same transcriptional activity was observed when cells were transfected with LIM-domains alone fused to GAL_{DBD} (Fig. 35)
Thus, when nTrip6 is directly recruited to the promoter, its N-terminus is dispensable for its co-activator function, and the co-activators are recruited via the LIM-domains.

3.3.3. nTrip6 forms homo-dimers

There is a discrepancy in these results: on the one hand the N-terminus is essential for nTrip6 function as a co-activator for AP-1 (Fig. 34), but on the other hand the N-terminus is dispensable for the autonomous co-activator function of nTrip6 (Fig. 35).

One hypothesis, which could explain these apparent contradictory results, is that nTrip6 dimerises through its N-terminus and that this dimerisation is essential for its co-activator function.

To study this hypothesis I used the BiFC protein-protein interaction assay to investigate whether nTrip6 dimerises in living cells. Therefore I fused Trip6$^{190\text{-}476}$ to the C-terminal and to the N-terminal part of YFP.

I first investigated if the fusion proteins localise properly within transfected cells, by an antibody staining against the HA-tag encoded in the fusion protein.

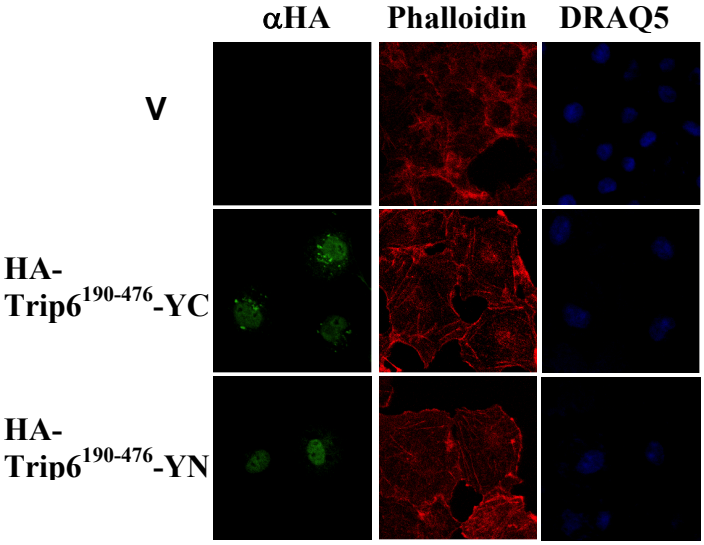

Fig. 36: Fusion of Trip6$^{190\text{-}476}$ to YC or YN does not hamper its localisation
Cos-7 cells were transfected with Trip6$^{190\text{-}476}$ fused to the C-terminal part of YFP (YC) or to the N-terminal half of YFP (YN) and probed with an antibody against the HA-tag. Phalloidin conjugated to rhodamine was used to show the cellular shape.The nuclei of live cells were stained with DRAQ5 and fluorescent images were acquired by confocal microscopy.

In cells transfected with empty vector alone (V), no specific signal was detectable, as expected. In cells transfected with either Trip6$^{190\text{-}476}$ fused to YC or Trip6$^{190\text{-}476}$ fused to YN, a specific staining was detectable in the nucleus, highlighted by staining with DRAQ5. Phalloidin rhodamine was used to illustrate the cell shape (Fig. 36).

The fusion of Trip6$^{190\text{-}476}$ to YC or YN did not hamper the proper expression nor the localisation of the protein. Thus I used the constructs to study the dimerisation of nTrip6 in a BiFC assay.

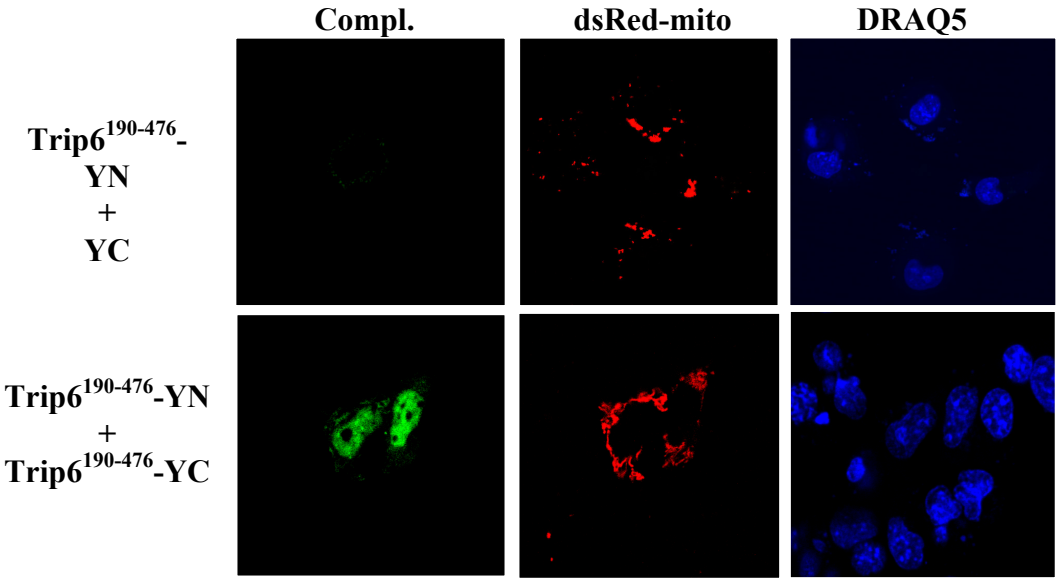

Fig. 37: nTrip6 homo-dimerises in the nucleus
HeLa cells were transfected with Trip6$^{190-476}$ fused the N-terminal half of YFP (YN) together with empty vector encoding YC, or together with nTrip6 fused to YC As a transfection marker cells were co-transfected with dsRed-mito. The nuclei of live cells were stained with DRAQ5. Fluorescent images were acquired by confocal microscopy.

Cells transfected with Trip6$^{190-476}$-YN and with the empty vector encoding YC, together with dsRed-mito as a transfection marker, did not show any fluorescence complementation, showing that no spontaneous complementation of YFP was occurring (Fig. 37).
After co-transfection of Trip6$^{190-476}$-YC together with Trip6$^{190-476}$-YN, a strong fluorescence was detected in the nucleus, highlighted by DRAQ5 staining. The complementation of YFP was observed in the nucleus of 70-80% of transfected cells, as judged by the expression of dsRed-mito. This result shows that nTrip6 dimerises, and this dimerisation occurs exclusively in the nucleus (Fig. 37).

Since nTrip6 is the short isoform of the cytosolic protein Trip6 (Kassel et al., 2004) one could hypothesise that Trip6 dimerises as well, and this would occur in the cytosol. To answer this question, I generated BiFC-constructs based on the Venus fluorescent protein fusing VN and VC N-terminally to Trip6. To ensure that the fusion did not hamper the localisation of Trip6, cells were transfected with either Trip6 fused to VC or transfected with Trip6 fused to VN, and stained by using an antibody against the HA-Tag (Fig. 38).

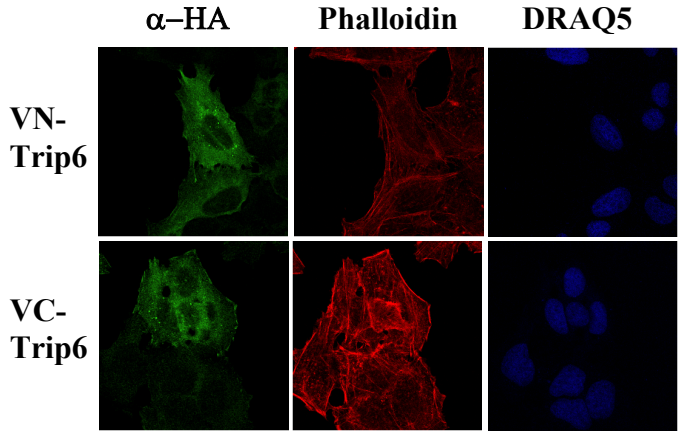

Fig. 38: Fusion of Trip6 to VC or VN does not hamper its localisation
HeLa-ells were transfected with Trip6 fused to the C-terminal half of Venus (VC) or to the N-terminal half (VN) and probed with an antibody against the HA-tag. Phalloidin was used to show the cellular shape. The nuclei of live cells were stained with DRAQ5 and fluorescent images were acquired by confocal microscopy.

In cells transfected with either Trip6 fused to VC or transfected with Trip6 fused to VN, a specific staining was detectable in the cytosol. The nuclei were highlighted by DRAQ5, and Phalloidin rhodamine was used to illustrate the cell shape (Fig. 38).
The fusion of Trip6 to VC or VN did not hamper the proper expression nor the localisation of the protein. These constructs were then used to study the dimerisation of Trip6 in the cytosol.

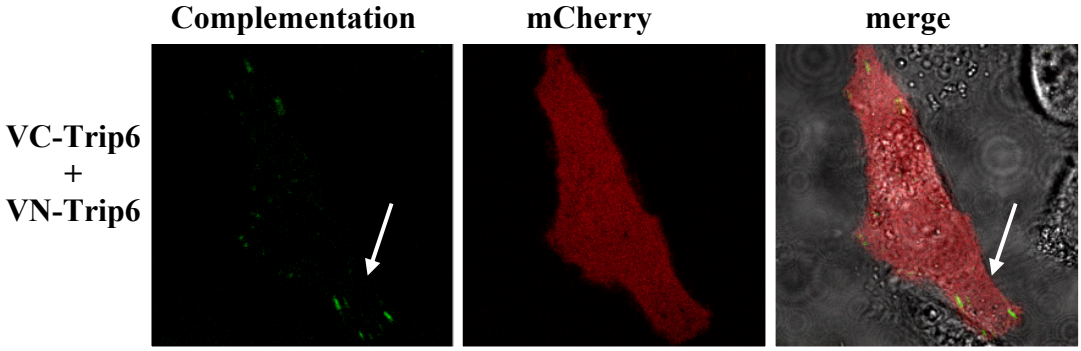

Fig. 39: Trip6 dimerises at focal adhesion sites
HeLa cells were transfected with Trip6 fused the N-terminal half of Venus (VN) together with Trip6 fused to VC, and with mCherry as a transfection indicator. Fluorescent images were acquired by confocal microscopy.

