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Faculty of Chemistry and Pharmacy
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Master Thesis for the degree

"Master of Science"

Functional Analyses of Compartmentalized Binary Myc Interactions

submitted by

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under the guidance of

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Abstract

c-Myc is a member of the bHLH-LZ protein family which needs to heterodimerize with Max to perform its critical functions as a transcription factor. Thereby it regulates growth, cell proliferation, DNA replication, cell cycle progression, differentiation, and metabolism. Deregulation of Myc expression contributes to the etiology and progression of cancer. Due to its huge impact on cellular processes, deregulated Myc signaling occurs in almost all types of human cancer. In addition to c-Myc, the variants v-Myc, N-Myc, L-Myc have been studied intensively. In this work, differences of the oncogenic potentials of the Myc variants were analyzed using transformation assays based on avian cell culture systems. v-Myc displayed the highest transforming potential followed by N-Myc, c-Myc, and L-Myc. The transformation potential correlates with the protein-protein interaction (PPI) pattern which was quantified using a protein fragment complementation assay (PCA). In addition to the studies of Myc:Max *Renilla* Luciferase (*R*Luc) PCA dynamics using cellular second messenger molecules, the subcellular localization using Venus YFP (Ven) PCA was determined. Besides nuclear localization of $Myc^{332-439}$:Max and Max:Max, we showed for the first time that Ven PCA tagged full length Myc:Max complexes are restricted to the nucleus of quail embryonic fibroblasts. Finally, a new PPI was analyzed which might link BASP1 functions to Myc signaling. The Myc target gene BASP1 is known to inhibit Myc-induced cell transformation. Calmodulin (CaM) interacts with BASP1 and could be functionally connected to BASP1-mediated Myc inhibition. We have characterized the binding interface of the Myc:CaM PPI, which involves the basic region of Myc. We believe that targeting known and new PPIs of Myc is a promising strategy to reduce Myc variant driven cell proliferation.

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1. Introduction

During the 1960s and 1970s four retroviruses: MH-2, MC29, CMII and OK10 were isolated from avian neoplasms. All of these viruses displayed the ability of transforming monocytes/macrophages which led to subsequent induction of myelocytomas, endotheliomas, kidney and liver tumors in chicken [1–4]. The mentioned viruses had a genetic element in common that was closely correlated with cell transformation, but not related to structural genes of the viruses [2–4]. It was the discovery of the gene *myc* (for *my*elo*c*ytomatosis) which was derived by retroviral transduction from the chicken cellular protooncogene *c-myc* [3, 5–8]. Soon after identifying *myc* as a major player in tumorigenesis two *myc* paralogs were found, designated as *N-myc* and *L-myc* [9–13]. *N-myc* plays a key role in normal development. In addition *N-myc* is frequently amplified in human neuroblastoma, a pediatric tumor of the sympathetic nervous system [14, 15]. Under physiological conditions, L-Myc is expressed during development, but it becomes deregulated in several types of cancer, such as small cell lung and ovarian carcinomas [16–18].

Structural and functional studies in the last 30-40 years showed that Myc acts as transcription factor belonging to the basic region/helix-loop-helix/leucine zipper (bHLH-LZ) protein family [2, 4, 11]. All Myc variants form heterodimers with the Max protein, being also a member of the bHLH-LZ family. By binding of the Myc-Max transcription factor complex to a specific DNA sequence, the so called E-box (CACGTG), the transcription of target genes is initiated [8, 11, 13, 19–22]. In the last 3 decades numerous target genes of Myc were identified. It is discussed that at least 15% of all human genes are directly regulated by Myc [2, 23].

The Myc protein contains several highly conserved regions which are similar to the corresponding regions in N-Myc and L-Myc (**Figure 1**). Parts of these conserved regions are the five Myc boxes (MB): MBI, MBII, MBIIIa, MBIIIb and MBIV. Both, MBI and II are mainly involved in transcriptional activation, whereas

MBII participates in promoting cell transformation in living cells [24], and driving tumorigenesis in animal models [25, 26]. In contrast to MBI and MBII little is known about MBIIIa/b and MBIV. Whether the missing MBIIIa in L-Myc is responsible for the reduced oncogenic potential [26–29] or if it contributes to other functions remains to be clarified [26]. Among other functions, MBIV is reported to be involved in the regulation of apoptosis [30]. All Myc variants, except of L-Myc, contain a nuclear localization signal (NLS) which enables the entry into the nucleus. Dimerization with the bHLH-LZ family member Max typically occurs through the two bHLH-LZ domains. The bHLH-LZ domains of Max and Myc are located at the centre or at the carboxy-terminal end of the proteins, respectively [2].

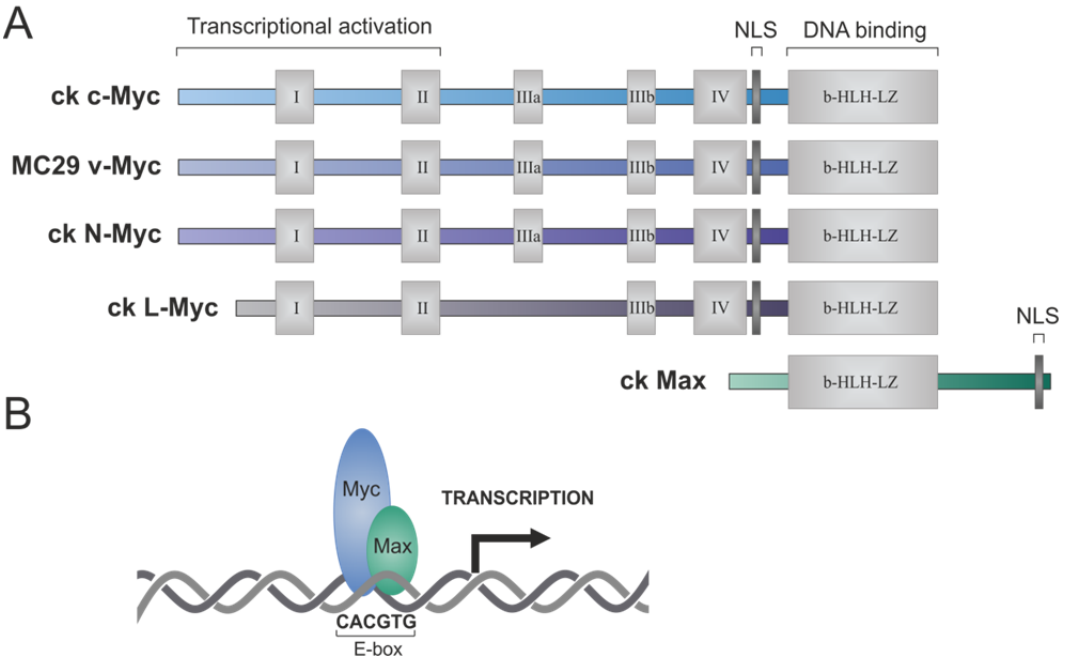


Figure 1. General structure of Myc family members. (A) Representation of the conserved regions (MBI-IV, BR-HLH-LZ) present in c-Myc, v-Myc, N-Myc, L-Myc and their binding partner Max. **(B)** Schematic depiction of the Myc:Max complex bound to a canonical E-box which initiates the transcription of target genes. ck; chicken; I-IV, Myc boxes I-IV; bHLH-LZ, basic region-helix loop helix-leucine zipper domain; NLS, nuclear localization signal.

In contrast to Myc, Max by itself has the ability to form homodimers. This could play an important role in regulating Myc activities. The Max homodimers are less prone to directly regulate transcription [31]. This assumption is based on the idea that no free Max monomeres are available to form PPI with Myc, a precondition for activation of gene expression. Another strategy to limit the rate of Myc:Max complex formation has been reported. It has been shown that Max heterodimerizes with other bHLH-LZ proteins like MXD1 and MNT1 [22, 32]. This also leads to limited availability of free Max proteins for a functional PPI with Myc.

As a transcriptional regulator Myc plays a key role in differentiation, cellular growth, metabolism, cell cycle progression and apoptosis [26]. This involvement in multiple cellular functions converts Myc into a potent oncoprotein following deregulation [8]. It is perhaps not surprising that Myc is involved in almost all types of cancers [4, 16, 26]. Unlike oncoproteins such as Ras, deregulation of Myc does not occur due to mutations within the coding region [26]. Rare exceptions of disease relevant Myc mutations are found in Burkitt's and AIDS-related lymphomas [33]. It has been reported that deregulation of Myc abundance is the driving force for its oncogenic potential [26, 34, 35]. This leads to the question: Is it possible to target just the oncogenic potential of Myc?

In general, there are different possibilities in targeting Myc-driven cancers: either by targeting Myc at the level of expression or by inhibiting the active binary Myc:Max complex [26]. Since Myc is expressed in all proliferating cells, inhibition might be associated with unacceptable toxicity [33]. However, there are also reports suggesting that Myc inhibition represents a safe and effective therapeutic option with only mild side effects [36, 37]

Continuous Myc expression is required to sustain tumor proliferation and viability [33]. Therefore, targeting of specific Myc functions in cancer cells which are necessary for the maintenance of the tumorigenic state is desirable.

Currently the application of small molecules seems to be the most promising strategy [26].

Unfortunately, there are several difficulties in designing a Myc inhibitor. To disrupt the PPI of Myc:Max, the BR-HLH-LZ domain needs to be targeted. The binding interface lacks targetable binding pockets for small molecules, like grooves and pockets which are found in enzymes [33, 38]. Therefore Myc widely earned the reputation to be “undruggable” [38].

However, recently the two Myc inhibitors KJ-Pyr-9 and KJ-Pyr-10 were identified in a Kröhnke pyridine library screen which specifically interfered with Myc:Max complex formation [38]. In addition, also differences among the binding of Max to the different Myc variants have been reported in literature [29]. According to these results, Max displayed the strongest interaction with N-Myc, followed by v-Myc, c-Myc and far behind L-Myc [29]. Testing of the two mentioned Myc inhibitors as well as quantifying the binding affinities of the different Myc variants was performed in our laboratory using a method called Protein-fragment complementation assay (PCA).

The general principle of PCAs is based on a rationally dissected enzyme or fluorescent molecule, for example *Renilla* luciferase (*R*Luc) (**Figure 2**) or Venus YFP (Ven) [39, 40]. The resulting fragments are then fused to two proteins of interest that are thought to bind to each other [41]. When the two proteins of interest interact, the two reporter fragments come into close proximity and reconstitution of a functional reporter protein occurs.

Using this PPI reporter based on *R*Luc PCA, binding affinities of Max with different Myc variants were determined [29]. An additional feature of PCA is the possibility to visualize PPI in localization experiments using a Ven-based PCA. The Ven-based PCA principle is the same as that for the *R*Luc-based PCA.

Besides details about direct Myc interaction partners, a plethora of Myc target genes have been described [16, 23, 42–46]. Myc regulates genes in a stimulatory

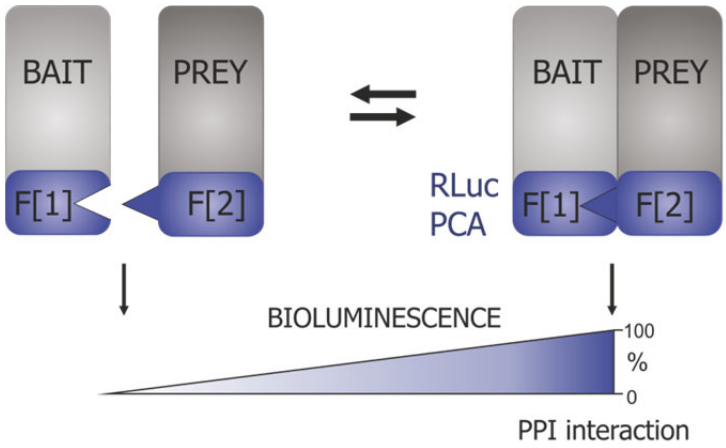


Figure 2. Schematic depiction of the *R*Luc-PCA based PPI reporter. The proteins of interest, referred to as bait and prey, are fused to *R*Luc fragment 1 and 2. Interaction of the proteins results in quantifiable bioluminescence upon addition of the substrate benzylcoelenterazine. *R*Luc, *Renilla* luciferase; PCA, protein-fragment complementation assay; F[1/2], fragment 1/2; PPI, protein-protein interaction

or repressive mode of action [47]. Recently BASP1 (CAP-23, NAP-22), which stands for brain acid-soluble protein 1, was identified as a Myc target gene in our laboratory [48]. Suppression of BASP1 by Myc was observed as well as the inhibiting effect of BASP1 on Myc-induced cell transformation, which defines BASP1 as a potential tumor suppressor [48]. The exact mechanism how BASP1 interferes with Myc driven transformations is still unknown. Therefore the molecular mechanism by which Myc is inhibited by BASP1 remains to be determined. What is known about BASP1? It is a 22 kDa, membrane bound protein which is specifically expressed in nervous tissue [49] and is known to be phosphorylated by protein kinase C (PKC). In addition, BASP1 binds to the Ca^{2+} binding protein Calmodulin (CaM) [50–52].

CaM is a small, highly conserved 149-amino acid protein containing 4 EF-hand motifs [53]. Each of the EF-hand motifs consists of two helices that can bind one Ca^{2+} ion which results in a conformational change of CaM leading to transduction of signals [54]. CaM is involved in numerous processes in the cell, like

proliferation, growth and movement [52]. It has been reported that Ca^{2+} loaded CaM binds to several transcription factors of the bHLH family. By interacting with the bHLH domain of TFs, CaM has the ability to inhibit the binding of DNA and thereby to influence transcription [55]. Recently, an interaction between the transcription factor complex vMyc:Max and CaM was identified in our laboratory. The exact site of interaction and the function of this PPI also in the context of BASP1 signaling remains to be determined.

2. Aims of the study

Deregulation of the Myc variants causes and contributes to the etiology and progression of the different forms of cancer. In the healthy and in the diseased state, Myc function depends on protein-DNA interactions and on localized and distinct PPIs.

The research efforts of this master thesis were divided into four specific aims:

1. Analyses of the different Myc variants regarding their oncogenic potential using two different *in vitro* transformation assays.
2. Cloning and subcellular visualization of Ven-PCA fused to Myc:Max complexes.
3. Effect of the second messenger molecule cAMP on *R*Luc PCA fused Myc:Max complexes.
4. Characterization of the Myc:CaM binding interface.

3 Materials

3.1 Bacteria and cells

Bacteria

- HB101

  HB101 is an *Escherichia coli* derived strain that is used for transformation with plasmid DNA.

  Genotype: F-, thi1, hsdS20 (rB-, mB-), supE44, recA13, ara-14, leuB6, proA2, lacY1, ga/K2, rpsL20 (strr), xyl-5, ntl-1

- XL10-Gold

  An *Escherichia coli* derived strain used for transformation of plasmid DNA

  Genotype: TetrD(mcrA)183D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lacHte [F´ proAB lacIqZDM15Tn10(Tetr) Amy Camr]

- Rosetta pLys-S

  RosettaTM-2(DE3)pLysS-Singles bacteria derived from the BL21 strain were designed to enhance the expression of eukaryotic proteins. DE3 refers to the host which is a lysogen of λDE3 phage that carries a T7 RNA polymerase gene which is under control of the lacUV5 promoter. Addition of IPTG induces the expression of target genes in this strain. Another advantage of this bacterial strain is the expression of T7 lysozyme, which suppresses basal expression of T7 RNA polymerase prior to induction with IPTG.

  Genotype: F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE (Cam^R)

Cells

- QEF

  Quail embryonic fibroblasts were prepared freshly from fertilized quail eggs after they have been incubated for nine days in an egg incubator with water-saturated atmosphere (>60% humidity) and automatic turning device.

- HEK293

  Human embryonic kidney 293 cells

  In 1973 the HEK293 cell line was generated from normal human embryonic kidney cells which were transformed with sheared adenovirus type 5 DNA [56].

- SW480

  The SW480 cell line derived from a human, Dukes' type C, grade IV, colorectal adenocarcinoma [57].