In cells co-transfected with VN-Trip6 and VC-Trip6 together with mCherry as a transfection marker, complementation of Venus protein was detected, showing that the cytoplasmic protein Trip6 is also able to dimerise (Fig. 39). Surprisingly, the complementation was only occurring at sites of focal adhesion, but not in the cytosol, where Trip6 is also present (Fig. 39).
This result suggests that Trip6 dimerisation might be a regulated event at focal adhesion sites. This mechanism of dimerisation would then be different to the dimer formation of the nuclear isoform, nTrip6, for which dimerisation is always observed throughout the nucleus (Fig. 37).

3.3.4 Dimerisation occurs at AP-1 regulated promoters

If the dimerisation of nTrip6 in the nucleus is required for the co-activator function of nTrip6 then it should occur at the promoter of activated AP-1 target genes.
To investigate this question in living cells, I made use of a cell line containing an array of amplified AP-1 dependent luciferase reporter gene (-517/+63Coll-Luc). The cell line was generated as previously described for the uPA and MMTV array cell lines and the clone 12C was chosen for further investigations.
As a first step, I confirmed the presence of the gene array by DNA fluorescent in situ hybridisation using a luciferase specific probe (Fig. 40).

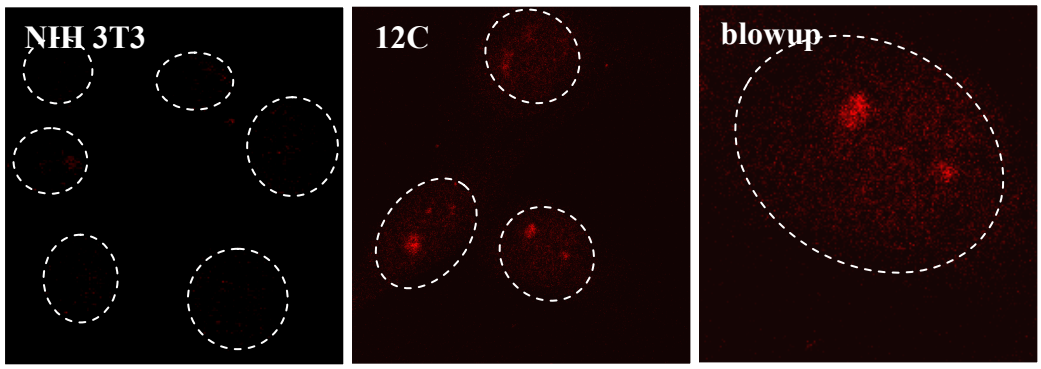

Fig. 40: DNA fluorescence in situ hybridisation (DNA-Fish) in highlights the -517/+63Coll-Luc gene array
Clone 12C and parental NIH-3T3 fibroblasts were subjected to DNA *in situ* hybridization using a fluorescently labelled cDNA probe complementary to the luciferase coding sequence. A single gene array is visible in 100% of the 12C cells.

A specific staining was detected in 1 to 2 spots within the nucleus of the 12C cells, and was present in 100% of the cells, whereas only background staining was visible in the parental NIH-3T3 cells (Fig. 40). To investigate if the gene array was functional, clone 12C cells were treated with TPA to activate AP-1 and the luciferase activity was measured.

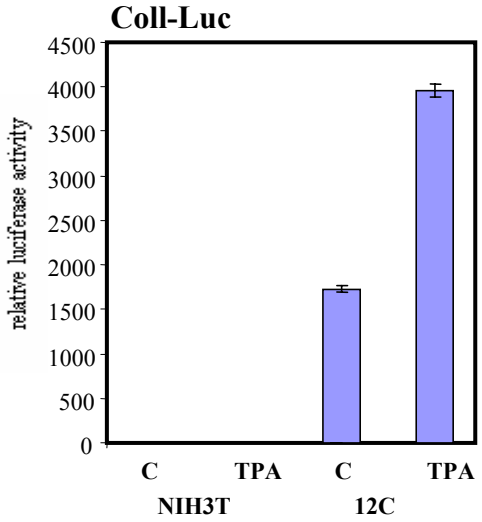

Fig.41: The -517/+63Coll-Luc gene array in clone 12C is functional
$1x10^4$ logarithmically growing 12C-cells as well as parental cells were seeded in a 96 well plate. 24h post seeding cells were serum-starved with 0,5% DCS-DMEM and treated with the phorbol ester TPA. Luciferase activity was determined 16h later, and was plotted relative to the untreated NIH3T3 control cells. Results are presented as fold induction, and are the mean ± SD of a representative experiment performed in triplicates.

Treatment of the parental cell line NIH3T3 with TPA did not result in any detectable luciferase activity, as expected. The clone 12C showed a significant basal luciferase activity, which was strongly increased upon treatment of cells with TPA (Fig. 41). This result shows that the array is functional. I then used this array cell line to study the dimerisation of nTip6 at an activated AP-1 dependent promoter by using the advanced BiFC (Fig. 42). Again, the recruitment of GFP-PolII was used as an indicator of transcriptional activity.

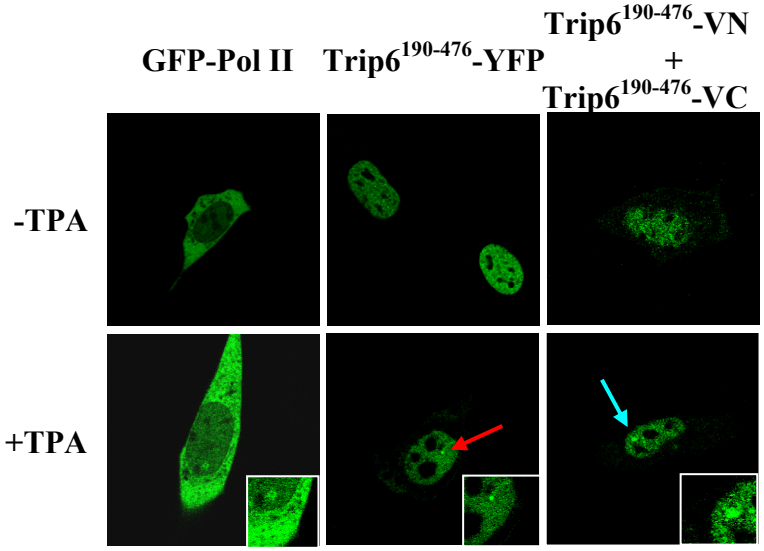

Fig. 42: nTrip6 dimerises at AP-1-regulated promoters
12C cells were transfected with, an expression vector for GFP-Pol II, an expression vector for Trip6$^{190-476}$ fused to YFP or expression vectors for Trip6$^{190-476}$ fused to the N-terminal part of Venus (Trip6$^{190-476}$-VN) together with nTrip6 fused to the C-terminal part of Venus (Trip6$^{190-476}$-VC). Cells were treated with TPA for 1h to activate AP-1 and imaged by confocal microscopy. Nuclei of representative cells are shown.
Small windows show a blowup of the array.

In cells transfected with GFP-PolII no recruitment was detectable to the array when AP-1 was not active, but after treatment with the phorbol ester TPA, RNA-PolII was recruited to the array. These enrichments were detectable in 70-80% of the transfected cells. These data further confirm that the array is functional and responds to TPA (Fig. 42). Transfection of YFP-fused Trip6$^{190-476}$ resulted in a homogeneous distribution in the nucleus of untreated cells. Upon treatment with TPA Trip6$^{190-476}$ was recruited to the array as indicated by an enrichment of Trip6$^{190-476}$-YFP in a single spot in the nucleus (Fig. 42). This result confirms that nTrip6 is recruited to the promoter of AP-1 target genes in an activation-dependent manner.

I then studied if nTrip6 is recruited to the promoter as a dimer using the BiFC system in this cell line. Although fluorescent complementation between Trip6$^{190-476}$-YN and Trip6$^{190-476}$-YC was detectable in the nucleus of transfected cells, no recruitment to the array was observed in control untreated cells. After treatment of the transfected cells with TPA, the fluorescence complementation was enriched at the array. This result shows that nTrip6 dimerises at the promoter of activated AP-1 target genes. To confirm this result, I used FRET (fluorescent resonance energy transfer) as another fluorescence microscopy based protein-protein interaction assay in the array cell line. As a FRET donor I used Trip6$^{190-476}$ fused to CFP (Trip6$^{190-476}$-CFP), as an acceptor I used Trip6$^{190-476}$ fused to YFP (Trip6$^{190-476}$-YFP) (Fig. 43).

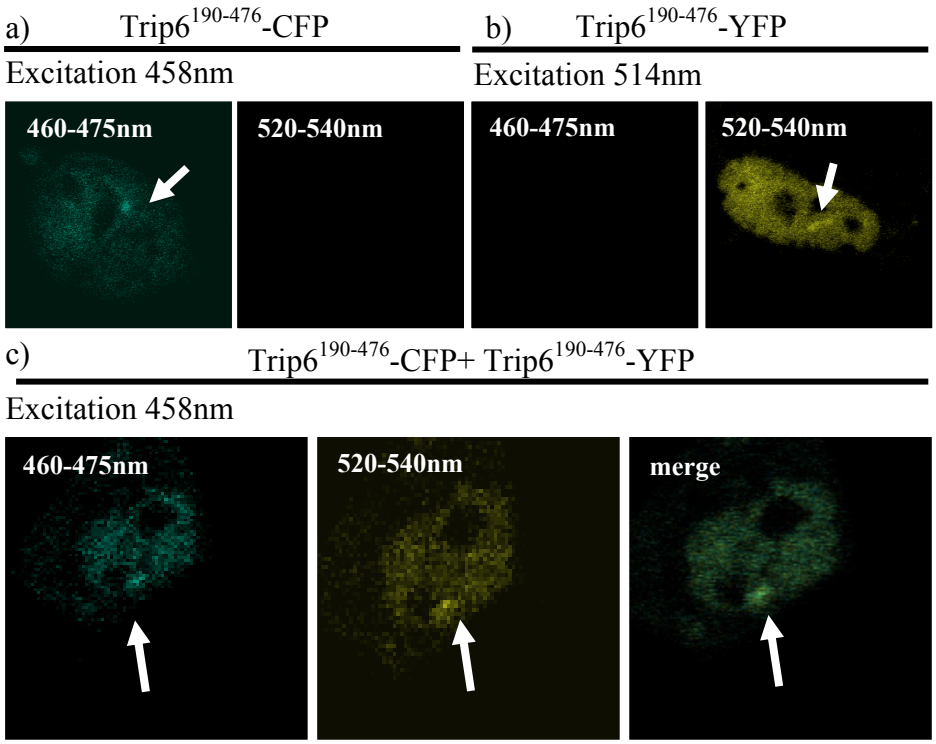

Fig. 43: Dimerisation of nTrip6 on the promoter can be detected via FRET
$1x10^4$ 12C cells were seeded in 8-well chamber slides and next day transfected with a) Trip6$^{190-476}$ fused to CFP (Trip6$^{190-476}$-CFP) alone, b) Trip6$^{190-476}$ fused to YFP (Trip6$^{190-476}$-YFP) or c) Trip6$^{190-476}$-CFP together with Trip6$^{190-476}$-YFP. 2h before observation at the confocal microscope cells were treated with TPA to induce AP-1.

a) CFP-channel setup for FRET: Trip6$^{190-476}$-CFP transfected cells were excited via a 458nm laser and emission was detected between 460nm-475nm. The detection channel was adjusted to YFP emission spectra between 520nm-540nm. In cells tranfected only with CFP-nTrip6 fluorescence was only detected in the CFP channel and the enrichment on the promoter was visualized (arrow).
b) YFP-channel setup for FRET: Trip6$^{190-476}$-YFP transfected cells were excited via a 514nm laser and no emission was detected between 460nm-475nm. In cells tranfected only with YFP-nTrip6 fluorescency was only detected in the YFP channel and the enrichment on the promoter was visualized (arrow).
c) Clone 12C was double-transfected with equal amounts of Trip6$^{190-476}$-CFP and Trip6$^{190-476}$-YFP and excited with a 458nm laser and emission was detected both in the CFP and YFP channel.

In transfected cells, Trip6$^{190-476}$-CFP alone showed a homogeneous distribution in the nucleus and the signal was only detectable in the CFP-specific detection-channel. After treatment with TPA, Trip6$^{190-476}$ was enriched at the array. No fluorescent signal was detectable within the YFP detection channel (Fig. 43 a)). Trip6$^{190-476}$-YFP transfected alone also showed a homogeneous distribution in the nucleus of transfected cells and the signal was only detectable in the YFP-specific detection-channel. Upon treatment with TPA Trip6$^{190-476}$-YFP was enriched at the array. No fluorescent signal was detectable in the CFP detection channel (Fig. 43 b)). In cells co-transfected with Trip6$^{190-476}$-CFP together with Trip6$^{190-476}$-YFP and treated with TPA, excitation at 458nm resulted in a specific fluorescent signal in the CFP channel (Fig. 43 c arrow). Moreover, a fluorescent emission of Trip6$^{190-476}$-YFP was

detectable in the YFP channel. This FRET signal was detectable as a result of the interaction between the two transfected proteins throughout the nucleus and enriched at the array (Fig. 43 c arrow).

The results of the BiFC and FRET experiments show that nTrip6 dimerises at the promoter of activated AP-1 target genes.

3.3.5. Mapping of nTrip6 dimerisation

As a first step to address the relevance of nTrip6 dimerisation for its co-activator function for AP-1 target genes, it was necessary to map the dimerisation domain.

3.3.5.1. The dimerisation occurs via the N-Terminus of nTrip6

To study through which domain (N-terminus or LIM-domains) nTrip6 dimerises, I used the improved BiFC-system based on Venus, using Trip6$^{190-476}$ lacking its LIM-domains (Trip6$^{190-476}$ΔLIM-VC) as well as Trip6$^{190-476}$ LIM-domains alone fused to the C-terminal half of Venus (LIM-VC), or Trip6$^{190-476}$ fused to VC (Trip6$^{190-476}$-VC) as a positive control. As an interaction partner I used Trip6$^{190-476}$ fused to VN (Trip6$^{190-476}$ -VN).
I first performed antibody stainings against the HA-epitope of the fusion proteins to investigate their localisation in transfected cells, using phalloidin rhodamine to visualize cell shape and DRAQ5 to highlight the nucleus. This localisation control was particular important to perform, since the localisation of the putative nuclear localisation signal (NLS) within Trip6 sequence is still unclear. Thus, the deletion of the N-terminus or of the LIM-domains could result in a loss of Trip6$^{190-476}$ localisation to the nucleus (Fig. 44).

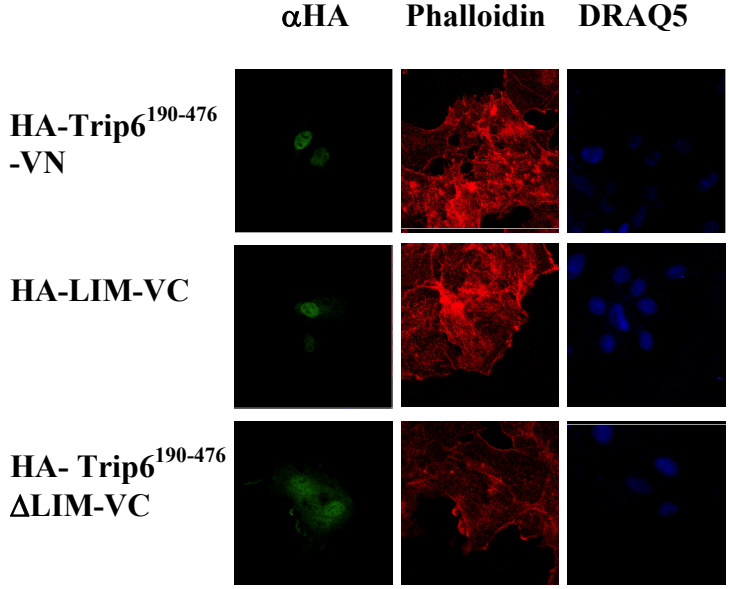

Fig. 44: Fusion of Trip6$^{190-476}$ΔLIM or LIM to VC or Trip6$^{190-476}$ to VN did not hamper the localisation
HeLa-ells were transfected with Trip6$^{190-476}$ΔLIM or LIM to fused to the C-terminal part of Venus (VC) or Trip6$^{190-476}$ to the N-terminal half VN and probed with an antibody against the HA-tag. Phalloidin was used to show the cellular shape. The nuclei of live cells were stained with DRAQ5 and fluorescent images were acquired by confocal microscopy.

The fusion of Trip6$^{190-476}$to VN or VC did not hamper the proper expression nor the localisation of the protein as illustrated by a positive antibody staining of the fusion protein in the nucleus of transfected cells (Fig. 44). In cells transfected with LIM-domains alone fused to VC (LIM-VC) a specific antibody staining was detectable in the nucleus of transfected cells illustrated by DRAQ5. In cells transfected with the LIM domain deletion mutant Trip6$^{190-476}$ΔLIM-VC, a specific staining was also detectable in the nucleus (Fig. 44).
The fusion of VC or VN to Trip6$^{190-476}$ or its deletion mutants only expressing the LIM-domains or N-terminus of Trip6$^{190-476}$ did not hamper the proper expression nor the localisation of the protein. Thus, there might be putative NLS sequences both within the LIM-domains and the N-terminus of Trip6. The constructs were thus suitable to use in a BiFC experiment (Fig. 45).

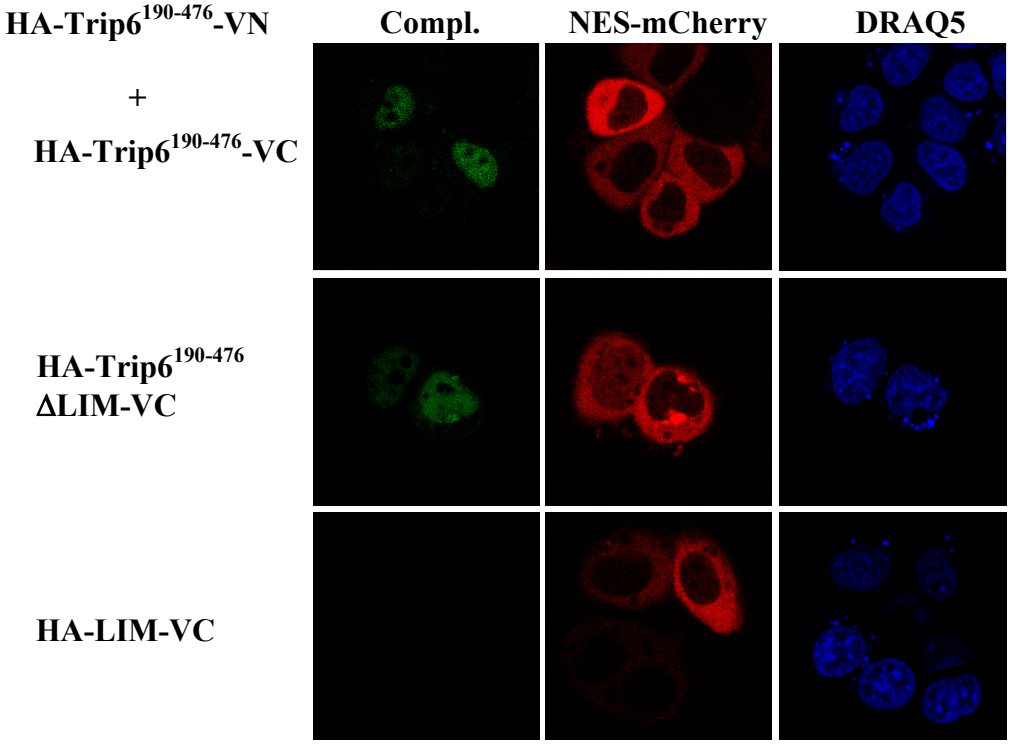

Fig. 45: nTrip6 dimerises via its N-terminus, not via the LIM-Domains
HeLa cells were transfected with Trip6$^{190-476}$ fused the N-terminal half of Venus (VN) together with either Trip6$^{190-476}$ fused to VC, Trip6$^{190-476}$ ΔLIM or LIM-domains alone fused to VC. As a tranfection marker cells were co-tranfected with mCherry fused to a nuclear export signal (NES). The nuclei of live cells were stained with DRAQ5 and fluorescent images were acquired by confocal microscopy.