3.2 Enzymes and buffers

Table 1. Enzymes and buffers

Enzyme	Buffer	Supplier
DNA Polymerase I (KLENOW Enzyme)	Klenow buffer (10x) 500 mM Tris (pH 7.5) 100 mM $MgCl_2$ 10 mM DTT 500 µg/ml BSA	Roche Applied Science
Phosphatase, alkaline (from calf serum) CIAP	CIAP buffer (10x) 500 mM Tris (pH 8.5) 1 mM EDTA	Roche Applied Science
Phusion High fidelity DNA Polymerase	Phusion HF buffer (5x) 125 mM Tris-HCl (pH 9.3) 250 mM KCl 7.5 mM $MgCl_2$ 10 mM 2-mercaptoethanol	New England Biolabs®
Restriction endonuclease *Age*I	NEBuffer 1.1 (10x) 10 mM Bis Tris Propane-HCl 10 mM $MgCl_2$ 100 µg/ml BSA (pH 7.0)	New England Biolabs®
Restriction endonuclease *Cla*I	NEBuffer CutSmart 50 mM Potassium acetate 20 mM Tris-acetate 10 mM Magnesium acetate 100 µg/ml BSA (pH 7.9)	New England Biolabs®
Restriction endonuclease *BamH*I	NEBuffer 3.1 100 mM NaCl 50 mM Tris-HCl 10 mM $MgCl_2$ 100 µg/ml BSA (pH 7.9)	New England Biolabs®
Restriction endonuclease *Bgl*II	NEBuffer 3.1 100 mM NaCl 50 mM Tris-HCl 10 mM $MgCl_2$ 100 µg/ml BSA (pH 7.9)	New England Biolabs®
Restriction endonuclease *BspE*I	NEBuffer 3.1 100 mM NaCl 50 mM Tris-HCl 10 mM $MgCl_2$	New England Biolabs®

	100 µg/ml BSA (pH 7.9)	
Restriction endonuclease *Not*I	NEBuffer 3.1 100 mM NaCl 50 mM Tris-HCl 10 mM $MgCl_2$ 100 µg/ml BSA (pH 7.9)	New England Biolabs®
Restriction endonuclease *Pvu*II	NEBuffer 3.1 100 mM NaCl 50 mM Tris-HCl 10 mM $MgCl_2$ 100 µg/ml BSA (pH 7.9)	New England Biolabs®
Restriction endonuclease *Xba*I	NEBuffer CutSmart 50 mM Potassium acetate 20 mM Tris-acetate 10 mM Magnesium acetate 100 µg/ml BSA (pH 7.9)	New England Biolabs®
T4 DNA Ligase	T4 DNA Ligase Reaction Buffer (10x) 500 mM Tris-HCl (pH 7.5) 100 mM $MgCl_2$ 100 mM DTT 1 mM ATP	New England Biolabs®
T4 DNA Polymerase	T4 DNA Polymerase buffer (10x) 100 mM Tris-HCl (pH 7.9) 500 mM NaCl 100 mM $MgCl_2$ 10 mM DTT	New England Biolabs®
T4 Polynucleotide Kinase	T4 Polynucleotide Kinase Reaction Buffer (10x)	New England Biolabs®

3.3 Reaction systems and kits

- QIAGEN® Plasmid Midi Kit 25 (Cat.No.12143)
  - Buffer P1, P2, P3
  - Buffer QBT
  - Buffer QC
  - Buffer QF
  - QIAGEN-tip-100

- QIAQUICK® Purification Kit
  - Buffer PE
  - Buffer PB
  - QIAquick® column

3.4 Molecular markers

3.4.1 Nucleotide marker

Table 2. Lambda *Hind*III nucleotide marker (Sigma Aldrich, #D9780-5MG) and pUC19 *Dde*I nucleotide marker

Lambda HindIII marker	pUC19 DdeI marker
[bp]	**[bp]**
23130	910
9416	540
6557	426
4361	409
2322	235
2027	169
564	

3.4.2 Protein size marker

Table 3. SDS 6H Molecular weight marker (Sigma Aldrich)

Protein	Molecular mass [kDa]
Myosin, porcine	200
β-Galactosidase, *E*. coli	116
Phosphorylase b, rabbit muscle	97
Albumin, bovine	66
Albumin, chicken egg white	45
Carbonic Anhydrase, bovine erythrocytes	29

Table 4. SDS 7H molecular weight marker (Sigma Aldrich)

Protein	Molecular mass [kDa]
Albumine, bovine	66
Albumin, egg	45
Glycerinaldehyde-3-phosphate Dehydrogenase, rabbit muscle	36
Carbonic Anhydrase, bovine	29
Trypsinogen, bovine pancreas	24
Trypsin inhibitor, soybean	20
α-Lactalbumin, bovine milk	

Table 5. Roti-Mark 10-150 molecular weight marker (Roth)

Protein	Molecular mass [kDa]
Roti-Mark 10-150 is a protein size marker which is composed of a set of genetically engineered and in vitro expressed proteins.	150 100 80 60 40 30 20 10

3.5 Oligodeoxynucleotides

Oligodeoxynucleotides were synthesized by Eurofins MWG Operon, Ebersberg, Germany.

Sequence analysis was done by Microsynth AG, Balgach, Switzerland.

Table 6. Oligodeoxynucleotides for polymerase chain reaction (fwd, forward; rev, reverse)

Primer		Sequence	[nt]
MC29 v-myc, c-myc (ck)	fwd	TACCCATACGATGTTCCAGATTACGCTCCGCTCAGCGCCAGCCTC	45
	rev	TAATTATGGATCCTACAGCAGAGCCGCGGGGTT	33
N-myc (ck)	fwd	TACCCATACGATGTTCCAGATTACGCTCCGGGAATGATCAGCAAGAACC	49
	rev	TTATATGGATCCTTAGCAAGTCCGCTTGTACTCTATTTTC	40
L-myc (ck)	fwd	TACCCATACGATGTTCCAGATTACGCTGAGCGGGACGCGTACCAGC	46
	rev	TTATATGGATCCTAGTGCCCCTTGAGCTGAGC	32
pET3d-p15-vmyc-ΔBR	A fwd	TAATACGACTCACTATAGGG	20
	B rev	GCAAAGAAACGCAGCTTCAGCTCATTGTCGTTCTCCTCTGAGTCTAACG	49
	C fwd	CGTTAGACTCAGAGGAGAACGACAATGAGCTGAAGCTGCGTTTCTTTGC	49
	D rev	GCTAGTTATTGCTCAGCGG	19

Table 7. Oligodeoxynucleotides for sequence analysis (fwd, forward; rev, reverse)

Primer		Sequence	[nt]
pcDNA3.1	fwd	CGCAAATGGGCGGTAGGCGTG	21
	rev	TAGAAGGCACAGTCGAGG	18
pRCAS(A)BP	fwd	TGAGCTGACTCTGCTGGTG	19
	rev	GGCCCGTACATCGCATCGAT	20
pET3d	fwd	TAATACGACTCACTATAGGG	20
	rev	GCTAGTTATTGCTCAGCGG	19

3.6 Buffers and Solutions

Acrylamide 30 % (w/v)/bisacrylamide 0.8 % (w/v) *filtrate (0.45 µm)* *store light-protected at 4 °C*	30g acrylamide 0.8 g bisacrylamide H2O to 100 ml
Alkaline lysis solution I *25 mM Tris HCl pH 8.0, 10 mM EDTA, 50 mM glucose* *autoclave, add 25 ml 1 M glucose,* *store at 4°C*	12.5 ml 1 M Tris HCl pH 8.0 10 ml 0.5 M EDTA H2O to 475 ml
Alkaline lysis solution II *0.2 M NaOH, 1 % (w/v) SDS* *prepare fresh,* *store at RT*	93 ml H2O 5 ml 20 % SDS 2 ml 10 M NaOH
Alkaline lysis solution III *3 M potassium acetate pH 4.8* *60 ml 5 M KOAc* *11.5 ml glacial acetic acid*	60 ml 5 M KOAc 11.5 ml glacial acetic acid 28.5 ml H_2O
Ammonium acetate 10 M *filter sterilize (0.22 µm),* *store at RT*	7.71 g ammonium acetate H2O to 10 ml
Ampicillin 100 mg/ml *filter sterilize (0.22 µm)* *store 1-ml aliquots at -20°C*	1 g ampicillin sodium salt H_2O to 10 ml
AP buffer *100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$*	50 ml Tris-HCl pH 9.5 10 ml 5 M NaCl 5 ml 1M $MgCl_2$ H_2O to 500 ml
APS 10% (w/v) *store 1-ml aliquots at -20°C*	1 g ammonium peroxodisulfate H_2O to 10 ml
ATP 100 mM *store 1-ml aliquots at -20°C*	1g adenosine triphosphate 18 ml H_2O

Avian cell culture medium (Q8 medium) *1 × HAM's F10 with glutamine, 0.295 % (w/v) TPB, 5 %(v/v) calf serum, 2 % (v/v) chicken serum, 1.875 % (w/v) NaHCO3, 0.5 % (v/v) DMSO, 1x antibiotics/antimycotics* *store at 4 °C*	50 ml 10 × HAM's F10 with glutamine 50 ml 2.95 % (w/v) tryptose phosphate broth (TPB) 25 ml calf serum 10 ml chicken serum (heat-inactivated at 56°C for 30 min) 12.5 ml 7.5 % (w/v) NaHCO3 2.5 ml dimethyl sulfoxide (DMSO) 5 ml 100x antibiotics/antimycotics 345 ml H2O
Bacto agar 1.8 % (w/v) *autoclave,* *store at 4 °C*	1.8 g Bacto agar H2O to 100 ml
Blocking solution (AP) *5 % (w/v) non-fat dry milk, 20 mM NaCl, 150 mM NaCl* *prepare fresh,* *store at RT*	7.5 g non-fat dry milk 1x TBS to 150 ml
Blocking solution (ECL) *5 % (w/v) non-fat dry milk, 10 mM sodium phosphate pH 7.2, 150 mM NaCl, 0.1 % (v/v) Tween® 20* *prepare fresh,* *store at RT*	7.5 g non-fat dry milk 150 µl Tween® 20 1x PBS to 150 ml
BRADFORD-reagent 4% (w/v) Serva Blue G 4% (v/v) EtOH 6.8% o-H_3PO_4 *prepare fresh*	1 ml Serva Blue G-solution 1 ml EtOH 400 ml o-H_3PO_4 21 ml H_2O
Bromophenol blue 1% (w/v) *store at 4°C*	10 mg bromophenol blue H_2O to 1 ml
BSA 10x *1 mg/ml BSA* *store at -20°C*	100 µL 100x bovine serum albumin supplied by New England BioLabs Inc. 900 µL H_2O
BSA 100x *10 mg/ml bovine serum albumine (BSA)*	*supplied by New England BioLabs Inc.* store at -20°C

Buffered F10 medium (HBF10) *1x HAM's F10 with glutamine, 1.875 % (w/v) NaHCO3, 25 mM HEPES pH 7.3* *store at 4°C*	50 ml 10x HAM's F10 with glutamine 12.5 ml 7.5 % (w/v) NaHCO3 12.5 ml 1 M HEPES pH 7.3 425 ml H2O
n-Butanol water saturated *store at RT*	100 ml n-butanol 100 ml H_2O
Chloroform/isoamylalcohol (24:1)	24 ml $CHCL_3$ 1 ml isoamylalcohol
CIAP (calf intestine phosphatase) buffer 10x *500 mM Tris HCl pH 8.5, 1 mM EDTA* *store at 4°C*	500 µl 1 M Tris HCl pH 8.5 2 µl 0.5 M EDTA 498 µl H2O
CIAP buffer 10x *0.5 M Tris-HCl pH 8.5, 1 mM EDTA* *store at 4°C*	500 µL 1 M Tris-HCl pH 8.5 400 µL 0.5 M EDTA pH 8.0 498 µL H_2O
Cloning bottom agarose *1x HAM's F10 with glutamine, 0.295 % (w/v) TPB, 3.5 %(v/v) calf serum, 1.2 5% (v/v) chicken serum, 0.1875 %(w/v) NaHCO3, 1.25 % (v/v) DMSO, 1x glutamine, 1xantibiotic-antimycotic, 0.625 % (w/v) Sea plaqueagarose*	1 vol 1.5 % (w/v) Sea plaque agarose 1.4 vol DC3 medium heat Sea plaque agarose in a microwave oven, then cool to 45 °C in a waterbath; mix with 45 °C warm DC3 medium
Cloning medium *1.25x HAM's F10 with glutamine, 0.369 % (w/v) TPB,12.5 % (v/v) calf serum, 5.75 % (v/v) chicken serum,0.25 % (v/v) DMSO, 1.875x vitamins solution, 1.875xfolic acid solution, 1.25x glutamine, 1.25x antibioticantimycotic* *store at 4°C*	50 ml 10x HAM's F10 with glutamine 50 ml 2.95 % (w/v) TPB 50 ml calf serum 23 ml chicken serum 1 ml dimethyl sulfoxide (DMSO) 7.5 ml 100x vitamins solution 7.5 ml 100x folic acid solution 5 ml 100x L-glutamine (200 mM) 5 ml 100x antibiotic-antimycotic 200 ml H2O

Cloning top agarose *1x HAM's F10 with glutamine, 0.295 % (w/v) TPB, 10 %* *(v/v) calf serum, 4.6 % (v/v) chicken serum, 0.2 % (v/v) DMSO, 1.5x vitamins solution, 1.5x folic acid solution, 1x glutamine, 1x antibiotic-antimycotic, 0.3 % (w/v) Sea plaque agarose*	1 vol 1.5 % (w/v) Sea plaque agarose 4 vol Cloning medium heat Sea plaque agarose in a microwave oven, then cool to 45 °C; mix with 45 °C warm Cloning medium and keep at 45 °C in a waterbath
Coomassie-Destaining solution *(SDS-PAGE)* *30% (v/v) MeOH, 10% (v/v) HOAc* *store at RT*	300 ml MeOH 100 ml acetic acid H_2O to 1000 ml
Coomassie-Staining solution (SDS-PAGE) 0.5% (w/v) Coomassie Serva G 50% (v/v) MeOH 10% (v/v) HOAc *store light-protected at RT*	5 g Coomassie Serva G 500 ml MeOH 100 ml acetic acid H_2O to 1000 ml
DC3 medium *1.71x HAM's F10 with glutamine, 0.507 % (w/v) TPB, 6.02 % (v/v) calf serum, 2.15 % (v/v) chicken serum, 0.323 % (v/v) NaHCO3, 2.15 % (v/v) DMSO, 1.71x glutamine, 1.71x antibiotic-antimycotic* *store at 4°C*	85.7 ml 10x HAM's F10 with glutamine 85.7 ml 2.95 % (w/v) Tryptose phosphate broth (TPB) 30.1 ml calf serum 10.75 ml chicken serum 21.5 ml 7.5 % (w/v) NaHCO3 10.75 ml dimethyl sulfoxide (DMSO) 8.6 ml 100x L-glutamine (200 mM) 8.6 ml 100x antibiotic-antimycotic 238.3 ml H2O
DNA ligation buffer 10x *500 mM Tris HCl pH 7.5, 100 mM MgCl2, 100 mM DTT, 10 mM ATP* *store 10-µl aliquots at -20°C*	50 µl Tris HCl pH 7.5 10 µl 1 M MgCl2 10 µl 1 M DTT 10 µl 100 mM ATP 20 µl H2O
DNA sample buffer 5x *20 mM EDTA* *30% (v/v) glycerol* *0.5% (w/v) SDS* *0.05% (w/v) bromophenol blue* *store at 4°C*	1 ml 0.5 M EDTA (pH 8.0) 7.5 ml glycerol 0.625 ml 20 % (w/v) SDS 1.25 ml 1 % (w/v) bromophenol blue H_2O to 25 ml

dNTP-mix *10 mM dATP* *10 mM dCTP* *10 mM dGTP* *10 mM dTTP* *store 10-µL aliquots at -20°C*	10 µL 100 mM dATP 10 µL 100 mM dCTP 10 µL 100 mM dGTP 10 µL 100 mM dTTP 60 µL H_2O
DTT 1 M *1M DTT* *10 mM sodium acetate pH 5.2* *filter sterilize (0.22 µm)* *store 1-ml aliquots at -20°C*	1.54 g DTT 33 µL 3M NaOAc pH 5.2 H_2O to 10 ml
Dialyse bag preperation *M_r cut-off: 10,000-20,000* *autoclave at 121°C for 15 min,* *store at 4°C,* *before use, rinse tubes with deionized H_2O*	Cut 60 tubes of 10 cm length and boil for 10 min in 1000 ml of 2% (w/v) $NaHCO_3$, 1 mM EDTA. Rinse in deionized H_2O and distribute into two 500 ml glass bottles containing each 500 ml 1mM EDTA
ECL-detection solution 1	supplied by ECL Western blotting system (GE Healthcare)
ECL-detection solution 2	supplied by ECL Western blotting system (GE Healthcare)
EDTA 0.5 M pH 8.0 *autoclave at 121°C for 15 min,* *store at RT*	93.05 g EDTA 400 ml H_2O adjust to pH 8.0 with NaOH (10 M) H_2O to 500 ml
Ethanol 70% (v/v)	70 ml EtOH absolute H_2O to 100 ml
Ethidium bromide 1 mg/ml *store light-protected at RT*	100 µL 10 mg/ml ethidium bromide 900 µL H_2O
Focus agar *1x HAM's F10 with glutamine, 0.295 % (w/v) TPB, 3.5 %(v/v) calf serum, 1.25 % (v/v) chicken serum, 0.1875 %(v/v) NaHCO3, 1.25 % (v/v) DMSO, 1x glutamine, 1xantibiotic-antimycotic, 0.75 % (w/v) Bacto agar*	1 vol 1.8 % (w/v) Bacto agar 1.4 vol DC3 medium heat Bacto agar in a microwave oven, then cool to 45 °C in a waterbath; mix with 45 °C warm DC3 medium

Folic acid solution 100x *1.81 mM folic acid, 1 M NaHCO3*	8.4 g NaHCO3 50 ml H2O 80 mg folic acid H2O to 100 ml
Freezing medium *0.55x HAM's F10 with glutamine, 0.162 % (w/v) TPB, 27.75 % (v/v) calf serum, 1.1 % (v/v) chicken serum, 1.031 % (v/v) NaHCO3, 20.275 % (v/v) DMSO, 0.55x antibiotics/antimycotics* *Store at 4°C*	5.5 ml Avian cell culture medium 2.5 ml calf serum 2.0 ml dimethyl sulfoxide
GIEMSA staining solution *heat to 80°C*	1 vol GIEMSA solution (azur-eosin-methylene blue) 9 vol H_2O
Glucose 1 M *filter sterilize (0.22 µm),* *store at 4°C*	4.95 g glucose H_2O to 25 ml
Glycerol 10% (v/v) *filter sterilize (0.22 µm)* *prepare fresh*	50 ml glycerol 450 ml H_2O
Growth medium (HBGM) *1x HAM's F10 with glutamine, 10 % (v/v) calf serum,* *1.875 % (w/v) NaHCO3, 25 mM HEPES pH 7.3,* *1x antibiotics/antimycotics* *store at 4°C*	50 ml 10x HAM's F10 with glutamine 50 ml calf serum 12.5 ml 7.5 % (w/v) NaHCO3 12.5 ml 1 M HEPES pH 7.3 5 ml 100x antibiotics/antimycotics 370 ml H2O
HBS (HEPES buffered saline) 2x *280 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4, 12 mMglucose, 50 mM HEPES pH 7.05* *filter sterilize (0.2 µm),* *store in 50-ml aliquots at -20°C*	6.55 g NaCl 0.296 g KCl 0.106 g Na2HPO4 × 2 H2O 0.95 g glucose × H2O 4.77 g HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) 360 ml H2O adjust to pH 7.05 with 0.5 N NaOH H2O to 400 ml

HEPES 1 M pH 7.9 *filter sterilize (0.22 µm)* *store at -20°C*	23.83 g HEPES [N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid] ~90 ml H_2O adjust to pH 7.9 with 10 N NaOH H_2O to 100 ml
Hoechst 33342	supplied by Sigma-Aldrich
Immunoblot transfer buffer *25 mM Tris, 190 mM glycine, 20 % (v/v) methanol p.a.* *store at RT*	3 g Tris 14.4 g glycine 200 ml methanol p.a. H2O to 1000 ml
Immunoblot washing buffer 5x *100 mM Tris HCl pH 7.5, 2.5 M NaCl, 2.5 % (v/v) Tween® 20, 0.25 % (v/v) Igepal® CA-630* *store at RT*	100 ml 1 M Tris HCl pH 7.5 146.1 g NaCl 25 ml Tween® 20 2.5 ml Igepal® CA-630 H2O to 1000 ml
Immunoblot washing buffer 1x *20 mM Tris HCl pH 7.5, 0.5 M NaCl, 0.5 % (v/v) Tween® 20, 0.05 % (v/v) Igepal® CA-630* *store at RT*	100 ml 5x Immunoblot washing buffer 900 ml H2O
IPTG 1 M *filter sterilize (0.22 µm)* *store at 4°C*	2.38 g isopropyl β-D-1-thiogalacto-pyranoside H_2O to 10 ml
KLENOW enzyme solution *1 unit/µl DNA polymerase 1 Klenow fragment (cloned) in 100 mM potassium phosphate pH 6.5, 10 mM 2-mercaptoethanol, 50 % (v/v) glycerol*	*supplied by the Amersham Megaprime™ DNA Labeling System, RPN1607, GE Healthcare*
KLENOWfragment	*supplied by the cDNA synthesis kit XY-003-00-04 (GE Healthcare)*
KLENOW polymerase buffer 10x *500 mM Tris-HCl* *100 mM MgCl2* *store at -20°C*	500 µL 1 M Tris-HCl pH 7.6 100 µL 1 M MgCl2 400 µL H_2O

LB-medium *170 mM NaCl* *10% (w/v) bacto-tryptone* *5% (w/v) yeast extract* *autoclave at 121°C for 15 min,* *store at 4°C*	10 g NaCl 10 g bacto-tryptone 5 g yeast extract H_2O to 1000 ml
LB medium agar plates *170 mM NaCl, 10 % (w/v) bacto-tryptone, 5 % (w/v)* *yeast extract, 15 % (w/v) agar agar* *store at 4 °C wrapped in plastic foil*	15 g agar agar 1000 ml LB medium autoclave, cool to 50 °C, pour ~25 ml per 80-mm dish and let harden for 1 d
LB-medium agar plates with ampicillin *170 mM NaCl* *10% (w/v) bacto-tryptone* *5% (w/v) yeast extract* *15% (w/v) agar agar* *100 µg/ml ampicillin* *store at 4°C wrapped in plastic foil*	15 g agar agar 1000 ml LB-medium autoclave at 121°C for 15 min, cool to 50°C add 1 ml 100 mg/ml ampicillin and mix pour ~25 ml per 80-mm dish and let harden for 1 day
Ligation buffer *store at -20 °C*	660 µl 1 M Tris HCl pH 7.6 20 µl 0.5 M spermidine HCl 100 µl 1 M MgCl2 150 µl 1 M DTT 200 µl 100x BSA H2O to 10 ml
Lysis buffer *150 mM NaCl* *10 mM sodium phosphate pH 7.2* *0.05% Triton-X100*	8.8 g NaCl 10 ml 0.1 M sodium phosphate pH 7.2 0.5 ml Triton-X100 H_2O to 1000 ml
Lysozyme 10 mg/ml *store in 1 ml aliquots at -20°C*	100 mg lysozyme 10 mM Tris-HCl pH 7.5 to 10 ml
MgCl2 1 M *autoclave,* *store at RT*	20.33 g MgCl2 × 6 H2O H2O ad 100 ml
$MgSO_4$ 1 M *autoclave at 121°C for 15 min,* *store at RT*	24.6 g $MgSO_4 \cdot 7H_2O$ H_2O to 100 ml

NaCl 5 M *autoclave at 121°C for 15 min,* *store at RT*	146 g NaCl H_2O to 500 ml
NaCl 1.6 M, 13 % (w/v) PEG *filtrate (0.45 µm),* *store at RT*	32 ml 5 M NaCl 13 g PEG 6000 H2O to 100 ml
NaOAc 3 M pH 5.2 autoclave at 121°C for 15 min, *store at RT*	24.61 g sodium acetate 80 ml H_2O adjust to pH 5.2 with acetic acid H_2O to 100 ml
NEBuffer 1 10x *100 mM Bis-Tris Propane HCl pH 7.0* *100 mM MgCl2, 10 mM DTT* *store at -20°C*	supplied by New England BioLabs Inc.
NEBuffer 2 10x *100 mM Tris HCl pH 7.9* *500 mM NaCl* *100 mM MgCl2, 10 mM DTT* *store at -20°C*	supplied by New England BioLabs Inc.
NEBuffer 3 10x *500 mM Tris HCl pH 7.9* *1 M NaCl* *100 mM MgCl2, 10 mM DTT* *store at -20°C*	supplied by New England BioLabs Inc.
NEBuffer 4 10x *200 mM Tris acetate pH 7.9, 500 mM KOAc, 100 mM magnesium acetate, 10 mM DTT* *store at -20°C*	supplied by New England BioLabs Inc.
PBS (phosphate buffered saline) 10x *autoclave at 121°C for 15 min,* *store at RT*	80 g NaCl 2 g KCl 14.4 g Na_2HPO_4 2.4 g KH_2PO_4 adjust to pH 7.4 H_2O to 1000 ml

PBS-T *10 mM sodium phosphate pH 7.2, 150 mM NaCl,* *0.1 (v/v) Tween® 20* *store at RT*	1000 ml PBS 1 ml Tween® 20
PCRx Amplification buffer	supplied by the PCRx Enhancer system (Invitrogen)
PCRx Enhancer solution	supplied by the PCRx Enhancer system (Invitrogen)
PGM (primary growth medium) *1x HAM's F10 with glutamine, 0.295 % (w/v) TPB, 8 %(v/v) calf serum, 2 % (v/v) chicken serum, 1.875 % (w/v) NaHCO3, 1x antibiotics/antimycotics* *store at 4°C*	50 ml 10x HAM's F10 with glutamine 50 ml 2.95 % (w/v) tryptose phosphate broth (TPB) 40 ml calf serum 10 ml chicken serum (heat-inactivated at 56°C for 30 min) 12.5 ml 7.5 % (w/v) NaHCO3 5 ml 100x antibiotics/antimycotics H2O to 500 ml
Phenol/chloroform/isoamyl alcohol (25:24:1)	12.5 ml ROTI-phenol 12 ml $CHCL_3$ 0.5 ml isoamyl alcohol
Phusion HF buffer 5x	supplied by the Phusion TM High-fidelity DNA Polymerase Kit (New England BioLabs Inc.)
Ponceau S-Staining solution *0.2% (w/v) Ponceau S* *3% (w/v) trichloro acetic acid* *store light-protected*	0.5 g Ponceau S [3-hydroxy-4-(2-sulfo-4-[sulfo-phenylazo]-phenyl-azo)-2,7-naphthalene-disulfonic acid] 7.5 g trichloro acetic acid H_2O to 250 ml
Potassium acetate 5 M *autoclave, store at RT*	147.23 g potassium acetate (KOAc) H2O to 300 ml

pUC19/DdeI marker 250 ng/µL *store in 1-ml aliquots at -20°C*	50 µL 1 µg/µL pUC19 plasmid DNA 35 µL H_2O 10 µL 10x restriction enzyme buffer 5 µL restriction enzyme *DdeI* Incubate for 1 hour at 37°C. Heat for 10 minutes at 70°C to stop reaction. Add 100 µL H_2O.
RNAseA solution 10 mg/ml *10 mM Tris-HCl pH 7.5* *15 mM NaCl* *store in 1-ml aliquots at -20°C*	100 mg RNAse (bovine pancreas) 100 µL 1 M Tris-HCl pH 7.5 30 µL 5 M NaCl H_2O to 10 ml Heat to 100°C for 15 min, slowly cool to RT.
SDS 20% (w/v) *store at RT*	20 g sodium dodecylsulfate H_2O to 100 ml
SDS gel-loading buffer 1x *60 mM Tris HCl pH 6.8, 3 % (w/v) SDS, 10 % (v/v)* *glycerol, 0.005 % (w/v) bromophenol blue, 5 % (v/v) 2-mercaptoethanol*	0.6 ml 1 M Tris HCl pH 6.8 1.5 ml 20 % (w/v) SDS 1 ml glycerol 50 µl 1 % (w/v) bromophenol blue H2O to 9.5 ml store at RT prior to use add 500 µl 2-mercaptoethanol
SDS- Protein sample buffer (Laemmli) 5x *125 mM Tris-HCl pH 6.8* *20% (w/v) SDS* *15% (w/v) DTT* *0.1% (v/v) bromophenol blue* *store in 1-ml aliquots at -20°C* *Colour has to be dark blue.*	2.5 ml 1 M Tris-HCl pH 6.8 ~5 ml H_2O 2 g SDS 1.5 g DTT 1 ml 1% bromophenol blue H_2O to 10 ml, dissolve components 10 ml glycerol
Sea plaque agarose 1.5 % (w/v) *autoclave, store at 4°C*	1.5 g Sea plaque agarose H2O to 100 ml
Separating gel acrylamide mix *filtrate (0.45 µm),* *store light-protected at 4 °C*	66 g acrylamide 1.8 g bisacrylamide (N,N'-methylene bisacrylamide) H2O to 200 ml

Separating gel solution 10 % (40 ml) *10 % (w/v) acrylamide, 0.27 % (w/v) bisacrylamide,0.375 M Tris HCl pH 8.8, 0.1 % (w/v) SDS* *degas for 5 min*	12.1 ml separating gel acrylamide mix 17.7 ml H2O 10 ml 1.5 M Tris HCl pH 8.8 200 µl 20 % (w/v) SDS
Separating gel solution 12.5 % (40 ml) *12.5 % (w/v) acrylamide, 0.34 % (w/v) bisacrylamide,0.375 M Tris HCl pH 8.8, 0.1 % (w/v) SDS* *degas for 5 min*	15.2 ml separating gel acrylamide mix 14.6 ml H2O 10 ml 1.5 M Tris HCl pH 8.8 200 µl 20 % (w/v) SDS
Separating gel solution 15 % (40 ml) *15 % (w/v) acrylamide, 0.41 % (w/v) bisacrylamide, 0.375 M Tris HCl pH 8.8, 0.1 % (w/v) SDS* *degas for 5 min*	18.2 ml separating gel acrylamide mix 11.6 ml H2O 10 ml 1.5 M Tris HCl pH 8.8 200 µl 20 % (w/v) SDS
Stacking gel acrylamide mix *20 % (w/v) acrylamide, 1 % (w/v) bisacrylamide* *filtrate (0.45 µm),* *store light-protected at 4 °C*	20 g acrylamide 1 g bisacrylamide (N,N'-methylene bisacrylamide) H2O to 100 ml
Stacking gel solution (10 ml) *4.5 % (w/v) acrylamide, 0.23 % (w/v) bisacrylamide,* *0.125 M Tris HCl pH 6.8, 0.1 % (w/v) SDS* *degas for 5 min*	2.25 ml stacking gel acrylamide mix 5.2 ml H2O 2.5 ml 0.5 M Tris HCl pH 6.8 50 µl 20 % (w/v) SDS
STE *10 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA* *autoclave, store at RT*	10 ml 1 M Tris HCl pH 7.5 2 ml 0.5 M EDTA 30 ml 5 M NaCl H2O to 1000 ml
Stripping solution *5 mM Tris HCl pH 8.0, 0.1x DENHARDT's reagent, 2 mM EDTA* *store at 4°C*	5 ml 1 M Tris HCl pH 8.0 2 ml 50x DENHARDT's reagent 4 ml 0.5 M EDTA H2O to 1000 ml
T4 DNA ligase	*supplied by the cDNA synthesis kit XY-003-00-04* *(GE Healthcare)*

TBE buffer 10x *890 mM Tris-HCl* *890 mM boric acid* *20 mM EDTA* *autoclave at 121°C for 15 min,* *store at RT*	108 g Tris base 55 g boric acid 40 ml 0.5 M EDTA pH 8.0 H_2O to 1000 ml
TBST 1.5 M 10x *1.5 M NaCl* *0.5% (v/v) Tween* *200 mM Tris-HCl pH 7.4* *store at RT*	88 g NaCl 5 ml Tween 200 ml 1 M Tris-HCl pH 7.4 H_2O to 1000 ml
TBST 2 M 10x *2M NaCl* *0.5% (v/v) Tween* *200 mM Tris-HCl pH 7.4* *store at RT*	117 g NaCl 5 ml Tween 200 ml 1 M Tris-HCl pH 7.4 H_2O to 1000 ml
TE *10 mM Tris HCl pH 8.0, 1 mM EDTA* *autoclave, store at RT*	10 ml 1 M Tris HCl pH 8.0 2 ml 0.5 M EDTA H2O to 1000 ml
TPB *2.95 % (w/v) tryptose phosphate broth* *autoclave, store at 4°C*	29.5 g tryptose phosphate broth H2O to 1000 ml
Transfer buffer *10 % (v/v) calf serum in TS buffer*	10 ml calf serum 90 ml TS buffer
Tris-Glycine Semidry-transfer buffer 5x *0.14 % (w/v) Tris base, 0.7% (w/v) glycine* *store at RT*	14.5 g Tris base 72 g glycine H_2O to 1000 ml
Tris-Glycine Semidry-transfer buffer 1x *20% MeOH* *store at RT*	600 ml H_2O 200 ml MeOH 200 ml 5x Tris-glycine Semidry-transfer- buffer
Tris HCl 1 M pH 7.4/7.6/8.0 *autoclave at 121°C for 15 min,* *store at RT*	60.55 g Tris base 400 ml H_2O adjust to pH 7.4/7.6/8.0 with HCl_{conc}. H_2O to 500 ml

Tris-Glycine electrophoresis buffer 10x 0.3% (w/v) Tris base 0.2% (w/v) glycine *store at RT*	30.2 g Tris base 188 g glycine dissolve in 800 ml H_2O add 100 ml 10% SDS H_2O to 1000 ml
Tris HCl 1 M, pH 7.5/7.8/8.0 *autoclave, store at RT*	60.55 g Tris base 400 ml H2O adjust to pH 7.5/7.8/8.0 with HCl conc. H2O to 500 ml
Trypsin-EDTA solution *1x Trypsin-EDTA (0.5 % trypsin, 5.3 mM EDTA) in* *TS-buffer* *prepare fresh*	5 ml 10x Trypsin-EDTA TS buffer to 45 ml

4 Methods

4.1 DNA

4.1.1 General methods

- Plasmid DNA can be stored at -4°C or at -25°C. Repeated freeze-thaw cycles should be avoided; this can cause shearing of the DNA.
- In general DNA samples should be kept on ice during the experiments.
- Overdrying of DNA as well as vigorous pipetting should be avoided.
- Concentration of DNA can be determined by optical-density measurements. The purity of DNA can be checked by calculating the A_{260}/A_{280} ratio. Pure DNA has a ratio of 1.8.