In the positive control situation, when I co-transfected Trip6$^{190-476}$-VN together with Trip6$^{190-476}$-VC, I detected complementation of Venus in the nucleus of transfected cells, confirming that Trip6$^{190-476}$ forms homodimers, and that this dimerisation only occurs in the nucleus. When I co-transfected Trip6$^{190-476}$ΔLIM-VC together with Trip6$^{190-476}$-VN, the complementation of Venus was still detectable in the nucleus (Fig. 45). However, no

complementation of the Venus protein was detectable in cells transfected with LIM-VC together with Trip6$^{190-476}$-VN (Fig. 45).
Thus, these results show that, as initially hypothesised, nTrip6 dimerisation occurs through its N-terminus, and not via its LIM-domains.

3.3.5.2. The dimerisation is mediated by two dimerisation-domains within the N-terminus of nTrip6

To elucidate the exact amino acid sequence required for the dimerisation of nTrip6, I performed an *in vitro* protein-protein interaction assay, the Peptide SPOT analyses.
The whole amino acid sequence of Trip6 was synthesised on a cellulose membrane in single spots composed of 10 amino acids. The neighbouring spot contained a two amino acid shift in its sequence, and this procedure was carried out until the whole sequence of Trip6 was synthesised. The membrane was then incubated with recombinant GST-Trip6$^{190-476}$. An interaction between nTrip6 and a specific amino acid sequence was identified by an anti-GST antibody, followed by ECL-detection (Fig. 46).

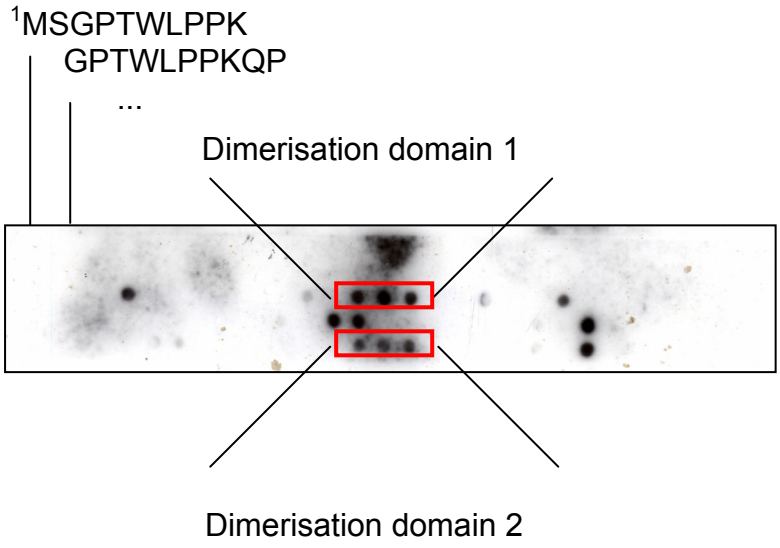

Fig. 46: nTrip6 dimerises *in vitro* via two sequences located in the N-terminus
Peptides covering the sequence of the whole Trip6 protein were spotted on a membrane. Each spot contains 10 amino acids, and each following spot has a 2 residues sequence-shift.
The membrane was incubated with a GST-nTrip6-fusion protein and the interaction was detected with an anti-GST antibody.

Two peptide sequences interacting with GST-Trip6$^{190-476}$ were identified, which I called dimerisation domain 1 (DD1) and dimerisation domain 2 (DD2) (Fig. 46). Both domains were within the N-terminus and not within the LIM domains and located after the NES (101-107). Dimerisation domain 2 was located within the N-terminus of Trip6$^{190-476}$. Surprisingly, DD1 is not within Trip6$^{190-476}$ which was used as a GST-fusion to detect the interaction. This strongly suggests that dimerisation through these domains is not mediated via homologous interactions, i.e. DD1 interacting with DD1 and DD2 with DD2, but rather via heterologous interactions, such as DD1 interacting with DD2.

Since the exact nature of nTrip6 was yet not identified when I performed these experiments, I focused on the dimerisation domain 2. This domain is present in our working tool, Trip6$^{190-476}$, whereas dimerisation domain 1 is not. I used the BiFC-system to study the *in vivo* relevance of the dimerisation motif.

Therefore I generated a deletion-mutant lacking DD2, and fused it to the C-terminal part of YFP (Trip6$^{190-476}$ΔDD2-YC), to test its interaction with Trip6$^{190-476}$-YN (Fig. 47).

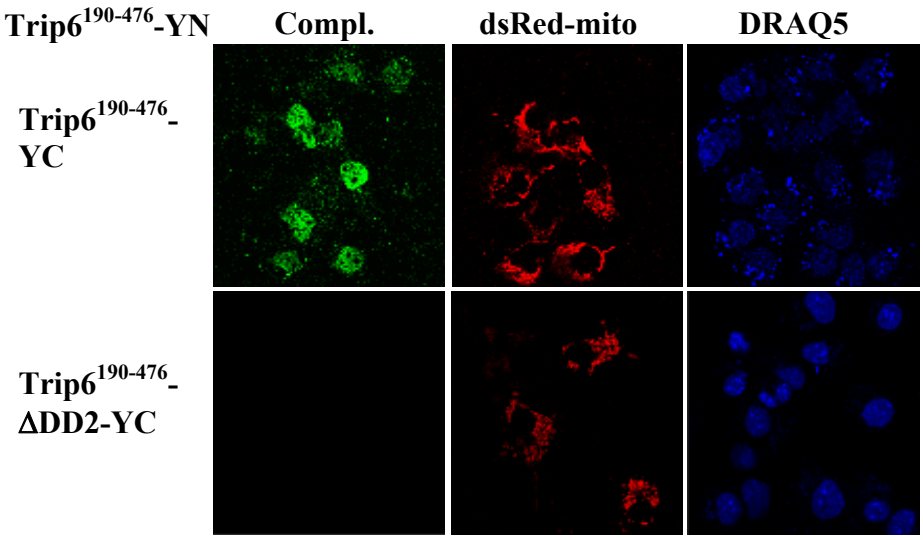

Fig. 47: Deletion of the dimerisation domain 2 abolishes nTrip6 dimerisation
HeLa cells were transfected with nTrip6 fused the N-terminal half of YFP (YN), together with either Trip6$^{190-476}$ fused to YC as a positive control or with Trip6$^{190-476}$ lacking dimerisation domain 2 (Trip6$^{190-476}$ΔDD2) fused to YC together with dsRed-mito as a transfection control.
16h post transfection cells were treated with DRAQ5 to highlight the nuclei and images were acquired by confocal microscopy.

In the positive control situation, when I co-transfected cells with Trip6$^{190-476}$-YN together with Trip6$^{190-476}$-YC, I detected complementation of YFP in the nucleus of transfected cells, confirming that Trip6$^{190-476}$ forms homodimers, and this dimerisation was only observed in the nucleus, highlighted by staining with DRAQ5 (Fig. 47). In cells co-transfected with the deletion mutant Trip6$^{190-476}$ΔDD2-YC together with Trip6$^{190-476}$-YN, no complementation of YFP was detectable, although cells were efficiently transfected as indicated by the expression of dsRed mito. This result shows the relevance of DD2 in the dimerisation of nTrip6.

3.3.6. nTrip6 dimerisation is essential for its co-activator function

3.3.6.1 Dimerisation deficient mutants do not act as co-activators for AP-1

The next step then was to address the relevance of nTrip6 dimerisation via the identified dimerisation domain in nTrip6 function as an AP-1 co-activator. To address this question, I performed a reporter gene assay using the AP-1 dependent -517/+63Coll-Luc l reporter gene.

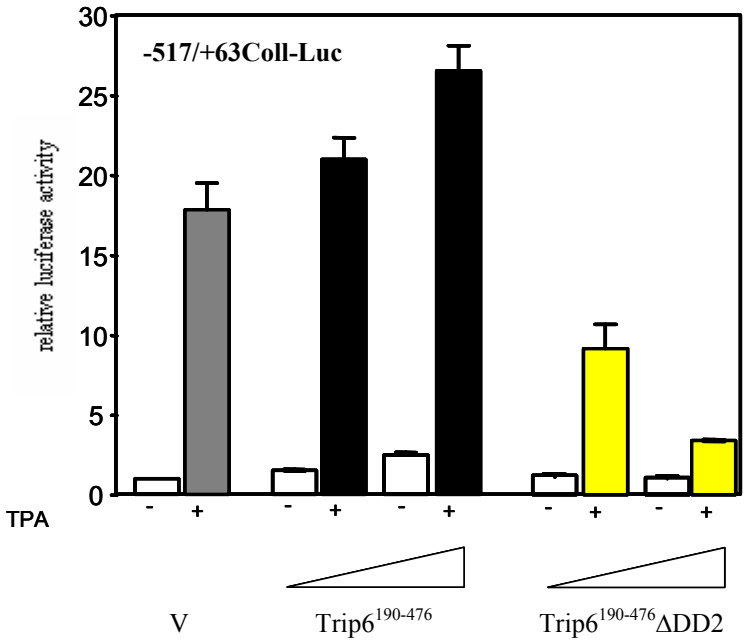

Fig. 48: Abolishing nTrip6 dimerisation results in loss of AP-1 function
HeLa cells were cotransfected with a luciferase reporter gene driven by a minimal AP-1-dependent collagenase-1 promoter (-517/+63Coll-Luc), together with either empty vector (V), an expression vector for Trip6$^{190-476}$ or for the Trip6$^{190-476}$ dimerization-mutant lacking the dimerisation domain 2 (Trip6$^{190-476}$ΔDD2). Cells were treated with TPA as indicated and luciferase activities were determined. Results are presented as fold induction, and are the mean ± SD of a representative experiment performed in triplicates.

Cells transfected with vector control alone (V) showed a low basal reporter activity when not treated with TPA. TPA treatment resulted in an increase in reporter gene activity (Fig. 48).
This AP-1 dependent transcriptional activity was further increased by the co-transfection of increasing amounts of Trip6$^{190-476}$, recapitulating the co-activator function of nTrip6. In cells co-transfected with the deletion mutant of Trip6$^{190-476}$ lacking DD2 (Trip6$^{190-476}$ΔDD2) after treatment with TPA, the AP-1 transcriptional activity was strongly reduced, in a Trip6$^{190-476}$ΔDD2 dose dependent manner. This dominant negative activity of Trip6$^{190-476}$ΔDD2 on AP-1 transcriptional activity shows that the dimerisation of nTrip6 via DD2 is essential for its function as a co-activator for AP-1, at least in the context of artificial constructs.