4.1.2 Agarose gel electrophoresis

- Measure an appropriate amount of agarose powder and add it to a 500 ml flask.
- Add an adequate amount of 1x TBE buffer.
- Melt the agarose in a microwave until the solution becomes clear.
- Let the solution cool to about 50°C.
- In the meantime prepare the casting tray by sealing the ends with two layers of tape.
- Place the combs in the gel casting tray.
- Pour the melted agarose solution into the casting tray and let it cool until is solid.
- Remove combs and tapes and place the tray into the electrophorsis chmaber containing 1x TBE buffer.
- Make sure that the agarose is overlaid with TBE buffer.
- Load the DNA samples dissolved in 1x DNA sample buffer and perform electrophoresis until the required separation is achieved (3 to 5 V/cm).
- Gel fragments can be visualized using UV light.

4.1.3 DNA transfer into bacteria

4.1.3.1 Preparation of competent HB101

- Streak bacteria from frozen stock of the desired *E.coli* strain (HB101) onto a LB medium agar plate and incubate at 37°C for 16-20 hours.
- Pick twenty ~2mm diameter colonies and disperse in 1 ml LB medium by vortexing.
- Inoculate the cells into a 2 l Erlmeyer flask containing 200 ml LB medium
- Shake at 200 rpm at 37°C until the OD_{600} has reached 0.4 corresponding to 4-8 x 10^7 viable cells/ml.
- Cool the bacteria on ice for 20 minutes and perform the following steps in a cold room at 4°C.
- Distribute the cells into four sterile 50 ml tubes.
- Centrifuge at 4°C for 15 minutes at 1,000 x g and completely remove the supernatant.
- Resuspend the pellet by moderate vortexing in 67 ml RF1 buffer and incubate on ice for 1 hour.
- Centrifuge the cells as above.
- Resuspend the pellet by moderate vortexing in 16 ml RF2 buffer and incubate on ice for 15 minutes.
- Distribute 425 µL aliquots into chilled 1.5 ml tubes and flash freeze in liquid nitrogen.
- Store the competent cells at -80°C.

4.1.3.2 Preparation of competent XL10 Gold

- Prepare Inoue transformation buffer and chill on ice before use.
- Streak out the desired *E.coli* strain (XL10-Gold) from frozen stock onto a LB-plate and incubate in a bacterial incubator at 37°C over night.
- Transfer a single bacterial colony into 25 ml of LB-medium in a 250 ml flask. Incubate the culture for 6 to 8 hours at 37°C by vigorous shaking (200 rpm).

- Use this starter culture to inoculate three 1 L-flasks, each containing 250 ml of LB-medium. The first flask received 10 ml of starter culture, the second received 4 ml and the third receives 2 ml. Incubate all three flasks overnight at 20°C by moderate shaking (111 rpm).
- The following morning, measure the OD_{600} of the three culture every 45 minutes.
- When the OD_{600} of one of the cultures reaches 0.55, transfer the flask to an ice-water bath for 10 minutes to rapidly cool the culture. The other two cultures can be discarded.
- Centrifuge at 2,500 rpm (Heraeus Biofuge 13) for 10 minutes at 4°C.
- Pour off the supernatant and store the open centrifuge bottle on a stack of paper towels for 2 minutes. Use a vacuum aspirator to remove any drops of remaining medium adhering to the walls of the centrifuge bottle or trapped in its neck.
- Resuspend the cells gently in 80 ml of ice-cold Inoue-transformation buffer.
- Centrifuge ar 2,500 rpm (Heraeus Biofuge 13) for 10 minutes at 4°C.
- Pour off the supernatant and use a vacuum aspirator to remove any drops of remaining liquid adhering to the walls of the centrifuge bottle.
- Resuspend the cells gently in 20 ml of ice-cold Inoue-transformation buffer.
- Add 1.5 ml DMSO and mix the bacterial suspension by swirling and then store it on ice for 10 minutes.
- Immediately aliquote 500 µL into pre-chilled 1.5 ml tubes, snap freeze the competent cells by immersing the tightly closed tubes in a bath of liquid nitrogen.
- Store the competent bacteria at -80°C until needed.

4.1.3.3 Chemical tranformation

- Quickly thaw an aliquot of competent XL10-Gold or HB101 cells at room temperature and then put on ice.
- Add 100 µL of competent bacteria to a cooled reaction tube containing 5 to 10 µL of ligation mixture or 5 to 10 ng plasmid DNA and mix carefully by pipetting.
- Incubate on ice for 30 minutes.
- Heat-shock the cells by transferring the tubes in a 42°C thermoblock for 90 seconds, and then chill on ice for 2 minutes.
- Add 900 µL of LB-medium (no antibiotics) and incubate at 37°C for 1 hour.
- Streak all of the cell suspension onto a pre-warmed LB-medium agar plate containing the appropriate antibiotic(s).
- Incubate at 37°C for 16 hours in a bacterial incubator.

4.1.4 Plasmid DNA isolation

4.1.4.1 Mini-preparation HB101 bacteria

- Inoculate a single colony of transformed bacteria in 3 ml LB-medium containing the appropriate antibiotic and shake over night at 37°C in a bacteria incubator at 200 rpm.
- The next day pour 2 ml of the overnight culture into a 2ml Eppendorf tube and centrifuge at 4°C for 1 min at 13,000 g.
- Aspirate the supernatant and re-suspend the bacterial pellet in 100 µl of ice-cold *Alkaline lysis solution I* followed by 200 µl of *Alkaline lysis solution II* and mix it gently by inverting the tubes 5 times.
- After 5 min of incubation at room temperature add 150 µl ice-cold *Alkaline lysis solution III* and mix thoroughly by inverting the tube several times. At this stage incubate the tube on ice for 5 min before centrifuging the tube at 13,000 g for 10 min at 4°C.
- Transfer the supernatant into a fresh 1,5 ml Eppendorf tube. Add an equal volume of ROTI-phenol:chloroform:isoamylalkohol (25:24:1). Mix

the organic and aqueous phase by vortexing and centrifuge at 13,000 g for 5 min at 4°C.

- Then aspirate the aqueous upper phase and transfer it into a new 1,5 ml Eppendorf tube.
- Repeat the extraction step with an equal volume of chloroform:isoamylalkohol (24:1) to remove remaining phenol from the aqueous phase.
- To precipitate the nucleic acids from the aqueous phase add 2 volumes of ethanol 100% and incubate at RT for 10 min. Collect the nucleic acid by centrifuging the tube at 13,000 g for 15 min at 4°C.
- Remove the supernatant and replace it by 1 ml of 70% ethanol to wash the DNA pellet. To recover the DNA pellet centrifuge the tube at 13 000 g for 15 min at 4°C.
- In order to get rid of residual ethanol dry the pellet in a Speed Vac for 5 min at RT.
- Dissolve the remaining DNA pellet in 20 µl TE pH 8.0 containing 100µg/ml RNAse A and incubate it for 30 min at 30°C to hydrolyse co-precipitated RNAs. Store plasmid DNA at -20°C.

4.1.4.2 Mini-preparation XL10-Gold bacteria

- Inoculate a single colony of transformed bacteria in 3 ml LB-medium containing the appropriate antibiotic and shake it overnight at 37°C in a bacteria incubator at 200 rpm.
- The next day pour 2 ml of the overnight culture into a 2ml Eppendorf tube and centrifuge at 4°C for 1 min at 13,000 g.
- Aspirate the supernatant and re-suspend the bacterial pellet in 250 µl of ice-cold *Alkaline lysis solution I*.
- Add 250 µl of *Alkaline lysis solution II* and mix it by gently inverting the tubes 5 times.

- After 5 min of incubation at room temperature add 300 µl ice-cold *Alkaline lysis solution III* and mix thoroughly by inverting the tube several times. At this stage centrifuge the tube centrifuged at 13,000 g for 10 min at 4°C.
- Transfer the supernatant into a fresh 1,5 ml Eppendorf tube and add 0,7 volume of Isopropanol to the tube and invert it 5 times.
- After a centrifugation step of 15 min at 13,000 g and 4°C remove the supernatant and fill the tube with 700 µl 70 % ethanol and centrifuge as before.
- In order to get rid of residual ethanol dry the pellet in a Speed Vac for 5 min at RT.
- Dissolve the remaining DNA pellet in 20 µl ddH_2O containing 100µg/ml RNAse A and incubate it for 30 min at 30°C to hydrolyse co-precipitated RNAs. Plasmid DNA was stored at -20°C.

4.1.4.3 Midi-preparation using QIAGEN® Plasmid Midi Kit

- Inoculate 25 ml (high copy) or 100 ml (low copy) of LB-medium containing the appropriate antibiotic(s) with 200 µL of a small-scale overnight-culture or with ~ 10 µL of a frozen glycerol stock.
- Harvest the cells at 6,000·g for 15 minutes at 4°C.
- Carefully remove the supernatant by aspiration; resuspend the bacterial pellet in 4 ml Buffer P1 (containing RNaseA solution) provided by the supplier (QIAGEN® Plasmid Midi Kit).
- Add 4 ml Buffer P2 (QIAGEN® Plasmid Midi Kit), mix thoroughly by vigorously inverting six times and incubate at room temperature for not more than 5 minutes.
- Add 4 ml pre-chilled Buffer P3 (QIAGEN® Plasmid Midi Kit), mix thoroughly by vigorously inverting six times and incubate on ice for 15 minutes.
- Centrifuge at 20,000·g for 30 minutes at 4°C.

- In the meantime, equilibrate a QIAGEN®-tip-100 by applying 4 ml Buffer QBT (QIAGEN® Plasmid Midi Kit) and allow the column to empty by gravity flow.
- After centrifugation, apply the supernatant to the QIAGEN®-tip and allow it to enter the resin by gravity flow.
- Wash the QIAGEN®-tip twice with 10 ml Buffer QC (QIAGEN® Plasmid Midi Kit) allowing to move through the QIAGEN®-tip by gravity flow.
- Elute the DNA with 5 ml Buffer QF (QIAGEN® Plasmid Midi Kit) into a clean 15 ml-vessel.
- Precipitate the DNA by adding 3.5 ml room-temperature isopropanol (absolute) to the eluted DNA and mix. Centrifuge at 15,000·g for 30 minutes at 4°C. Carefully decant supernatant.
- Wash the DNA pellet with 2 ml room-temperature 70% (v/v) ethanol and centrifuge at 15,000·g for 10 minutes at 4°C. Carefully decant supernatant.
- Air-dry the remaining pellet for 5 to 10 minutes and re-dissolve the DNA in 200 µL H_2O. Store the plasmid DNA solution at -25°C.
- The amount and purity of DNA can be visualized using a photometer.

4.1.4.4 Maxi-preparation

- Inoculate 250 ml of LB medium containing the appropriate antibiotic with 250 µl of a 3-ml over-night bacteria culture and shake it over-night at 200 rpm at 37°C.
- The next day transfer the bacterial culture into GSA-beakers and chill on ice for 10 min, before centrifuging it at 6,000 g for 15 min at 4°C.
- Decant the supernatant and the suspend the pellet in 10 ml Alkaline lysis solution I.
- Add 1ml of 10 mg/ml Lysozyme solution and incubate at RT for 10 min
- Add 20 ml of Alkaline lysis solution II and mix thoroughly by inverting five times and incubate it at RT for 10 min.

- Add 15 ml of Alkaline lysis solution III and mix it by vigorously inverting five times followed by an 15 min incubation step on ice.
- After a centrifugation step allowing the rotor to stop without braking at 20,000g for 30 min at 4°C decant the supernatant through four layers of cheesecloth into a fresh GSA-beaker.
- Add 0.6 volume of isopropanol and store it at RT for 15 min after mixing it.
- Centrifuge the tube at 12,000 g for 15 min at RT to recover precipitated DNA.
- Decant the supernatant and rinse the pellet with 70% ethanol. Aspirate the ethanol again and let it dry for some minutes.
- Proceed either with "Precipitation with polyethylen glycol" or with "Equilibrium centrifugation is CsCl-ethidium bromide gradient", depending on the desired quality of DNA.

4.1.4.5 Equilibrium centrifugation in CsCl-ethidium bromide gradient

- Dissolve the damp pellet of nucleic acid in 6.3 ml *TE*.
- Add 6.3 g cesium chloride and dissolve at 30°C.
- Prepare polyallomer tubes (16 × 76 mm) and insert a syringe attached to a 18 gauge needle.
- Press the DNA solution through the syringe into the tube and overlay with 200 µl of 10 mg/ml *Ethidium bromide* solution.
- Fill the tube with paraffin and remove all air bubbles.
- Seal tubes, check their masses and spin in a ultracentrifuge (Ti70.1 rotor) at 60,000 rpm for 24 h at 20 °C.
- After the run pierce a hole in the top of the tube using a needle.
- With a syringe attached to a 18 gauge needle draw the lower band representing supercoiled plasmid DNA out of the tube and transfer into a 2-ml microfuge tube.

- To remove the ethidium bromide extract the DNA solution five times with the same volume of CsCl/H_2Osaturated isopropanol by gentle shaking, then separate the phases by centrifugation at 2,000 × g for 1 min at RT, and discard the upper phase each time.
- Dialyze in 1 l *TE* at 4°C in pretreated dialysis bags (for preparation see 1.5 DNA fragment isolation). Change the buffer after 6-12 hrs and continue dialysis for another 6-12 hrs.
- Adjust the volume to 0.3 M *Sodium acetate pH 5.2*.
- Precipitate the nucleic acids by adding 2 volumes of ethanol at RT. Mix the solution and allow to stand for 10 min at RT.
- Collect the precipitated nucleic acids by centrifugation at 13,000 × g for 15 min at 4°C in a microfuge.
- Remove the supernatant by gentle aspiration and wash the pellet with 1 ml of 70 % (v/v) ethanol.
- Recover the DNA by centrifugation as above.
- Remove the supernatant and dry the pellet in a Speed Vac for 5 min at RT
- Redissolve the DNA in 300 µl H2O. Store the DNA at -20 °C.
- Measure the concentration and check quality and identity as described above.

4.1.4.6 Precipitation with polyethylen glycol

- Dissolve the damp pellet of nucleic acid in 3 ml of *TE* and transfer into a 15 ml Corex tube.
- Add 3 ml of ice cold *5 M Lithium chloride*, mix and centrifuge at 16,000 g (10,000 rpm in a Sorvall HB-6 swing-out rotor) at 4 °C for 10 min to precipitate high molecular RNA.
- Transfer the supernatant into a 30-ml Corex tube. Add an equal volume of isopropanol (6 ml), mix and leave at room temperature for 20 min.

- Spin at 16,000 g at 4°C for 20 min. Decant the supernatant and wash the pellet with 70 % (v/v) ethanol. Repeat centrifugation and decant the supernatant.
- Dry the pellet and dissolve in 500 µl TE.
- Add 10 µl of 10 mg/ml RNAseA and transfer the solution into a microfuge tube.
- Incubate at RT for 30 min.
- Add 500 µl of *1.6 M NaCl/13 % (w/v) polyethylen glycol* solution.
- Mix, store on ice for 5 min, then spin at 13,000 g at 4°C for 5 min in a microfuge.
- Remove the supernatant, and dissolve the (almost not visible) pellet in 400 µl TE.
- Add an equal volume of ROTI-phenol and vortex.
- Place on ice for 1 min and then repeat vortexing.
- Centrifuge at 13,000 × g for 5 min at RT and transfer the upper aqueous phase into a fresh tube.
- Repeat the extraction one more time each with phenol/chloroform/isoamylalkohol (25:24:1) and chloroform/isoamylalkohol (24:1).
- Add 100 µl *10 M Ammonium acetate* and 1 ml ethanol and store at RT for 10 min.
- Centrifuge at 13,000 × g at 4°C for 15 min. Wash the pellet with 70% ethanol and dissolve in 300 µl TE. Store the DNA at 4 °C.
- Determine the OD at 260 and 280 nm for a sample diluted 1:100; for pure DNA the OD260:OD280 ratio is 1.8.
- Calculate the DNA concentration by using the correlation 1 OD260 = 50 µg/ml. Yields vary between 50 and 500 µg DNA per 250 ml bacteria culture depending on the copy number of the plasmid.

- Check 0.5 µg uncut DNA by agarose gelelectrophoresis and confirm the identity of the plasmid by restriction enzyme analysis and DNA sequencing.

4.1.5 Modification and cloning of DNA

4.1.5.1 Restriction enzyme digest

- The DNA should be digested in a total volume of 10 to 20 µL per µg of plasmid DNA.
- Add the appropriate amount of the recommended 10x restriction enzyme buffer to the plasmid DNA and if necessary 1/10 volume of 10x BSA.
- Adjust to the total volume minus the volume of the enzyme(s) with H_2O.
- Add 2 to 3 units of restriction enzyme per µg DNA. One unit of restriction endonuclease is the amount of enzyme required to completely digest 1 µg template DNA in 1 hour. The volume of the enzyme should not exceed 10% of the total volume.
- Incubate for 1 to 2 hours at 37°C (adequate temperature for most enzymes). Terminate the reaction by inactivation of the enzyme(s) adding 5x DNA sample buffer to a final concentration of 1x.
- Store the samples at 4°C or load an agarose gel.

4.1.5.2 Partial digest

- Add the appropriate amount of the recommended 10x restriction enzyme buffer to the plasmid DNA and if necessary 1/10 volume of 10x BSA.
- Dilute the restriction enzyme 1:20.
- Take 1 µl of the diluted enzyme and add to the reaction tube.
- Add H_2O to a final volume of 15 µl. Incubate for 7 minutes at 37°C.
- Inactivate the enzyme for 10 minutes at 65°C (adequate temperature for most enzymes).
- Store the samples at 4°C or load on an agarose gel.

4.1.5.3 Generation of blunt ends

- To fill in 5´-overhangs or to remove 3´-overhangs 2 µl of 10x KLENOW polymerase buffer and 2 µl of 10 mM dNTP mix is add to 1 µg of purified DNA fragment. H_2O is adjusted to a volume of 15 µl.
- Add 1 µl of KLENOW DNA polymerase (2U/µl) and incubate for 1 hour at 37°C.
- The enzyme can be inactivated by incubating at 70°C for 10 minutes.

4.1.5.4 Phosphorylation

For the cloning of PCR products and synthetic double-stranded oligonucleotides into a dephosphorylated vector the insert needs to be phosphorylated.

- For the phosphorylation reaction dissolve the DNA in 15 µL H_2O and add 2 µL of 10x Polynucleotide Kinase (PNK) buffer, 2 µL 10 mM ATP and 1 µL of T4 Polynucleotide Kinase (PNK) (1U/µL).
- Incubate the reaction solution at 37°C for 1 hour.
- Inactivate the enzyme by heating the sample at 65°C for 10 minutes.
- Otherwise terminate the reaction by adding 5x DNA sample buffer to a final concentration of 1x.

4.1.5.5 Dephosphorylation

Removing of 5'-terminal phosphate groups results in a lower background from re-ligation of vector DNA.

- For dephosphorylation dissolve the DNA in 17 µL H_2O and add 2 µL of 10x calf intestine alkaline phosphatase buffer and 1 µL of calf intestine alkaline phosphatase (CIAP) (1U/µL).
- Incubate the reaction solution at 37°C for 1 hour.
- Terminate the reaction by adding 5x DNA sample buffer to a final concentration of 1x.

4.1.5.6 Ligation

T4 DNA Ligase catalyzes the formation of phosphodiester bonds between 3'-OH and 5'-P ends in double-stranded DNA.

- Mix 10 to 50 ng of linearized and dephosphorylated vector-DNA with 100 to 200 ng phosphorylated insert-DNA (sticky or blunt ends) in H_2O to a total volume of 17 µL.
- Add 2 µL of 10x T4 DNA Ligase buffer and 2 to 5 units of T4 DNA Ligase and incubate at 16°C over night.
- It is possible to use 2 µL of 10x FAST ligation buffer; in this case incubation for 1 hour at room temperature is sufficient.
- Use 5 to 10 µL of the reaction mixture to transform competent *E.coli* prepared for transformation.

4.1.5.7 Purification of DNA fragments by affinity chromatography using QIAquick Purification columns

- Add 5 volumes of the PB buffer (QIAquick®) to 1 volume of reaction solution and mix.
- Place a QIAquick® column in a 2 ml-tube.
- To bind the DNA, apply the sample to the QIAquick® column and centrifuge for 1 minute at 13,000 rpm (Heraeus Biofuge Pico) until all the samples have passed through the column. Discard the flow-through and place the QIAquick® column back into the same tube.
- To wash the column, add 750 µL PE buffer (QIAquick®) and centrifuge for 1 minute at 13,000 rpm (Heraeus Biofuge Pico). Discard the flow-through again and place the QIAquick® column back into the same tube.
- Centrifuge one more time in the provided 2 ml-collection tube for 1 minute to remove residual wash buffer.
- Place the QIAquick® column in a clean 1.5 ml-tube.

- To elute the DNA, add 20 µL H_2O (pH 7.0 to 8.5) to the center of the QIAquick® membrane and centrifuge the column for 1 minute at 13,000 rpm (Heraeus Biofuge Pico).

4.1.5.8 DNA fragment isolation by electroelution

- Pour a 1 % (w/v) TBE agarose gel (for the separation of DNA fragments smaller than 0.5 kbp increase the agarose concentration accordingly).
- Load DNA digest in 1x DNA sample buffer onto the gel. Load 1 µg of Lambda DNA Hind III digest as a size marker in a separate lane (heat to 55 °C before loading). Run the gel overnight at constant voltage (30 V) for ~ 600 Vh.
- Visualize the DNA under UV light (366 nm), and cut out the agarose slice containing the desired DNA fragment using a clean scalpel.
- Document the position of the excised band by photography of the residual gel.
- Wash pretreated dialysis tubing with sterile H2O and close one end using a dialysis clip. Carefully transfer the agarose slice into the dialysis bag and fill with 1x TBE.
- Squeeze out excess buffer and air bubbles, then clip the other end. Put the bag into the gel tank and electroelute the DNA fragment at constant voltage (130 V) for 2 h.
- Open the bag and transfer the solution into a 1.5-ml microcentrifuge tube.
- Wash the dialysis tubing with a small volume of 1x TBE , and determine final volume of total DNA solution (e.g. 450 µl).
- Add 50 µl of 3 M sodium acetate pH 5.2, 1 ml of cold absolute ethanol, and leave at -80 °C for 30 min.
- Centrifuge at 13,000 × g / 4 °C for 30 min, wash the DNA pellet with 70 % (v/v) ethanol, and dry briefly in a Speed Vac.
- Dissolve the DNA fragment in 10 µl of sterile H2O and store at -20 °C.

4.1.6 Polymerase chain reactions (PCR)

4.1.6.1 High fidelity PCR

- For high fidelity PCR mix (in a 0.2 ml-reaction tube) 35.5 µL H_2O, 10 µL of 5x Phusion HF buffer (containing $MgCl_2$), 1 µL 10 mM dNTP, 1 µL forward primer (25 pmol), 1 µL reverse primer (25 pmol), 1 µL of template (1 ng plasmid DNA/ 1 µg genomic DNA) and add 0.5 µL Phusion DNA polymerase (2U/µL) to a final volume of 50 µL.
- Insert the sample into a thermocycler and perform an initial denaturation step at 95°C for 30 seconds.
- Perform 30 cycles under the following conditions:
    - 95°C for 30 seconds (denaturation)
    - 50 to 60°C (T_m of the lower primer) for 30 seconds (annealing)
    - 72°C for 2 minutes (extension)
- Add a final extension step at 72°C for 5 minutes.
- Cool the reaction to 4°C.
- Add 12.5 µL of 5x DNA sample buffer and load on an agarose gel containing 0.5 µg/ml ethidium bromide for DNA fragment isolation.

4.1.6.2 Enhanced PCR

- In a 0.2 ml-reaction tube mix 29 µL H_2O, 5 µL 10x PCRx Amplification buffer, 1.5 µL 50 mM $MgSO_4$, 10 µL 10x PCRx Enhancer solution, 1 µL 10 mM dNTP, 1 µL forward primer (25 pmol), 1 µL reverse primer (25 pmol), 1 µL of template (1 ng plasmid DNA/ 1 µg genomic DNA), and 0.5 µL of Phusion Polymerase (2U/µl) (final volume: 50 µL).
- Insert the sample into a thermocycler and perform an initial denaturation step at 95°C for 2 minutes.
- Perform 30 cycles under the following conditions:
    - 95°C for 45 seconds (denaturation)
    - 50 to 60°C (T_m of the lower primer) for 60 seconds (annealing)
    - 68°C for 1 min/kbp (extension)

- Cool the reaction to 4°C.
- Add 12.5 µL of 5x DNA sample buffer and load on an agarose gel containing 0.5 µg/ml ethidium bromide for DNA fragment isolation.

4.1.7 Site directed mutagenesis

- For site directed mutagenesis mix 10 µL of 5x Phusion HF buffer (containing $MgCl_2$), 1 µL 10 mM dNTP, 1 µL phosphorylated forward primer (10 pmol), 1 µL phosphorylated reverse primer (10 pmol), 1 µL of template (1 ng), 0.5 µL Phusion DNA polymerase (2U/µL) and add H_2O to a final volume of 50 µL. Mutagenesis occurs through the oligonucleotide primers containing the desired mutation.
- Insert the sample into a thermocycler and perform an initial denaturation step at 98°C for 30 seconds.
- Perform 25 cycles under the following conditions:
    - 98°C for 10 seconds (denaturation);
    - 50 to 60°C (T_m of the lower primer) for 30 seconds (annealing);
    - 72°C for 2 minutes (extension)
- Add a final extension step at 72°C for 15 minutes (2 min/kb of plasmid length).
- Cool the reaction to 4°C.
- Add 1 µL of *DpnI* restriction enzyme and 2 µL of NEBuffer 4 to the reaction (it is used to digest the parental DNA template), mix properly and incubate for 1 hour at 37°C.

4.2 Protein

4.2.1 General methods

- Repeated freeze- and thaw- cycles should be avoided.
- Protein sample should be kept on ice while working to reduce protease activity.
- To prevent denaturation of the proteins het, vigorous vortexing and pipetting should be avoided.
- For long term storage proteins could be kept in liquid nitrogen.

4.2.2 SDS polyacrylamide gel electrophoresis of proteins (SDS-PAGE)

- Assemble two clean glass plates separated by spacers according to the manufacturer's instructions.
- Determine the volume of the gel mold. In an ERLENMEYER flask prepare the appropriate volume of *Separation gel solution* (e.g. 8 ml) containing the desired acrylamide concentration (e.g. 10 %, 12%, or 12.5 %).
- Add 40 µl APS and 8 µl TEMED (for a 8 ml solution), swirl the flask and pour the solution into the gap between the glass plates. Leave sufficient space for the stacking gel (length of the comb teeth plus 1 cm).
- Carefully overlay the Separating gel with water-saturated 1-butanol.
- After complete polymerization (>1 h) pour off the overlay and wash the top of the gel with deionized water.
- Drain excess fluid from the top of the gel and remove any water using a paper towel.
- Prepare an appropriate volume of *Stacking gel solution* (e.g. 10 ml). Add 50 µl APS and 10 µl TEMED (for a 10 ml solution) pour it directly onto the surface of the polymerized separating gel.

	Separating Gel 10%	**Separating Gel 12%**	**Separating Gel 12.5%**	**Stacking Gel**
Separating gel solution	2.44 ml	2,92 ml	3.04 ml	-
Stacking gel solution	-	-	-	1.13 ml
H_2O	3.52 ml	3.04 ml	2,92 ml	2.6 ml
1.5 M Tris-HCl pH 8.8	2 ml	2 ml	2 ml	-
0.5 M Tris-HCl pH 6.8	-	-	-	1.25 ml
20% SDS	40 µl	40µl	40µl	25 µl
TEMED	8 µl	8 µl	8 µl	5 µl
10% APS	40 µl	40 µl	40 µl	25 µl

- Immediately insert a clean Teflon comb into the Stacking gel avoiding trapping air bubbles and fill the spaces of the comb completely.
- After polymerization (>1 h) carefully remove the comb and rinse the slots with deionized water using a hypodermic needle attached to a syringe.
- Mount the gel in the electrophoresis apparatus and fill top and bottom reservoirs with *Tris-glycine electrophoresis buffer*. Remove any bubbles at the bottom of the gel using a bent hypodermic needle attached to a syringe.
- Prepare the samples by heating them to 95 °C for 5 min in *1x SDS gel-loading buffer* to denature the proteins.
- Depending on the gel electrophoresis system load up to 40 µl of the samples in a predetermined order into the bottom of the wells using a HAMILTON microliter syringe that is washed with *Tris-glycine electrophoresis buffer* after each sample is loaded.
- Include an appropriate size marker and load an equal volume of *1x SDS gel-loading buffer* into any unused wells.

- Attach the electrophoresis apparatus to an electric power supply and apply a constant voltage of 7.5 V/cm to the gel. After the dye has moved into the separating gel increase the voltage to 15 V/cm and run the gel until the bromophenol blue reaches the bottom of the separating gel (~ 4 h).
- Remove the glass plates and separate the plates using a spatula and discard the stacking gel using a scalpel.
- The gel can now be processed for staining with Coomassie Brilliant Blue or for establishing an immunoblot.

4.2.3 Immunoblot (Western blot)

- During SDS-gelelectrophoresis, cut a nitrocellulose or a PVDF membrane and six sheets of Whatman 3MM CHR paper in the size of the gel
- PVDF membrane needs to be equilibrated in 100% ethanol.
- Wet the membrane in deionized H_2O, and then in *Immunoblot transfer buffer* for at least 15 min.
- Soak the gel briefly in deionized water, then in *Immunoblot transfer buffer* for 5 min.
- Transfer the proteins onto the membrane using either a semi dry blot- or a wet blot-transfer apparatus.
- For semi dry blotting wet the electrodes with deionized water and layer one piece of Whatman paper equilibrated in *Immunoblot transfer buffer* onto the bottom electrode (anode). Then add two more papers exactly aligned to the first one followed by the nitrocellulose or PVDF. Layer the gel onto the nitrocellulose/PVDF filter followed by three more Whatman papers. Avoid bubbles between all layers and close the device with the top electrode (cathode). Set up the strength of the electric current to filter square cm × 0.8 and transfer for 3 h.
- For wet blotting soak the sponges of the apparatus in *Immunoblot transfer buffer* and assemble Whatman papers, nitrocellulose or PVDF

filter, and gel as described above. Set up the strength of the electric current to 150 mA (3 mA per square cm) and transfer at 4 °C for 4 h.

- After the transfer stain the membrane for 2 min in *Ponceau S staining solution*, rinse with water and label protein marker positions with a ball pen. Destain the membrane with *1* × *TBS* for 30 min.