3.3.6.2 The dimerisation of endogenous nTrip6 is essential for its co-activator function

A direct way to address the relevance of the dimerisation of endogenous nTrip6 for its co-activator function is to interfere with dimer formation. A strategy to block the dimerisation of endogenous nTrip6 is to use peptides that would bind to the dimerisation domain, and thus block or compete with the dimerisation.

Blocking peptides are able to abolish dimerisation of nTrip6

Since both the nuclear and the cytosolic isoform of Trip6 do form dimers, the inhibition of dimerisation must be limited to the nucleus to specifically address the effect of nTrip6 dimerisation. I therefore generated vectors expressing peptides corresponding to the

dimerisation domains, DD1 and DD2, and fused them to the nuclear localisation signal (NLS) from SV40 virus to direct them exclusively to the nucleus. As a control peptide, I used the same constructs, but with scrambled versions of DD1 and DD2 ($DD1_{SCR}$ and $DD2_{SCR}$), which have exactly the same amino acid sequence, but should not block nTrip6 dimerisation. Finally, all blocking peptides were fused to mCherry. This fusion allowed me to observe their expression and to track their subcellular localisation.

HeLa-cells were transfected with vectors encoding for these peptides and their expression as well as localisation was observed 18h post transfection in living cells using a confocal microscope (Fig. 49).

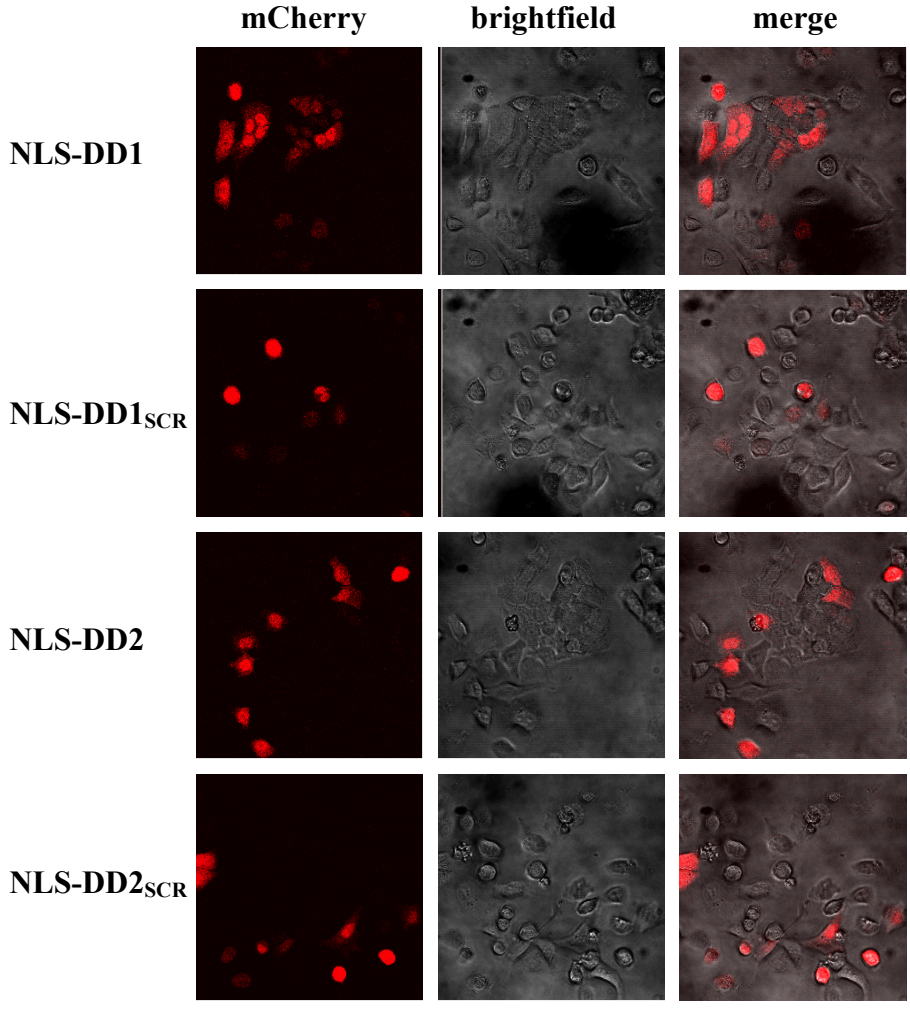

Fig. 49: Expression and subcellular localisation of genetically encoded blocking peptides
Hela cells were tranfected with expression vectors for the blocking peptides, DD1 and DD2, as well as their respective scrambled controls ($DD1_{SCR}$ and $DD2_{SCR}$) fused to a NLS (nuclear localisation signal) and imaged 18h post tranfection with a confocal laser scanning microscope.

Both, mCherry-NLS-DD1 and mCherry-NLS-DD2, as well as their respective scrambled controls, were expressed and detectable only in the nucleus of transfected cells (Fig. 49).

In a next step I was interested in the properties of the designed peptides. Would they be able to interfere with the dimerisation of nTrip6 in the nucleus? To answer this question, I used again the *in vivo* interaction assay BiFC. HeLa cells were transfected with $Trip6^{190-476}$- VC and $Trip6^{190-476}$-VN together with either mCherry as a control, mCherry-NLS-DD1 or DD2, or with their scrambled versions (Fig. 50).

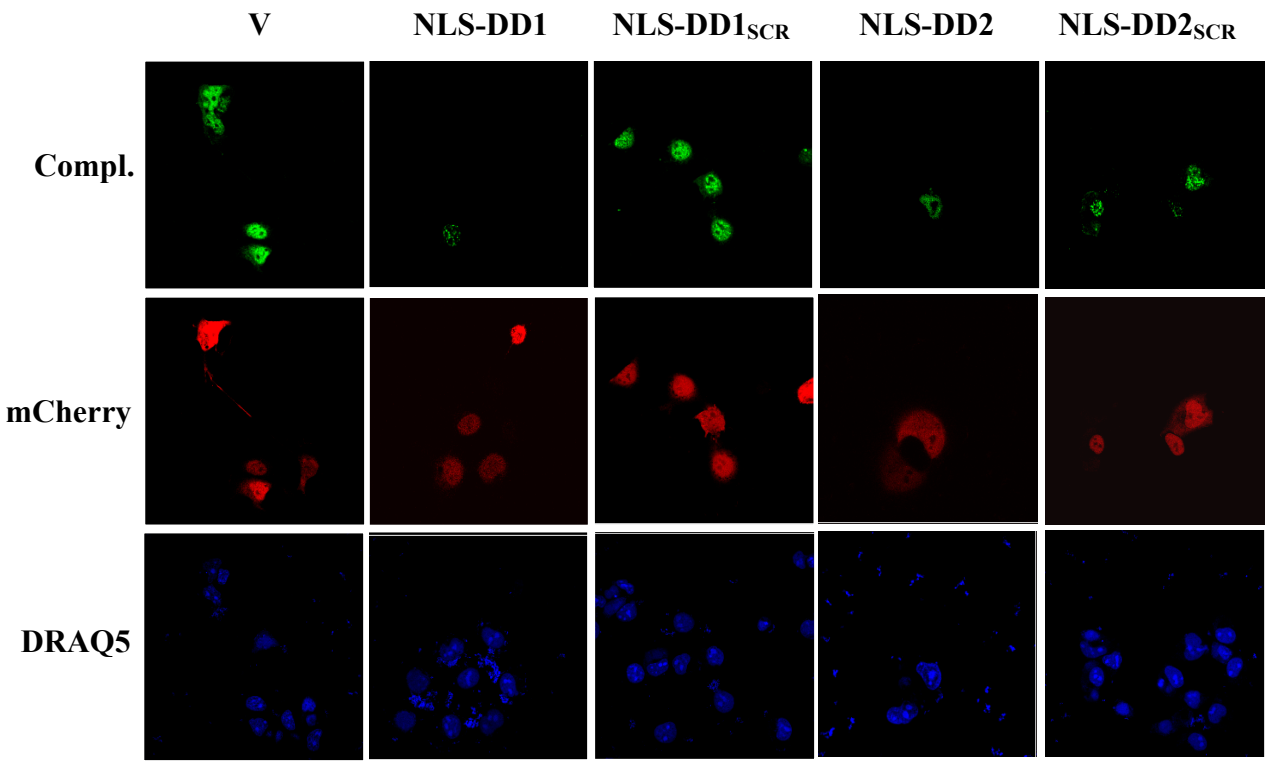

Fig. 50: Genetically encoded peptides block the dimerisation of nTrip6
HeLa cells were transfected with Trip6$^{190-476}$ fused to the N-terminal half of Venus (VN), together with Trip6$^{190-476}$ fused to VC together with expression vectors for the blocking peptides, DD1 and DD2, as well as their respective scrambled controls (DD1$_{SCR}$ and DD2$_{SCR}$) fused to a NLS (nuclear localisation signal) and imaged 18h post tranfection with a confocal laser scanning microscope.

Cells transfected with Trip6$^{190-476}$-VC together with Trip6$^{190-476}$-VN showed Venus complementation within the nucleus, highlighted by DRAQ5, in efficiently transfected cells demonstrated by the expression of mCherry (Fig. 50).
By co-expressing mCherry-NLS-DD1 the dimerisation of Trip6$^{190-476}$ was drastically reduced. In the nucleus of co-transfected cells only a very weak or even no complementation was observed. By co-expressing mCherry-NLS-DD2, the complementation was also reduced, but not as strongly as with mCherry-NLS-DD1. Co-expressing the scrambled control peptides mCherry-NLS-DD1$_{SCR}$ and mCherry-NLS-DD2$_{SCR}$ had no effect on Venus complementation, although the peptides were properly expressed and localised within the nucleus, as judged by the mCherry fluorescence and DRAQ5 staining (Fig. 50).

To estimate the efficiency of the blocking peptides and to compare the relative efficiency of DD1 and DD2, I counted among the transfected, mCherry positive cells, the number of nuclei showing Venus complementation (Fig. 51).