4.2.4 Detection with enhanced chemiluminiscence (ECL)

- Depending on the filter size place the membrane in 30-100 ml of *Blocking solution (ECL)* over night at 4 °C.
- Wash the filter 4 × 10 min with *TBS-T* (30-100 ml).
- Apply the first antibody diluted in a range between 1:100 and 1:1000 in 10-30 ml *Blocking solution (ECL)* for 3 h at RT on a rocking platform.
- Wash the filter 4 × 10 min with *TBS-T* (30-100 ml).
- Apply the second antibody (e.g. anti rabbit coupled to horseradish peroxidase) diluted to 1:2500 or 1:5000 in 10-30 ml of a solution containing one part *Blocking solution (ECL)* and two parts *TBS-T* for 90 min.
- Wash the filter 4 × 10 min with *TBS-T* (30-100 ml), then briefly with TBS.
- Mix equal volumes of *ECL-detection solution 1* and *ECL-detection solution 2* (1-5 ml each).
- Drain excess buffer from the washed membrane and place them on a piece of Saran wrap, protein side up.
- Add the detection reagent to cover the membrane completely.
- Incubate for 1 min at room temperature, and then drain off excess liquid.
- Wrap membrane in Saran Wrap and gently smooth out air pockets, but avoid pressure on the membrane.
- Visualize the proteins using Fusion Fx7 (peqlab) imager.

4.3 Cells

4.3.1 Preparation and culture of avian fibroblasts

4.3.1.1 Preparation of quail embryo fibroblasts (QEF)

- Incubate fertilized quail eggs at 37.8°C in an egg incubator with a water-saturated atmosphere (>60% humidity) and automatic turning device for 9 d.
- Place a 50 ml tube containing 10 ml PGM on ice.
- Clean the egg with ethanol and open at the shallow site using a spatula
- Remove the broken shell with a sterile forceps, then the membrane under the egg shell with another sterile forceps.
- Gently pull out the embryo with closed forceps under its neck.
- Put the embryo onto a petri dish. Remove head, arms, legs and transfer the body into a 50 ml tube.
- Wash the body with 10 ml TS buffer(37°C), shake briefly, decant the supernatant and thoroughly homogenize the body with the round end of a spatula for 5 min.
- Add 10 ml TR solution (37°C), shake gently, incubate for 5 minutes at RT, then carefully pipett 5 ×up and down using a 10 ml wide mouth pipette.
- Let clumps settle down for 5 min, then decant the supernatant into the prepared tube containing PGM.
- Repeat this washing step with following solutions kept at 37°C: 5 ml TS buffer, 5 ml TS buffer, 5 ml TR solution, 5 ml TS buffer, 5 ml TS buffer.
- Finally, mix cell suspension by inverting the 50 ml tube with the cells and centrifuge at 150 ×g at 4°C for 20 min.
- Decant the supernatant, add 20 ml PGM to the pellet and pipett 25 × up and down with a wide mouth pipette.
- Let the clumps settle down for 5 min and pipett 19 ml of the supernatant into a fresh 50 ml tube.
- From this supernatant remove 0.5 ml and dilute to 5 ml with TS bufferfor determination of the cell number.

- Rinse a hematocytometer and its cover slip with ethanol, then put the cover slip onto the chambe.
- Pipett one drop of the diluted cell suspension at the edge of the cover slip and put the device onto a microscope.
- Count the cells except the dark and smooth blood cells in the four fields encompassing 16 squares (0.1 mm^3) each and determine the average cell number of one field [calculation of the cell number/ml: N (number of cells in one field) ×10 (dilution factor) × 10^4]. The total number of cells should be in the range of 5 ×$10^7$to $10^8$for a 9 day old embryo.
- Plate 6 ×10^6 cells per 100-mm dish containing 10 ml PGM.
- Incubate cells at 37°C in a 5% CO_2 humidified atmosphere and replace the medium after three days with 12 ml PGM per dish.

4.3.1.2 Passaging of cells: QEF

- Culture the cells at 37°C (5% CO_2) in a water-saturated atmosphere.
- For transfer, wash the cells of one dish with 5 ml *TS-buffer*, aspirate, add 2 ml *Trypsin-EDTA solution*, and aspirate.
- Incubate at 37°C for 5-10 min, then suspend the cells in 2 ml of *Transfer buffer* and transfer into a conical 10-ml tube.
- Wash the dish with 3 ml of culture medium and add to the cell suspension.
- From the final volume of 5 ml dilute 0.1 ml with 9.9 ml of ISOTON® II Diluent.
- Count the cells using a Coulter counter, determine the number of cells/ml and the total cell number of the trypsinized dish.
- Calculate the volume of cell suspension (3-6 × 10^6) to be seeded into 100-mm dishes containing 10 ml of *Avian cell culture medium.*
- Change the medium after 2-3 days.

4.3.1.3 Freezing of cells: QEF

- Immortalized cells can be stored frozen at -80°C for several years and much longer in liquid nitrogen at approximately -150°C.
- Trypsinize cells as described above. Centrifuge the final volume of 5 ml at 50 × g for 5 min.
- Decant the supernatant and resuspend the cell pellet in 0.5 ml cold *Avian cell culture medium.*
- Add 0.5 ml cold *Freezing medium* and mix.
- Transfer the cell suspension into a cryo tube and freeze at -80°C. The next day transfer the vial into a liquid nitrogen tank.
- To revive cells quickly thaw the content of the cryo tube at 37°C and transfer the cell suspension into a 100-mm dish.
- Dropwise add 10 ml *Avian cell culture medium.*
- Change the medium after 2 h.

4.3.2 Culture of human cells: HEK293, SW480

4.3.2.1 Passaging of cells

- Aspirate all the DMEM-medium of a 100 mm-Petri dish and wash the cells twice with 5 ml 1x TS.
- Add 1 ml of 1x trypsin-EDTA to the attached cells and distribute equally on the dish, put the dish for 5 minutes in the incubator until the cells are released from the dish.
- Resuspend the cells in 5 ml of DMEM-medium and transfer the cell suspension to a 15 ml-Falcon-tube.
- Pellet the cells by centrifugation for 2 minutes at 1,000 rpm (Heraeus Biofuge 13) and resuspend the obtained pellet in fresh DMEM-medium.
- Dilute the cells 1:5 and distribute the cells to five new 100 mm-Petri dishes containing each 10 ml DMEM, supplemented with foetal bovine serum and penicillin-streptomycin.

4.3.3 Gene transfer into avian fibroblasts

4.3.3.1 Calcium phosphate-mediated DNA transfection

- Prepare a cell suspension from avian fibroblasts.
- Dilute the cell suspension with *Growth medium* to a concentration of 1-2 × 10^6 cells/ml depending on the size of the dish used for transfection.
- Seed cells into appropriate dishes containing 2 ml, 4 ml, or 10 ml of *Growth medium* per MP-6 plate (0.75 × 10^6 cells), 60-mm plate (1.5 × 10^6 cells), or 100-mm plate (4.0 × 10^6 cells), respectively.
- Incubate over night at 37°C (5 % CO_2). The cells should be confluent the next day.
- Before transfection replace the medium with fresh *Growth medium* and incubate at 37 °C for 1 h (5 % CO_2).
- To prepare the transfection cocktail add 100 µl of *2.5 M* $CaCl_2$ to 900 µl of H_2O containing 10 µg of Cesium chloride-purified plasmid DNA. Drop this mixture into a conicial tube containing 1 ml of *2 × HBS*. Vortex at 1800 rpm for 2 s.
- After the solution has become turbid due to calcium phosphate precipitation, slowly drop the transfection mixture into the medium of the seeded cells. Use 0.3 ml (1.5 µg DNA), 1 ml (5 µg DNA), or 2 ml (10 µg DNA) of the transfection cocktail for cells grown on MP-6, 60-mm, or 100-mm dishes, respectively.
- Gently swirl the dishes and incubate at 37 °C for 4 h.
- Aspirate, then add *Avian cell culture medium* (2 ml, 4 ml, or 10 ml for MP-6, 60-mm, or 100-mm dishes, respectively) and incubate the cells at 37 °C (5 % CO_2).

4.3.4 Gene transfer into human cells

4.3.4.1 Transfectin transfection

- Transfection of cells is carried out one day after cell passing according to the supplier's instruction. For transfection the TransFectin™ Lipid reagent (BIORAD) transfection reagent is used.
- Prepare 1.5 ml-tubes with 50 or 100 µL of DMEM-medium (no PS or fbs) and an appropriate amount of TransIT-LT1 reagent (recommended ratio DNA:reagent 1:3).
- Add the appropriate amount of DNA and incubate for 20 minutes at room temperature.
- Add the DNA/transfection reagent complexes to the cells followed by incubation for 24 to 48 hours in water-saturated 5% CO_2-containing atmosphere at 37°C.

4.3.5 Cell transformation assays with avian fibroblasts

4.3.5.1 Test for focus formation

- Transfect cells seeded on 100-mm dishes using the calcium phosphate method.
- After the glycerol shock cultivate the cells in 10 ml *Growth medium* at 37°C (5% CO_2).
- The next day remove the medium from the confluent cells and add 10 ml of liquid *Focus agar.*
- Let the agar harden for 1 h at RT, then incubate the cells at 37°C (5% CO_2).
- Overlay cells every 2-3 d with 5 ml of *Focus agar*. Foci consisting of transformed cells are detectable after 1-2 weeks.
- To pick single foci, prepare a flexible plastic tube attached to a PASTEUR pipette and MP-24 wells filled with each 1 ml of *Avian cell culture medium.*

- Under the microscope detach the focus and carefully suck until the focus is visible in the pipette tip.
- Transfer the focus into the MP-24 well and rinse the pipette with culture medium.
- Incubate at 37°C (5% CO_2) for 1 d until cells have been attached to the dish. Replace medium.
- After cells have grown to confluency, transfer cells from the entire well to an MP-6 well, then to a 60-mm dish, and finally to a 100-mm dish to propagate cells derived from a single focus.
- To score the number of foci, remove the agar block from the cells and wash twice with *TS buffer.*
- Add 5 ml of absolute ethanol to fix the cells. Incubate for 2 min at RT.
- Aspirate ethanol, cover the cells with 5 ml of *GIEMSA staining solution*, and incubate for 30 min at RT (do not rock the dishes).
- Remove the staining solution and wash 3 × with water. Air-dry the dishes and score foci by counting and photographing.
- Determine the number of foci per microgram of transfected DNA to quantify the transformation efficiency.

4.3.5.2 Test for agar colony formation

- Transfect cells plated on 60-mm dishes using the calcium phosphate method.
- 2-3 d after transfection transfer the cells into a 100-mm dish.
- Cultivate the cells by transferring them every 4-7 d until they have adopted a transformed morphology (3-6 passages).
- Prepare MP-6 dishes filled with each 1 ml of liquid *Cloning bottom agarose* per well. Let the agar harden for 30 min.
- Prepare an adequate amount of *Cloning top agarose* and keep at 45°C in a water bath.

- Transfer trypsinized cells suspended in 5 ml of *Transfer buffer* into a conical tube.
- Determine the cell number and transfer each 10,000 to 50,000 cells into 3-ml tubes.
- Add each 2 ml of liquid *Cloning top agarose* (45°C) into the tubes and mix with a 2-ml wide-mouth pipette.
- Pour the mixtures into the prepared MP-6 wells. Let the agar harden for 2 h at RT, then incubate at 37°C (5% CO_2).
- Overlay the cells every 2-3 d with 1 ml of *Cloning top agarose* per well. Colonies consisting of transformed cells are detectable after 1-2 weeks.
- Determine the number of colonies formed by 1,000 cells to quantify the transformation efficiency.
- To generate cell clones derived from single colonies, prepare a flexible plastic tube attached to a PASTEUR pipette and MP-24 wells filled with each 1 ml of *Avian cell culture medium.*
- Localize the colony under the microscope and carefully suck until the colony is visible in the pipette.
- Hold the pipette into the MP-24 well and rinse with culture medium until the colony has been transferred into the well.
- Incubate at 37°C (5% CO_2) for 1 d until cells have been attached to the dish. Replace medium.
- After cells have grown to confluency, transfer cells from the entire well to an MP-6 well, then to a 60-mm dish, and finally to a 100-mm dish to propagate cells derived from a single agar colony.

5 Results

5.1 Oncogenic potential of Myc variants

To pursue their functions as transcription factors, all Myc variants need to dimerize with their interaction partner Max. Dimerization with Max and binding to DNA initiates the transcription of target genes. Deregulated expression of Myc is a crucial driving force in most human cancers. The difference in the amino acid (aa) sequences of Myc variants might reflect the various patterns of target gene expression [58–60] along with differences in the transformation potential [26–28]. Changes in aa sequence as well as highly conserved regions among the Myc variants are shown in **Figure 3**.

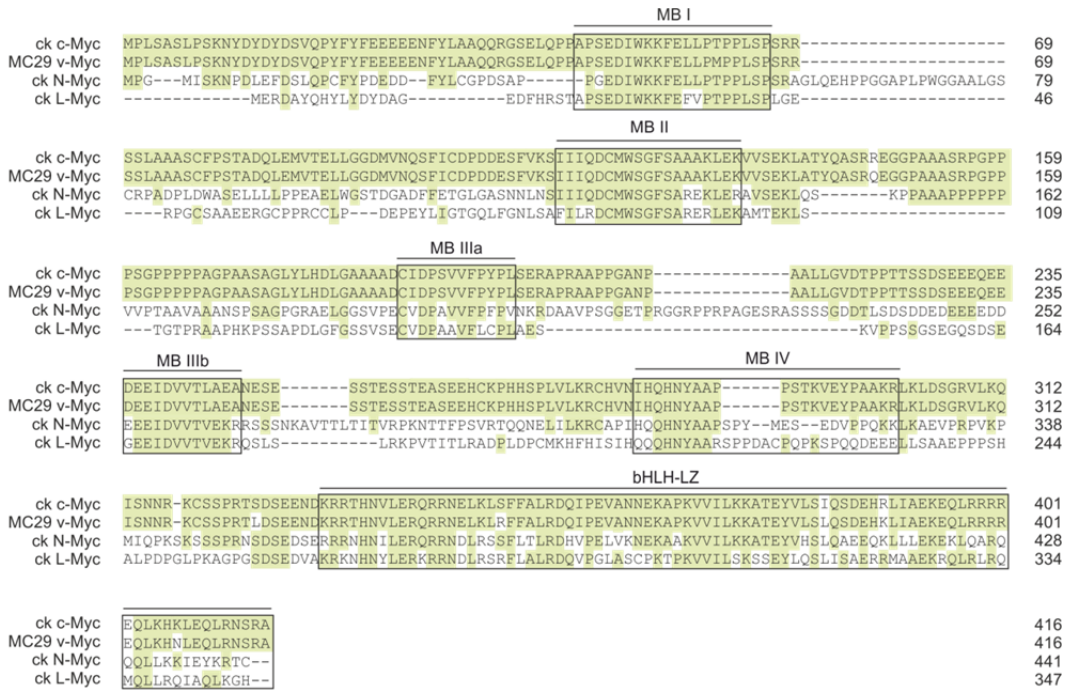


Figure 3. Amino acid sequence alignment of Myc variants. The sequences of c-Myc, v-Myc, N-Myc, and L-Myc (GenBank accession numbers: ck c-Myc, NP_001026123; MC29 v-Myc, p06295.2; ck N-Myc, chicken genome contig NM_001471673, nt 3920044-3922476; ck L-Myc, XP_425790) were aligned using ClustalW with additional manual adjustments. Identical residues in c-Myc are highlighted, dashes indicate gaps, Myc boxes and the bHLH-Zip region are framed. MB, Myc box; bHLH-LZ, basis region-helix loop helix-leucine zipper domain; ck, chicken.

5.1.1 Cloning of expression vectors for Myc variants (c-*myc*, v-*myc*, N-*myc*, L-*myc*)

In order to investigate the transformation potential of the Myc variants c-Myc, v-Myc, N-Myc and L-Myc, the coding regions were cloned into the replication-competent retroviral pRCAS vector [61] which is used in transformation assays. Below we illustrate the cloning procedure for pRCAS(A)BP-HA-*c-myc*(ck) (**Figure 4**). Cloning of N-*myc*, L-*myc* and v-*myc* was performed using the same strategy.