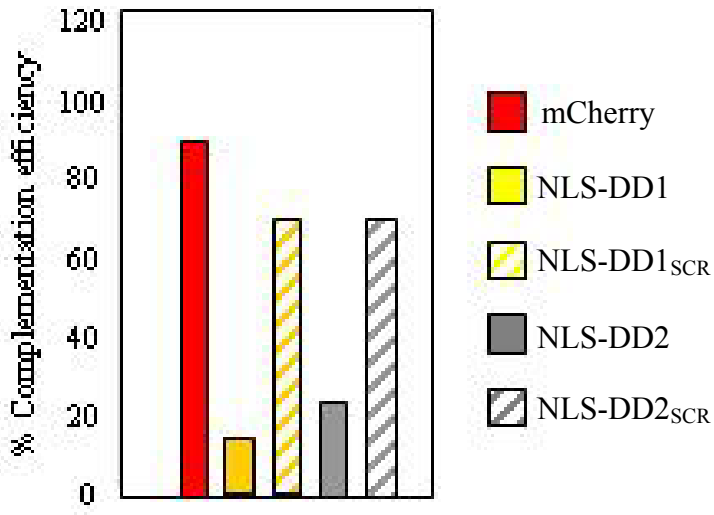

Fig. 51: Quantification of the effect of the blocking peptides on nTrip6 dimerisation
HeLa cells were transfected as indicated in the legend of Fig. 50.

The efficiency was calculated as the percentage of transfected cells (mCherry positive) showing Venus complementation.

In cells co-transfected with mCherry alone, 89% of the nuclei showed complementation of Venus protein (Fig. 51). After co-transfection of DD1, the complementation efficiency was strongly reduced (Fig. 51) whereas the scrambled DD1 peptide had only a minor effect on the dimerisation of nTrip6 (Fig. 51). In cells co-transfected with mCherry-NLS-DD2, the complementation within the nuclei of transfected cells was reduced, but to a weaker extend compared to DD1. The scrambled DD2 control peptide also had only a minor effect on the complementation efficiency. These results show that the blocking peptides specifically inhibit the dimerisation of Trip6$^{190\text{-}476}$ and that DD1 is more efficient then DD2. I therefore decided to continue using DD1 as a blocking peptide.

Blocking peptides are able to block AP-1 function

The next step was to study if blocking the dimerisation of endogenous nTrip6 by the DD1 blocking peptide would have an effect on nTrip6 co-activator function for AP-1.
To test this hypothesis, I transfected HeLa-cells with the AP-1 dependent -517/+63CollI-Luc-reporter gene and co-transfected increasing amounts of either mCherry-NLS-DD1 or its scrambled control (Fig. 52).

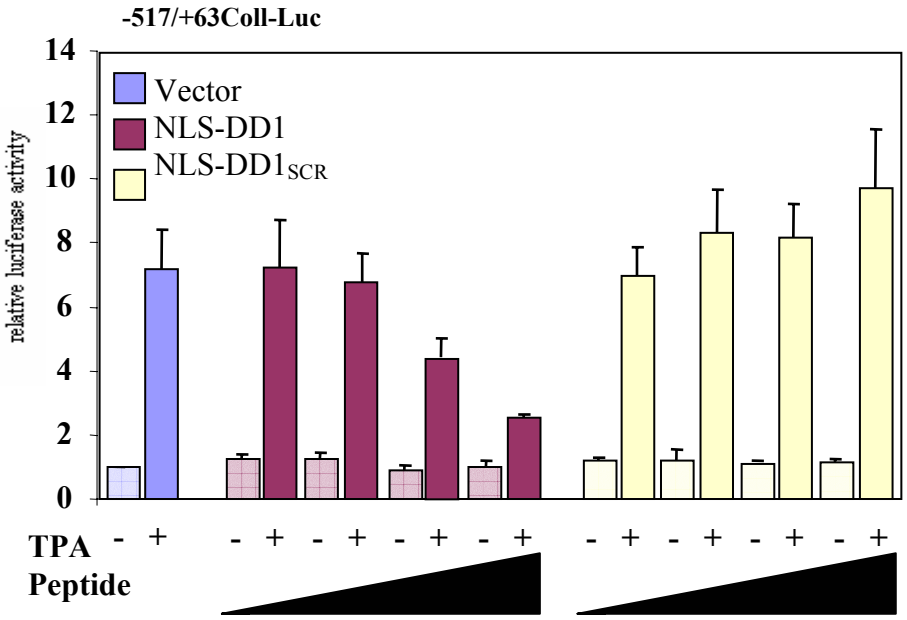

Fig. 52: Inhibition of nTrip6 dimerisation results in repression of AP-1 target genes
HeLa cells were cotransfected with a luciferase reporter gene driven by a minimal AP-1-dependent collagenase-1 promoter, together with either empty vector (V) or increasing amounts of controll-peptide (NLS-DD1$_{SCR}$) or blocking peptide (NLS-DD1). Cells were treated with TPA as indicated and luciferase activities were determined. Results are presented as fold induction, and are the mean ± SD of a representative experiment performed in triplicates.

The transcriptional activity of vector control transfected cells (V) showed a weak level, which was strongly increased after treatment with TPA leading to activation of AP-1. Co-transfection of increasing amounts of mCherry-NLS-DD1 had no effect on the basal reporter activity, but after TPA treatment, a strong inhibitory effect on AP-1 function was observed as compared to vector transfected cells, and this effect was dose-dependent. The scrambled control peptide transfected cells showed no alteration in the reporter gene activity after treatment with TPA, as expected (Fig. 52).
Thus, the dimerisation of endogenous nTrip6 is essential for its co-activator function for AP-1.

3.3.7. nTrip6 dimerisation promotes the recruitment of other proteins to AP-1 activated promoters

Since nTrip6 does not contain any known functional domain, which could account for its co-activator like function, we postulated that it functions by recruiting other co-activators to the promoters. I now have shown that nTrip6 dimerisation is essential for its co-activator function. It is thus reasonable to assume that the dimer formation is essential for the recruitment of the other co-activators, and that the blocking-peptides will compete with these recruitments.

To address this question I used the DNA-pulldown method. A biotinylated DNA-fragment containing a TRE-site, a TATA-box and a part of the luciferase coding sequence, was used as a template to allow the assembly of AP-1, nTrip6 and the co-activator complexes *in vitro* after incubation with nuclear extracts of TPA treated HeLa cells, or of untreated cells as control. The incubation was performed in the presence of increasing concentrations of synthetic blocking peptide DD1. Then the proteins assembled on the DNA template were eluted, separated on an SDS-PAGE and revealed by silver staining (Fig. 53).

Fig. 53: The blocking peptide DD1 interferes with protein assembly on the promoter of AP-1 target genes *in vitro*
A biotinylated DNA bait composed of a TRE, a TATA box and a fragment of luciferase coding sequence was incubated with nuclear extracts of control cells (lane 1) or of cells treated with TPA (lane 2-6) in the presence of increasing amounts (from 0,01mM lane 6 to 1mM lane 3) of synthetic DD1 peptide. The DNA-protein complexes where purified by using streptavidin coated magnetic beads , and the bound proteins were eluted and resolved by SDS-page and visualized by silver staining.

By incubation of the biotinylated DNA template with nuclear extracts of control untreated cells, only a weak recruitment of proteins to the DNA-bait was observed. Incubation of the template with TPA treated nuclear extracts led to the assembly of multiple proteins. This presumably includes AP-1 co-activators.
After co-incubation of the TPA treated nuclear extracts with increasing amounts of the blocking peptide DD1, some discreet protein bands were reduced in their intensity in a DD1 dose-dependent manner, reflecting a lower amount of these proteins recruited to the promoter (Fig. 53).

Thus, the dimerisation of nTrip6 is required for the recruitment of specific proteins to an AP-1 dependent promoter, at least *in vitro*. Only the identification of these proteins, by mass spectrometry, will allow to conclude that they are nTrip6-dependent AP-1 co-activators.

4. Discussion

4.1. nTrip6 acts as a transcriptional co-activator

The AP-1 family of heterodimeric transcription factors has been extensively studied regarding the physiological and pathological functions of the different dimers (Eferl and Wagner, 2003; Hess et al., 2004). However, little is known about the specific regulation of different dimers when bound to DNA. In particular, an open question is whether their function requires dimer-specific co-activators. Some known AP-1 co-activators exert their function independently of the dimer composition. For instance, CBP/p300 and Src1 interact with and co-activate c-Jun, c-Fos as well as ATF2 (Arias et al., 1994; Bannister et al., 1995; Duyndam et al., 1999; Lee et al., 1998). For other AP-1 co-activators, the specificity has not been addressed. The first question addressed in this work concerned the specificity of nTrip6 co-activator function for the different AP-1 dimers.

Based on *in vitro* protein-protein interaction studies, Christine Heilbock (Heilbock, 2004) could show that nTrip6 selectively interacts only with members of the Fos family, respectively c-Fos, Fra 1 and 2. Although based on *in vitro* interaction assays with AP-1 monomers, these results strongly suggested that nTrip6 might be a selective co-activator for Fos containing dimers. This hypothesis was strengthened by the observation that increasing the protein-level of nTrip6 leads to an increased transcriptional activity of c-Jun:c-Fos, but not of c-Jun:ATF2.

During this PhD work I showed that nTrip6 selectively interacts *in vivo* with c-Jun:c-Fos but not with c-Jun:ATF2. Furthermore in the uPa-promoter gene array cell line nTrip6 was only recruited to the promoter when the promoter was bound by c-Jun:c-Fos, but not when bound by c-Jun:ATF2, although this dimer was functionally recruited to the promoter.

Together, these results show that nTrip6 is a selective co-activator for Fos containing dimers (Diefenbacher et al., 2008). nTrip6 does not harbour any known co-activator function and serves as a bridging factor, leading to the recruitment of other yet unknown co-activators or even co-activator complexes. Given that nTrip6 exerts this function selectively for Fos-containing AP-1 dimers, it is likely that other co-activators or co-activator complexes are also recruited in an AP-1 dimer selective manner. However, one cannot exclude the possibility that another protein exerts the same function, i.e. recruits the same co-activators, for dimers not containing Fos, like for example another LIM domain protein.

Since nTrip6 is a specific co-activator limited to AP-1 dimers containing Fos family members the selective manipulation of this interaction could be used to affect only the function of certain AP-1 dimer pairs. c-Jun:Fra2 and c-Jun:ATF2 dimers play distinct, complementary roles in oncogenesis by inducing either anchorage independence or growth factor independence, respectively. v-Jun:ATF2 rather than v-Jun:Fra2 triggers the development of primary fibrosarcomas in the chicken wing (van Dam and Castellazzi, 2001). These results

were obtained by using artificial AP-1 family members with altered or modified properties. Therefore targeting nTrip6, for instance by the usage of blocking peptides, might allow us to discriminate the functions of endogenous AP-1 dimers containing or not containing Fos family members.