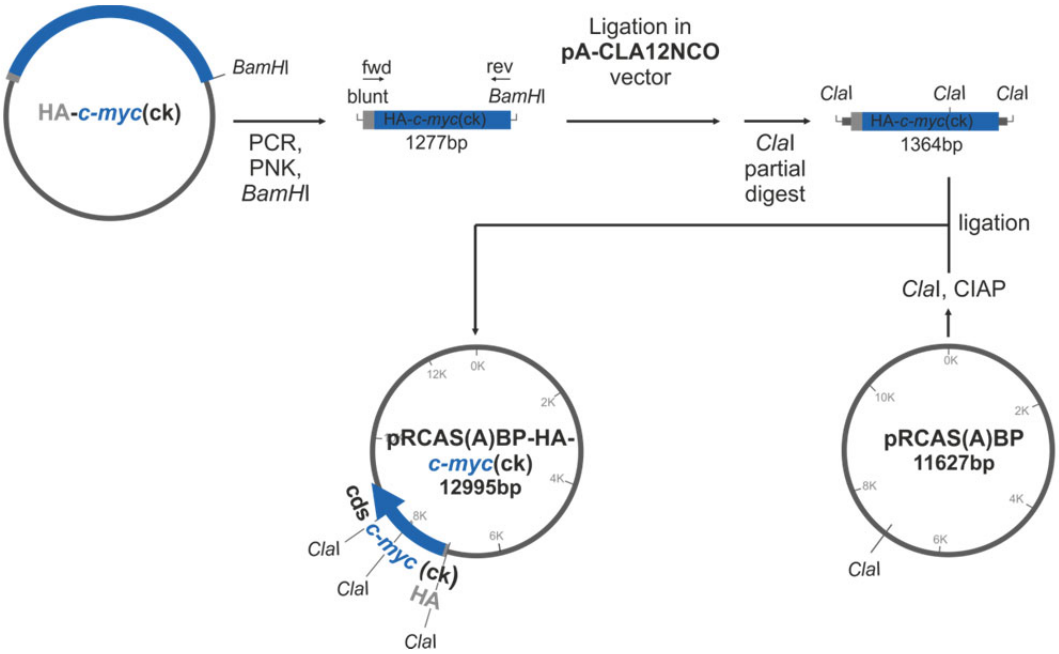


Figure 4. **Cloning strategy for the construction of *c-myc*(ck) expression vector.** Phosphorylated and *BamH*I digested HA-*c-myc*(ck) insert was cloned into pA-CLA12NCO vector followed by a partial *Cla*I digest. HA-*c-myc*(ck) insert was then ligated into ClaI digested and dephosphorylated pRCAS(A)BP vector. The same procedure was done for the construction of pRCAS(A)BP-HA-*v-myc*, pRCAS(A)BP-*L-myc* and pRCAS(A)BP-*N-myc*. PCR, Polymerase chain reaction; PNK, T4 Polynucleotide Kinase; fwd, forward primer; rev, reverse primer; CIAP, calf intestine alkaline phosphatase; HA, HA-tag; *BamH*I and *Cla*I, restriction endonucleases.

In order to generate the double-stranded DNA fragment encoding for HA-*c-myc*(ck) (1277 nucleotides), primers were designed and a polymerase chain reaction (PCR) was performed (**Table 8**). The 3´-primer contains a *BamH*I restriction site.

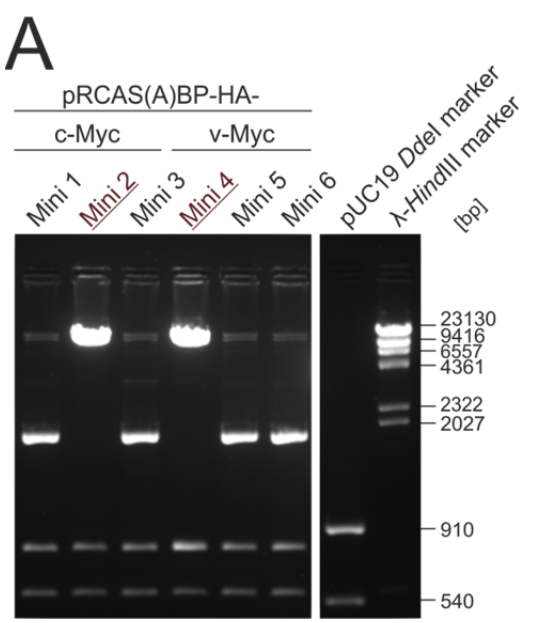
After PCR amplification, purification of the fragments with the predicted size was performed. Phosphorylation and subsequent *BamH*I (5´G/GATCC-3´) restriction digest led to a 5´-blunt ended and a 3´-*BamH*I ended HA-*c-myc*(ck) DNA fragment. In parallel we performed the pA-CLA12NCO vector preparation starting with a *Nco*I (5´-C/CATGG-3´) digest followed by a *Klenow* reaction in order to generate blunt ends. Next we digested with *BamH*I (5´G/GATCC-3´) followed by the CIAP reaction. CIAP is a dephosphorylating enzyme that removes 5´-terminal phosphate groups to prevent re-ligation of the vector. The 1277 bp HA-c-myc(ck) fragment was ligated into the pA-CLA12NCO vector using the T4 DNA ligase. This enzyme catalyses the formation of a phosphodiester bond between juxtaposed 5´phosphate and 3´hydroxyl termini in duplex DNA. The pA-CLA12NCO vector acts as an adaptor plasmid. It provides the insert with an eukaryotic leader sequence (KOZAK consensus sequence) and an initiator ATG which ensures efficient expression of the mRNA from the RCAS vector [62].

Table 8. Synthetic oligodeoxynucleotids for *myc* cDNA amplification. Sequences are written in 5´ to 3´ direction. fwd, forward; rev, reverse; ck, chicken.

Primer		Sequence	[nt]
MC29 v-*myc*, c-*myc* (ck)	fwd	TACCCATACGATGTTCCAGATTACGCTCCGCTCAGCGCC AGCCTC	45mer
	rev	TAATTATGGATCCTACAGCAGAGCCGCGGGGTT	33mer
N-*myc* (ck)	fwd	TACCCATACGATGTTCCAGATTACGCTCCGGGAATGATC AGCAAGAACC	49mer
	rev	TTATATGGATCCTTAGCA-AGTCCGCTTGTACTCTATTTTC	40mer
L-*myc* (ck)	fwd	TACCCATACGATGTTCCAGATTACGCTGAGCGGGACGCG TACC-AGC	46mer
	rev	TTATATGGATCCTAGTGCCCCTTGAGCTGAGC	32mer

To clone HA-*c-myc*(ck) into the pRCAS(A)BP expression vector, we started with a *Cla*I restriction digest of the backbone vector followed by CIAP reaction. The HA-*c-myc*(ck) DNA sequence is flanked by two *Cla*I restriction sites but due to an internal *Cla*I site a partial digest had to be performed. The resulting *myc* DNA fragment possessed two *Cla*I-sticky ends and had a calculated length of 1364 bp. The same strategy was used for creating the full-length *c-myc* insert fragment. In contrast to the c-*myc* cloning strategy no partial *Cla*I digest was needed for *N-myc* and *L-myc*, due to a missing internal *Cla*I cleavage site. Each of the different *myc* DNA inserts were ligated into pRCAS(A)BP expression vector.

The ligation mixture was transformed into the chemical competent *Escherichia coli* (*E.coli*) strain XL10-Gold using a heat shock protocol. To sort out bacteria which do not contain the plasmid of interest (pRCAS(A)BP-HA –*c-myc*/ -*v-myc*/ -*N-myc*/ -*L-myc*) the cells were plated onto ampicillin-containing lysogeny broth (LB) -agar plates. Bacterial colonies that contain the right plasmid provide ampicillin resistance and grow on the selection plates.



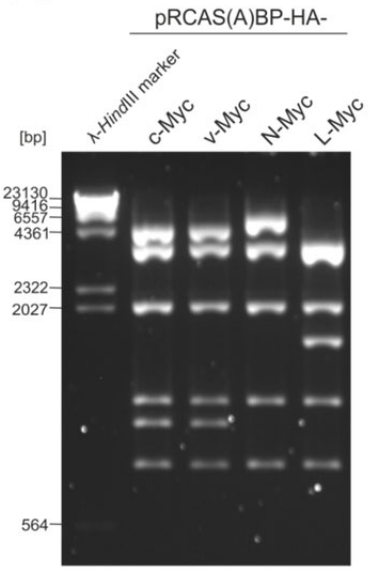
B

pRCAS(A)BP-HA-	
c-Myc	**v-Myc**
6005	6005
5622	5622
795	795
570	569

Figure 5. Restriction mapping of pRCAS(A)BP-HA-c-Myc/-v-Myc. (A) Restriction digest of indicated DNA preparations using *Cla*I restriction endonuclease. Lambda *Hind*III and pUC19 *Dde*I markers were used as size reference. Mini 2 and Mini 4 showed the expected DNA fragment pattern and were used for further DNA preparation steps. **(B)** Theoretical size in base pairs (bp) of the obtained fragments resulting from *Cla*I digest.

Next we isolated plasmid DNA from bacteria colonies using an alkaline lysis protocol. The isolated DNA was digested using *Cla*I restriction endonuclease to identify positive clones (**Figure 5A**). Clones that display the correct amount and size of fragments (**Figure 5B**) after the restriction digest were used for large scale DNA preparation.

A



B

pRCAS(A)BP-HA-			
c-Myc	**v-Myc**	**N-Myc**	**L-Myc**
4408	4407	5482	3582
3582	3582	3582	3466
2074	2074	2074	2074
1136	1136	1136	1634
999	999	793	1136
793	793		793
			86
			14

Figure 6. Restriction mapping of pRCAS(A)BP constructs containing different Myc variants with *Pvu*II. (A) Restriction digest of indicated DNA preparations using *Pvu*II endonuclease. Lambda *Hind* III marker was used as a size reference. **(B)** Theoretical size in base pairs (bp) of the obtained fragments resulting from *Pvu*II digest.

Following large scale DNA preparation we confirmed the integrity of pRCAS(A)BP-HA-*c-myc*, -*v-myc*, -*N-myc* and -*L-myc* constructs. By defined restriction mapping all constructs displayed the right number and sizes of fragments after *Pvu*II restriction digest (**Figure 6A**). DNA-cycle sequencing was performed to confirm sequence integrity of the different Myc constructs. According to the sequencing result the constructs displayed 100% identity with the theoretical DNA sequence.

5.1.2 Overexpression of different Myc variants in QEFs

Next step was the testing of the different *myc* constructs using an avian cell culture system. Therefore quail embryo fibroblasts (QEFs) were seeded on 60-mm cell culture dishes one day before calcium mediated DNA transfection. For transfections 2 µg of each construct was used per dish. In the focus assay cells were overlaid with agar the next day and grown 2-3 weeks. In the colony assay cells were passaged several times after transfection. Fully transformed cells were embedded in agar and grown for 2-3 weeks. Both assays required an agar overlay every 2-3 days.

Due to contact inhibition normal, untransformed cells become growth inhibited when they encounter other cells which results in a monolayer only [63, 64]. In contrast cancerous cells have lost this property. We tested cells which were transfected with replication-competent retroviral pRCAS(A)BP vector containing the different Myc variants to test for their oncogenic potentials. Transformed cells form foci, or colonies in an agar-matrix. All cells from a focus or a colony originate from a single transformed cell. Untransformed QEF cells display a flat, wide spread phenotype, whereas Myc-transformed cells have a more rounded morphology and grow closely spaced.

Colony and focus formation assays showed that v-Myc had the highest potential to induce colony formation, followed by N-Myc and c-Myc (**Figure 7A,B**). In contrast L-Myc overexpression only induced a small amount of colonies. These findings correlate with the rate of proliferation of the different Myc variants [Doubling times after 25 passages: v-Myc (21h±1), N-Myc (22h±1), c-Myc (23h±1), L-Myc (27h±1), QEF (49h±1)]. It also correlates with the binding affinities of Myc-variants to Max as determined by PPI [29]. Again N-Myc, v-Myc and c-Myc showed the highest affinities for Max, L-Myc the lowest [29]. However all Myc variants significantly elevated cell proliferation.

To control the protein expression levels, immunoblot analysis was performed. Two weeks after DNA transfection, the same time point when the cell pictures

were taken, cell lysates were prepared. For the detection of Myc proteins an antibody directed against the HA-tag was used. To confirm that correct amounts of protein have been loaded, an antibody directed against α-tubulin was used as a control. Similar expression levels of all Myc proteins were observed (**Figure 7C**). Interestingly additional bands were present in the v-Myc and L-Myc samples which may result from protein degradation.

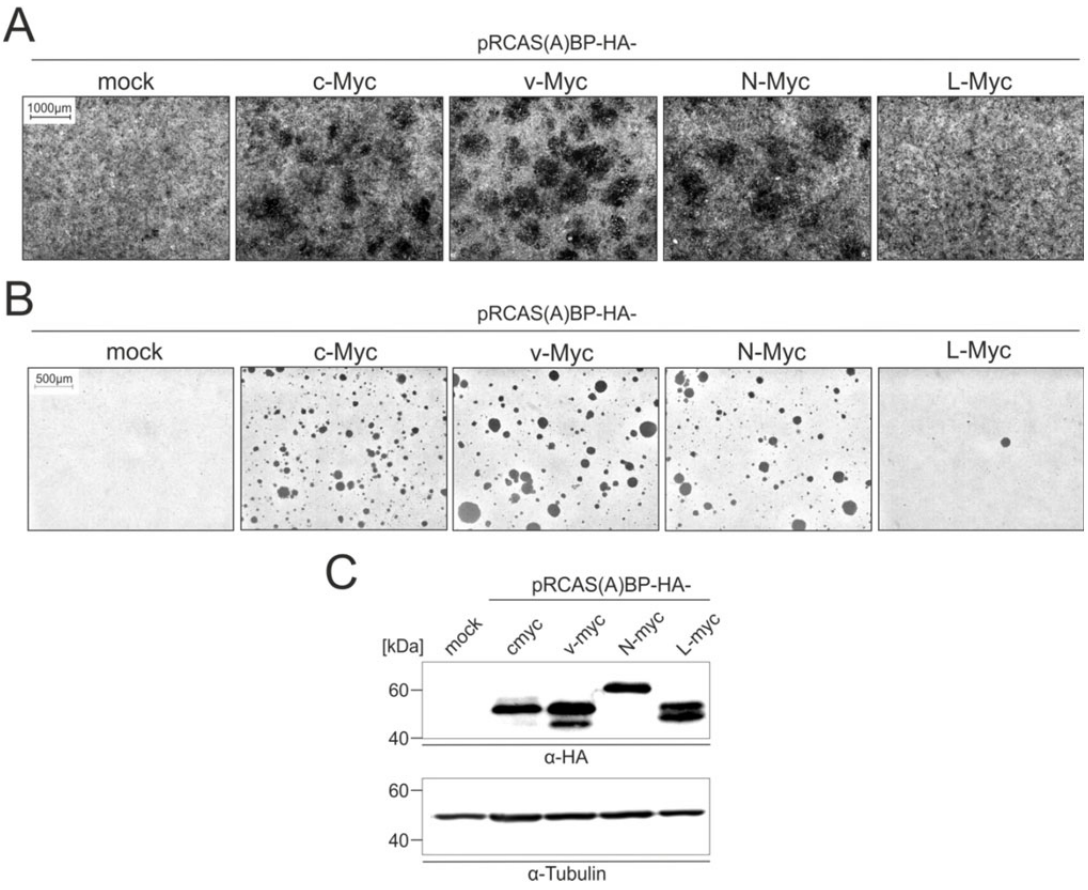


Figure 7. Transformation potential of different Myc variants. (A) Focus assay of *myc* transfected QEF cells. QEFs were transfected with 2 µg aliquots of different *myc* constructs in combination with the empty pRCAS vector DNA in 60 mm cell culture dishes. The cells were kept under agar overlay for 2 weeks and were then stained with Giemsa azur eosin methylene blue; representative of n=2 independent experiments. **(B)** Colony assay of *myc* transfected QEF cells that were passaged multiple times. Then an equal number of cells (5 x 10^4) were seeded in soft agar on 6-well cell culture plates. After two weeks of constantly overlaying the cells with nutrient agar, bright-field micrographs of agar colonies were taken; representative of n=3 independent experiments. **(C)** Cell lysates from different Myc variant expressing cell lines were analyzed by immunoblotting experiments using HA- and α-Tubulin antibodies.