However, interfering with the co-activator function of nTrip6 to discriminate between the different functions of the different AP-1 dimers might not be specific enough. Manipulating nTrip6 function might also interfere with the transcriptional activity of other transcription factors. nTrip6 is also a co-activator for NF-κB (Kassel 2004), and I report here that nTrip6 also serves as a co-activator for GR.

During this PhD work I showed that nTrip6 interacts *in vivo* with the glucocorticoid receptor in the nucleus upon activation. Furthermore I could confirm *in vivo* the interaction domain of nTrip6, the LIM-domain 3, as the essential protein-protein interaction surface with GR. This interaction is functional since overexpression of nTrip6 increases the transcriptional activity of GR. In the 7m gene array cell line nTrip6 was recruited to the promoter when only GR was bound to the promoter. Additionally I was able to show that nTrip6 and GR form a complex and are present at a promoter containing a GRE.

Together, these results show that nTrip6 is a co-activator for GR. As in the case of AP-1, this co-activator function most likely results from the recruitment of other co-activator or co-activator complexes. The different classes of co-activators have been extensively studied for their effect on GR (Kassel and Herrlich, 2007; Liberman et al., 2007). Which of these co-activators are recruited in a nTrip6 dependent manner to GR occupied promoters remains to be addressed.

nTrip6 is not the only LIM-domain protein fulfilling the function of a co-activator for GR. The LIM-domain protein Hic-5, first identified as androgen receptor co-activator 55 (ARA55) (Yang et al., 2000), is capable of regulating the transcriptional activity of all steroid receptors, including GR (Guerrero-Santoro et al., 2004; Heitzer and DeFranco, 2006a, b; Kim-Kaneyama et al., 2002). Following the recruitment of Hic-5, different other co-activators are recruited in a Hic-5 dependent manner, like CBP/p300 or TIF-2, to a GR-occupied promoter (Heitzer and DeFranco, 2006b). This might suggest that different LIM-domain proteins would be responsible for the recruitment of different co-activator complexes during the course of the sequential activation of transcription by GR. A similar recruitment of different complexes by different LIM-domain proteins might also hold true for AP-1. Indeed, there are also other LIM-domain proteins which serve as transcriptional co-activators for AP-1. For example, the "four-and-half-LIM domain protein 2", FHL2, associates with both Jun and Fos *in vitro* and *in vivo*. FHL2 stimulates Fos- and Jun-dependent transcription, thereby acting as a co-activator of AP-1 function (Morlon and Sassone-Corsi, 2003). Given that FHL2 consists of only LIM domains, it is obvious that its co-activator function stems from its ability to interact with and recruit other co-activators to the promoter. FHL2 has indeed been shown to interact with CBP/p300 (Labalette et al., 2004). Thus, the function of LIM domain proteins in

transcriptional regulation is most likely to mediate the sequential recruitment of different co-activator complexes to target promoters.
Does a given LIM-domain protein mediate the recruitment of always the same co-activator complex, independent of the transcription factor? This is probably not the case, as illustrated by the different functions that the same LIM-domain protein can exert for different transcription factors. For example, the LIM only protein LMO4 is, on the one hand, a transcriptional co-activator for Smad transcription factors (Lu et al., 2006), on the other hand LMO4 is involved in the negative regulation of estrogen receptor alpha trans-activation function, in a histone deacetylases-dependent manner (Singh et al., 2005). The LIM-domain protein Hic-5, which is able to function as a transcriptional co-activator for AR and GR, mediates the repression of other transcription factors. The activation of Wnt/beta-catenin target genes is regulated by a heterodimer of beta-catenin and the high mobility group box transcription factors of the lymphoid enhancer factor (LEF)/T-cell factor (TCF) family. Hic-5 was identified to interact with these transcription factors and to act as a negative regulator of a subset of LEF/TCF family members (Ghogomu et al., 2006). These examples strongly suggest that the proteins recruited to promoters by LIM-domain proteins also depend on the transcription factor, or on the chromatin context.

4.2. nTrip6 is essential for the "crosstalk" between GR and other transcription factors

nTrip6 does not only serve as a transcriptional co-activator for AP-1, NF-κB and GR, it is also involved in the negative crosstalk between these transcription factors, namely the trans-repression of AP-1 and NF-κB by GR, and the trans-repression of GR by AP-1 and NF-κB. Here, nTrip6 mediates the tethering of the repressing transcription factor to the chromatin-bound repressed transcription factor.
During this PhD work I showed that the ability of GR to tether to and to trans-repress different AP-1 dimers strictly depends on the interaction of the AP-1 dimer with nTrip6. In the 2uPa gene array cell line, nTrip6 was recruited to c-Jun:c-Fos bound promoters, but not to c-Jun:ATF2 bound promoters. Only if nTrip6 was recruited was GR able to tether to the promoter and to repress transcription (Diefenbacher et al., 2008). Thus, nTrip6 is responsible for the very first step in trans-repression, by providing a platform for the tethering of the repressing transcription factor.

AP-1 and NF-κB are not only trans-repressed by GR, these transcription factors are also able to trans-repress the nuclear receptor as well (Burkhart et al., 2005; Hofmann and Schmitz, 2002; Lucibello et al., 1990; McKay and Cidlowski, 2000; Schule et al., 1990). By making use of the 7m-gene array cell line, I could demonstrate that c-Fos is recruited to the promoter-bound GR, and that nTrip6 is present at this promoter at the same time. Upon the recruitment

of c-Fos to the promoter the transcriptional activity of GR was repressed. Furthermore, after mutation of LIM-domain 1 of nTrip6, which is essential for the interaction with c-Fos or p65 (Kassel et al., 2004), c-Fos was no more able to repress the transcriptional activity of GR (Heilbock, 2004), confirming the involvement of nTrip6 in the crosstalk. Taken together, these observations strongly suggest that nTrip6 mediates the tethering of c-Fos to promoter-bound GR, as a first step in the trans-repression of GR by c-Fos. Thus the trans-repression of GR by AP-1 uses the same mechanisms, at least for the first step, than the trans-repression of AP-1 by GR: the nTrip6-dependent tethering of the repressing transcription factor to the promoter-bound repressed transcription factor.

nTrip6 is able to influence the transcriptional activity of its interaction partners in a protein-protein interaction dependent context. It mediates on the one hand the activation, on the other hand it is essential for the repression of its interacting transcription factors. Another LIM-domain family member shows the same features, the context dependent involvement in activation and repression: Hic-5. As mentioned above, Hic-5 is able to increase the transcriptional activity of several nuclear receptors, including GR (Yang et al., 2000). But it is also mediating the repression of a subset of LEF/TCF family members (Ghogomu et al., 2006). Furthermore, like nTrip6, Hic-5 is essential for the repression of glucocorticoid-driven transcription through LEF/TCF family members, which again could be allocated to specific LEF/TCF subtypes (Ghogomu et al., 2006). Thus, the ability of multiple LIM-domain proteins to interact with several transcription factors to positively or negatively influence their transcriptional activity depending on the cellular context provides a mechanism for the fine tuning of the transcriptional response.

GR can trans-repress and is trans-repressed by transcription factors other than AP-1 and NF-κB. For example, GR trans-represses the pituitary specific factor Pit-1 (Nalda et al., 1997). Glucocorticoids were proposed to repress proopiomelanocortin (POMC) gene expression via the trans-repression by GR of the orphan nuclear receptor NGFI-B (Martens et al., 2005). The Spi-1/PU.1 protein, an Ets-related transcription factor, can specifically repress the glucocorticoid-induced activation of promoters carrying a glucocorticoid response element (GRE). Conversely, GR represses Spi-1/PU.1-mediated transcriptional (Gauthier et al., 1993). The repression by GR of the expression of TGF-beta responsive genes containing binding sites for the transcription factors Smad3/4 has been attributed to the interaction between GR and Smad3 (Song et al., 1999). GR trans-represses and is trans-repressed by the T cell transcription factor T-bet (Liberman et al., 2007). In lymphocytes, glucocorticoids suppress IL-4-induced trans-activation of a signal transducers and activators of transcription 6 (STAT6)-responsive promoter without affecting IL-4-stimulated STAT6 DNA-binding. This is mediated via a physical association between GR and STAT6. Reciprocally, STAT6 overexpression increased the IL-4 inhibitory effect on glucocorticoid-induced MMTV trans-activation. (Biola et al., 2000).

Since the reciprocal trans-repression between AP-1, NF-κB and GR requires nTrip6 as a tethering factor, one might also hypothesise a similar role for nTrip6, or for other LIM-domain proteins in the crosstalk between GR and these other transcription factors.

Similarly, other nuclear receptors than GR can trans-repress AP-1 and NF-κB. For example, the thyroid hormone (T3) receptor (TR) is capable of interfering with the transcriptional activity of AP-1, and vice versa, the transcriptional activity of the thyroid receptor can be inhibited by AP-1 (Zhang et al., 1991). Given that Trip6 was first identified as a TR interacting protein (Lee et al., 1995), one could also hypothesise a role for nTrip6 in the crosstalk between TR and AP-1. Another nuclear receptor that can repress AP-1 is the androgen receptor (AR) (Fronsdal et al., 1998; Kallio et al., 1995; Saatcioglu et al., 1994). Furthermore, the transcriptional activity of the AR is repressed by c-Fos (Mora and Mahesh, 1996). *In vitro* interaction studies could show an interaction between AR and nTrip6 (Heilbock, 2004). Thus nTrip6 might be the tethering protein between AR and AP-1. Conversely, *in vitro*, nTrip6 does not interact with the mineralocorticoid receptor (MR) (Heilbock, 2004), and this receptor does not trans-repress AP-1 (Gougat et al., 2002).
Thus, the interaction with LIM-domain proteins such as nTrip6 might be a general requirement for the crosstalk between nuclear receptors and other transcription factors.

Another still open question is the mechanism of trans-repression. The first step is the tethering of the repressing transcription factor to the promoter-bound transcription factor. But how is a transcriptional active complex turned into an inactive state after the recruitment of another active transcription factor? What is the role of nTrip6? Is it a general key-player in the crosstalk between GR and different transcription factors? We could show that nTrip6 functions as a co-activator for the different transcription factors, via the recruitment of other co-activators. Thus, one possibility might be that upon tethering of the repressing transcription factor via its interaction with nTrip6, it blocks the recruitment of the other co-activators. Alternatively, the tethered repressing transcription factor might mediate the recruitment of other factors with repressing properties.

4.3. nTrip6: Mechanism of transcriptional regulation

Since nTrip6 does not carry any known co-activator domain or function, we hypothesised that it exerts its action by mediating the recruitment of other co-activators, acting as a "co-activator platform". The three LIM domains of nTrip6 are the only known protein interaction domains within the protein, making them good candidates for the interaction with other co-activators. Indeed, during this work I could show that the LIM-domains of nTrip6 are sufficient, and the N-terminus dispensable for its autonomous co-activator function. However, I also demonstrated that the N-terminal part of nTrip6, which does not harbour any known co-

activator like domain, is also required for the co-activator function: when this part of nTrip6 was deleted, the transcriptional activity of AP-1 was not further increased. Because of this discrepancy I raised the hypothesis that nTrip6 might have to dimerise via its N-terminus to exert its co-activator like function. Based on *in vivo* interaction assays in combination with the gene array cell line 12C, I could demonstrate that nTrip6 forms a homo-dimer, and this dimer is recruited to the activated AP-1-dependent promoter. Two dimerisation domains, DD1 and DD2, were identified. Both domains are within the N-terminus of Trip6 and are located after the NES. Deletion of the dimerisation domain led to a loss of dimerisation and resulted in the loss of the co-activator function for AP-1. Interestingly, the deletion mutant had a dominant negative effect on the transcriptional activity of AP-1. This might be due to the squelching of co-activators away from the promoter by the dimerisation deficient protein, which still contains its LIM-domains. By using genetically encoded blocking peptides composed of the dimerisation domain fused to a nuclear localisation signal I was able to interfere with the dimerisation of nTrip6. Furthermore, by using these blocking peptides I was able to interfere with the dimerisation of endogenous nTrip6 resulting in an inhibition of AP-1 target genes.

These results clearly demonstrate that nTrip6 dimerisation through its N-terminus is essential for its co-activator function for AP-1. The role of nTrip6 dimerisation for its co-activator function for other transcription factors such as NF-κB and GR remains to be confirmed. Since the interaction domains of nTrip6 are its LIM-domains, the possible protein interactions at the same time are limited by the amount of LIM-domains available. The LIM-domain 1 is required for interaction with the transcription factors c-Fos or NF-κB/p65, whereas the LIM-domains 2 and 3 are required for the interaction with GR (Kassel et al., 2004). Thus, when nTrip6 is tethered to a promoter through its interaction with a transcription factor, its LIM domains are engaged in the interaction, and not available to recruit additional co-activators. Through dimerisation three additional LIM-domains are present at the promoter, and free to interact with other co-activators. Thus, although not formally proven, nTrip6 dimerisation is most likely essential for the recruitment of other co-activators to the regulated promoters. In favour of this hypothesis, I showed that nTrip6 dimerisation is required for the recruitment of several proteins on an AP-1- regulated promoter *in vitro*. It now remains to be demonstrated that these proteins are indeed co-activators, and to confirm that they are recruited in a nTrip6 dimerisation-dependent manner to AP-1-regulated promoters *in vivo*, using the gene array cell line for example. Another LIM-domain protein, Hic-5, was shown to interact both with GR and with co-activators like CBP/p300 on activated GR-dependent promoters. Furthermore, deletion of the LIM domain 4 of Hic-5 interfered with the co-activation properties of p300, suggesting a functional interaction between these co-activators. Hic-5 is also recruited to the *c-fos* promoter, however, here a different subset of co-activators is recruited in a Hic-5 dependent manner (Heitzer and DeFranco, 2006b). Thus, the set of co-activators recruited

through an interaction with a LIM-domain protein does not depend only on the nature of the LIM-domain protein, but also on the nature of the regulated transcription factor.

Also notably is the oligomerising ability of Hic-5. This dimerisation is different from the homo-dimerisation observed for nTrip6: it is attributed to the most C-terminal LIM domain, LIM domain 4, which is uniquely multifunctional. In addition to serving as an interface for interaction with itself and with the LIM-domain protein PINCH and ILK, it is implicated in the scaffold function of Hic-5 in the nucleus to assemble transcriptional complexes (Shibanuma et al., 2004) and in the association of Hic-5 with the nuclear matrix (Guerrero-Santoro et al., 2004). These functions of Hic-5 mediated by LIM domain 4 might be coupled with its homo- and hetero-oligomerisation (Mori et al., 2006). Only when able to form homo- or heterodimers Hic-5 is capable to serve as a transcriptional co-activator for GR (Mori et al., 2006). Since Hic-5 does hetero-dimerise with other LIM-domain proteins (Mori et al., 2006), it would be interesting to investigate whether nTrip6 is capable of interacting with other LIM-domain proteins as well. Based on sequence alignments the first candidate to test this hypothesis would be the closely related LIM-domain protein LPP which shares similar domains with nTrip6, including the dimerisation domains, and also functions as a co-activator (Petit et al., 1996). Another interesting interaction partner would be LMO4 (Lu et al., 2006). Both, LMO4 and nTrip6 interact with GR, GR represses TGF-beta signalling by repressing Smad 3/4 (Song et al., 1999) and LMO4 functions as a co-activator for Smads (Lu et al., 2006). Is the crosslinking of the activating and repressing transcription factors mediated via a hetero-dimer between nTrip6 and LMO4? To test both hypothesis, the interaction between nTrip6 and LPP or LMO4, *in vivo* interaction assays like described in this work are suitable to answer that question. Furthermore, by performing chromatin immunoprecipitations (ChIP) or by generating gene array cell lines containing Smad REs the complex formation between the different proteins on Smad-regulated promoters could be identified. And finally, the use of the blocking peptides would enable to investigate the role of heterodimer formation.

Conclusion

In this work I showed that nTrip6 act as a selective co-activator for a subset of AP-1 dimers. Moreover, the interaction of AP-1 dimers with nTrip6 determines the ability of GR to trans-repress these AP-1 dimers, since GR is tethered to the promoter via its interaction with nTrip6. Similarly, nTrip6 acts as a co-activator for GR, and mediates the tethering of AP-1 and NF-κB to promoter-bound GR, as a first step in the trans-repression of GR by AP-1 and NF-κB. The co-activator function of nTrip6 necessitates its dimerisation through two dimerisation domains in its N-terminus. This dimerisation enables the recruitment of other proteins, presumably co-activators, to the promoter. These properties of nTrip6 are most likely common to other LIM-domain proteins. The ability of transcription factors to interact with several LIM-domain proteins enables fine tuning of the transcriptional response, by the recruitment of different co-activator complexes, and by the possibility of repressing crosstalks with other transcription factors.

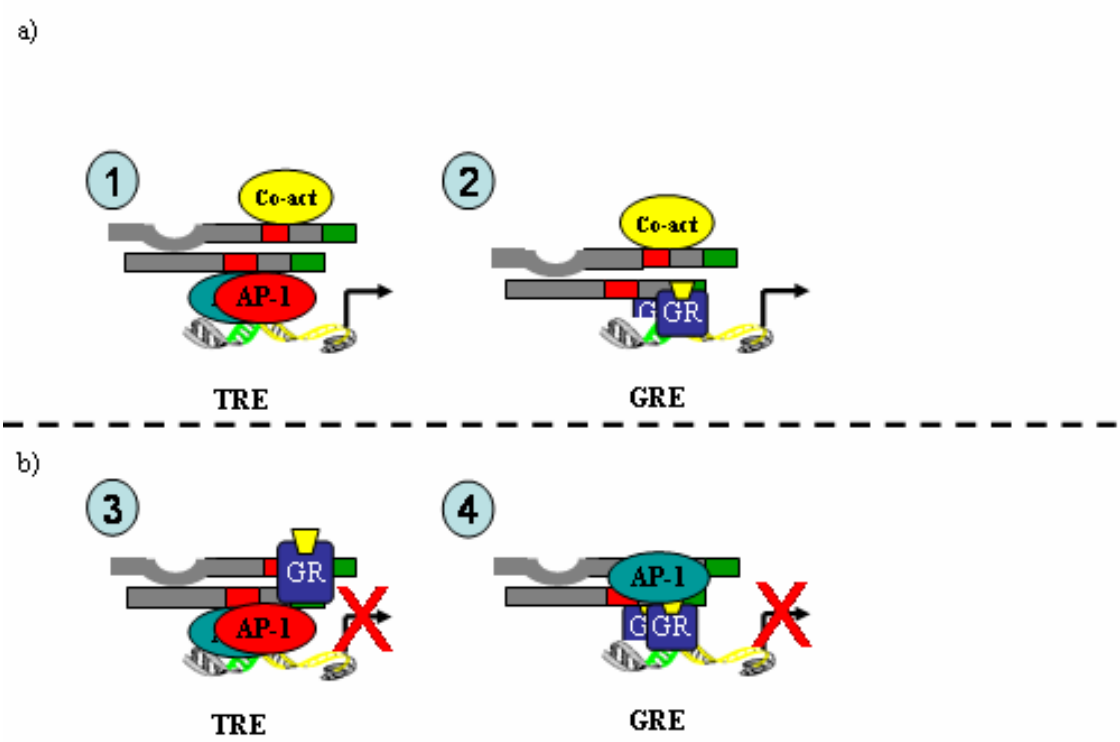

Fig. 54: The role of nTrip6 in the regulation of AP-1 and GR transcriptional activity
a) nTrip6 functions as a transcriptional co-activator
1) nTrip6 functions as a selective co-activator for Fos-family member containing AP-1 dimers. nTrip6 is recruited as a dimer to AP-1-occupied promoters, and increases the transcriptional activity by recruiting other co-activators
2) nTrip6 is a novel co-activator for GR, Similarly, nTri6 acts as a co-activator for GR. Here the dimerisation of nTrip6 has not been demonstrated and is only putative

b) nTrip6 mediates the crosstalk between different transcription factors
3) GR is tethered to promoters bound by AP-1 in a nTrip6 dependent manner, as a first step in the trans-repression of AP-1 activity.
4) Similarly, AP-1 trans-represses GR by tethering to the promoter via an interaction with nTrip6
TRE: TPA response element; GRE: glucocorticoid response element

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