5.2 Oncogenic potential of Myc hybrid proteins

v-Myc (MC29) and c-Myc differ on seven amino acid positions in their primary structure (**Figure 8**). Despite of these few sequence differences, a significant difference in the transformation potentials of both oncoproteins was observed. This raised the question, which of these amino acid exchanges could be responsible for this enhanced oncogenic potential of v-Myc. Some of these v-Myc mutations have been studied extensively. The T61M amino acid exchange is known to enhance the stability of the protein by affecting the rate of proteasomal degradation of v-Myc [65]. Insertion of I383L into a Myc mutant, which is defective for Max binding can substantially restore the complex formation of Myc and Max [66]. In order to map which part of v-Myc is critical for cell transformation, Myc hybrids were cloned. We decided to swap the N-terminal and the C-terminal part of v-Myc and c-Myc.

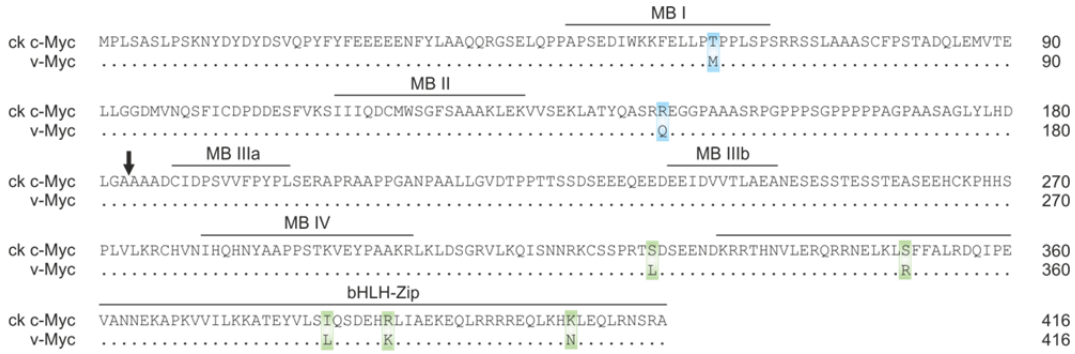


Figure 8. Amino acid sequence alignment of c-Myc and v-Myc. Highlighted residues show differences in the amino acid composition. The arrow bar indicates point of dissection for construction of Myc hybrids. MB, Myc box; bHLH-Zip, basic helix-loop-helix Leucine zipper domain.

5.2.1 Cloning of Myc hybrid expression constructs

For generating a v/c-Myc and a c/v-Myc hybrid a simple exchange of the C-terminal part of both coding sequences of c-Myc and v-Myc had to be carried out. Both Myc-variants contain an internal *Not*I restriction site which simplified the exchange of the C-termini. First a *Age*I/*Not*I (5´-A/CCGGT-3´)/(5´-GC/GGCCGC-3´) restriction digest was carried out (**Figure 9**). We purified the desired DNA inserts consisting of: N-terminal hemagglutinin (HA) tagged cds of v-Myc or c-Myc with a 5´-*Age*I end and a 3´-*Not*I end with a size of 2821 bp.

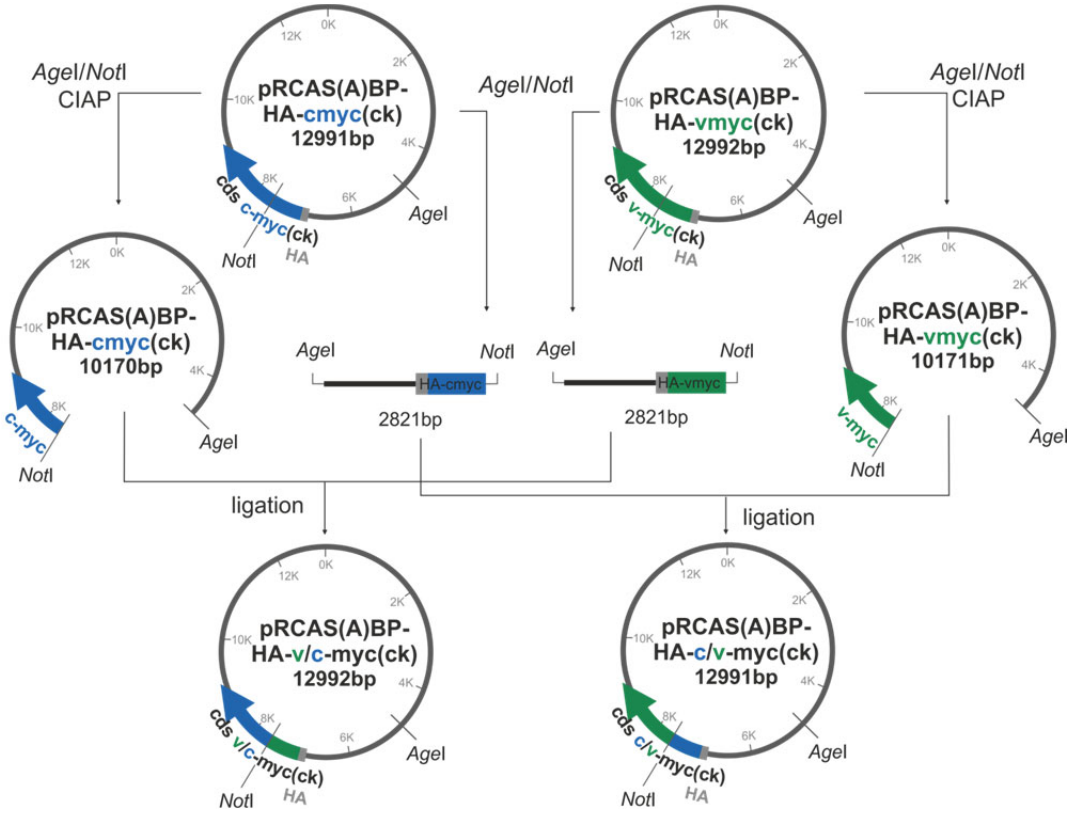


Figure 9. Cloning strategy for the construction of myc-hybrid constructs. After *Age*I and *Not*I digest inserts containing the N-terminal parts of v-*myc* or c-*myc* were ligated into the *Age*I and *Not*I digested pRCAS(A)BP vector containing the C-terminal part of v-*myc* or c-*myc*, creating a pRCAS(A)BP-HA-v/c-myc hybrid and a pRCAS(A)BP-HA-c/v-myc hybrid. CIAP, calf intestine alkaline phosphatase; HA, HA-tag; AgeI and NotI, restriction endonucleases.

For generation of the vector-backbone, pRCAS(A)BP-HA-cmyc(ck) or pRCAS(A)BP-HA-vmyc were used as template DNA. Since the vector-backbone should contain

the C-terminal part of either c-Myc or v-Myc an *Age*I/*Not*I restriction digest had to be done. In order to prevent re-ligation of the vector a CIAP reaction was performed. The resulting opened vector contained the C-terminal part of either c-Myc or v-Myc with *Not*I and *Age*I sticky ends.

The 2821 bp N-terminal HA-cmyc(ck) and HA-vmyc(ck) fragments were ligated into the pRCAS(A)BP vector containing the opposite, complementary C-terminal Myc variant using T4 DNA ligase.

The ligation mixture was then transformed into the competent *E.coli* strain XL10-Gold using a heat shock protocol. To sort out bacteria which did not contain the plasmid of interest (pRCAS(A)BP-HA-v/c-myc(ck) and pRCAS(A)BP-HA-c/v-myc(ck)) the cells were plated onto ampicillin containing LB-Agar plates. Bacterial colonies transformed by the plasmids are resistant against ampicillin and grow on the selection plate.

Next plasmid DNA was isolated from bacteria colonies using an alkaline lysis protocol. The isolated DNA was digested using *AgeI/NotI* restriction endonucleases to identify positive clones. Clones that displayed the correct amount and size of fragments after the restriction digest were used for large scale DNA isolation.

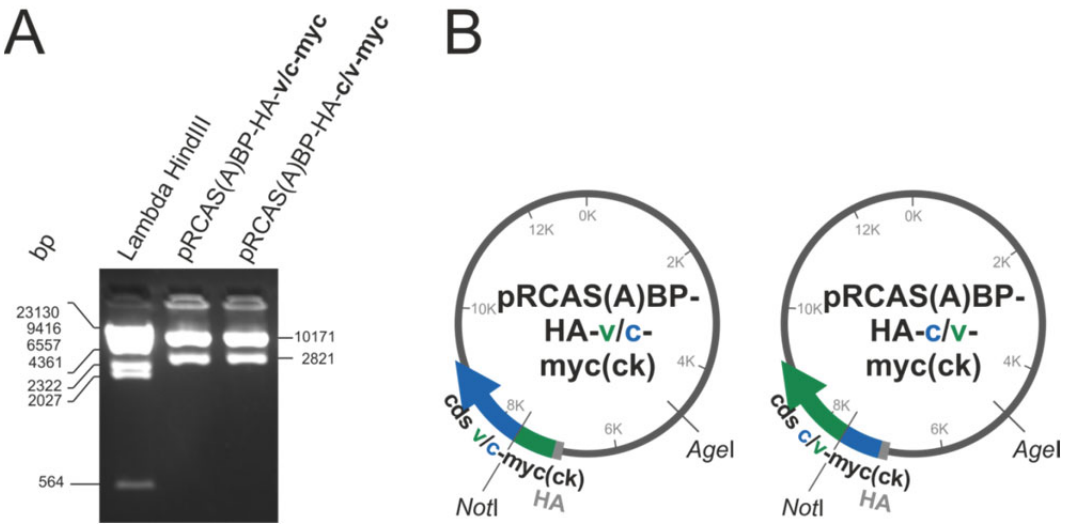


Figure 10. Restriction mapping of the two Myc hybrid constructs. (A) Restriction digest of indicated DNA preparations using *Age*I/*Not*I endonucleases. Lambda *Hind*III marker was used as a size reference for identification of the approximate sizes of the fragments. **(B)** Illustration of the digested Myc constructs. The restriction sites are indicated and a digest should result in theoretical fragment sizes of 10271 bp and 2821 bp.

AgeI/NotI digested constructs pRCAS(A)BP-HA-v/c-myc and pRCAS(A)BP-HA-c/v-myc were separated on a 1,5% agarose gel (**Figure 10**A). As indicated the resulting fragments were predicted to have a size of 10271 bp and 2821 bp, which was confirmed by the control digest. DNA-cycle sequencing was performed to confirm sequence integrity of the different *myc* constructs. According to the sequencing result the constructs displayed 100% identity with the theoretical DNA sequence.

5.2.2 Overexpression of Myc hybrid proteins in QEFs

The next step was the testing of the *myc* hybrid constructs in the avian cell culture system. Therefore QEFs were seeded one day before calcium phosphate-mediated DNA transfection was performed. In the focus assay cells were overlaid with agar the next day and grown 2-3 weeks. For carry out a colony assay the cells were passaged several times after transfection until they looked fully transformed and finally single cells were embedded in agar and grown further for 2-3 weeks. Every 2-3 days an agar overlay was necessary.

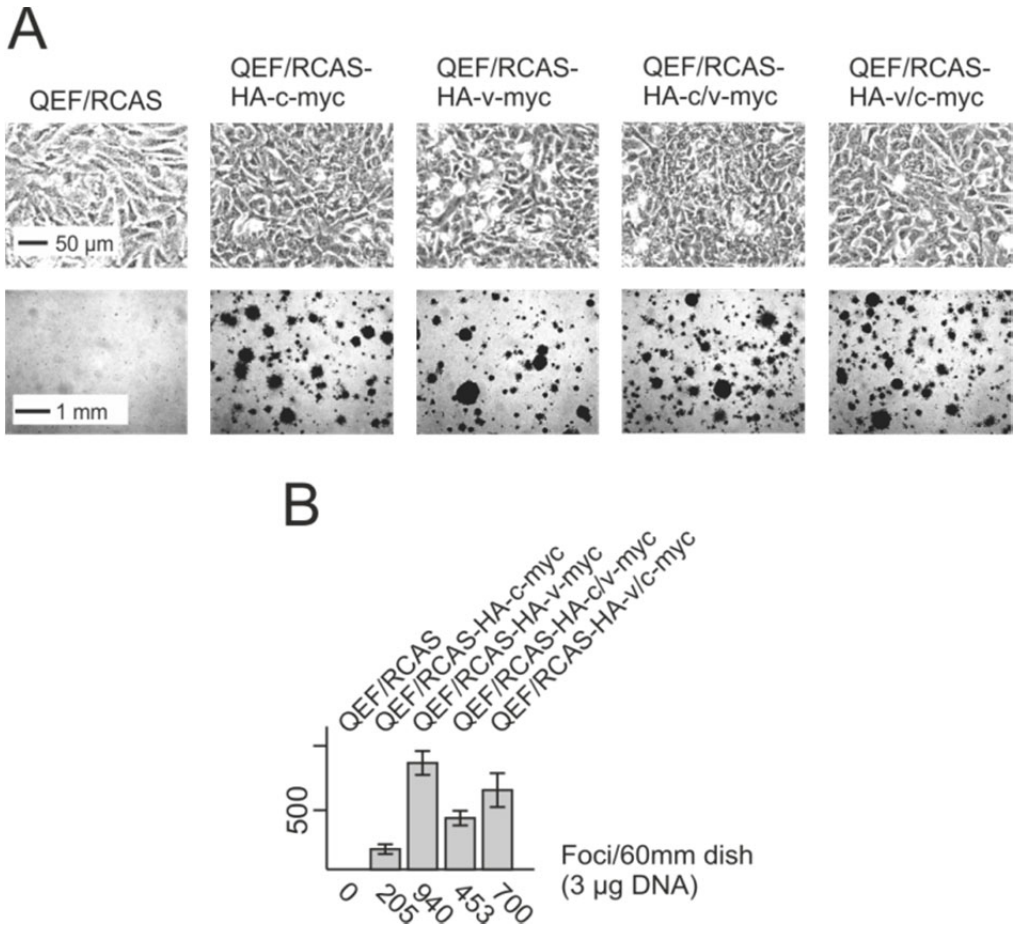


Figure 11. Transformation potential of Myc hybrids. (A) Colony assay of *myc* transfected QEF cells that were passaged multiple times. Then an equal number of cells were seeded in soft agar on 6-well cell culture plates and incubated for 2 weeks. Phase-contrast micrographs of cultured cells, bright-field micrographs of agar colonies are shown. **(B)** QEFs were transfected with 3µg aliquots of indicated DNA constructs, kept under overlay for 2 weeks, and stained with Giemsa azur eosin methylene blue. Quantification of foci scored on 60mm dishes is shown.

Transfection of QEF cells, colony assay, and quantification of focus assay was performed by Markus Hartl (**Figure 11**). This experiment confirmed that v-Myc has the strongest oncogenic potential as seen in **Figure 7A, B**. Also c-Myc showed transforming potential but not as strong as v-Myc. Concerning the Myc hybrids, he observed that the v/c-Myc hybrid has a higher tendency to form colonies compared to the c/v-Myc hybrid but both hybrids are not as strong as the full-length v-Myc but at least stronger then c-Myc.

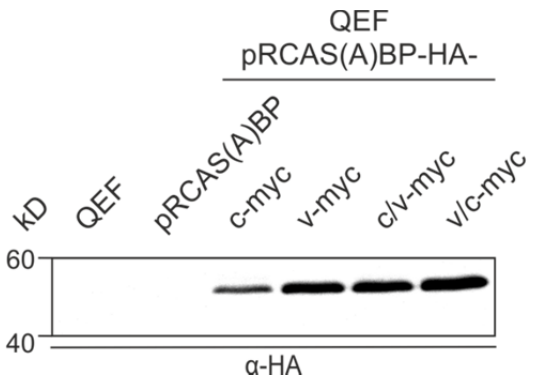


Figure 12. Protein expression of Myc hybrids. HA antibody was used to detect the amount of Myc protein expression. As controls cell-lysates of untransfected QEF cells were taken as well as cells which were transfected with the empty pRCAS(A)BP vector.

An immunoblotting analysis was performed, using cell lysates which were taken two weeks after the colony assay has been started, to compare the level of protein expression with the obtained results from the colony assay. As seen in **Figure 12** the amount of expressed Myc protein was approximately the same for all Myc variants expressing cell lines.

5.3 Subcellular localization of Myc:Max complexes

Protein fragment complementation assay (PCA) is a technique to localize or to quantify cellular PPI. The general principle of PCA is based on a reporter protein which was rationally dissected into two fragments covalently linked to two proteins of interest. Interaction of the two proteins of interest (bait and prey protein; cf. **Figure 2**) brings the fragments of the reporter into close proximity resulting in a functional reporter protein [41, 67]. In our lab we apply the *Renilla* luciferase based PCA for studying Myc:Max complexes directly in living cells. Using this technique, a strong interaction was observed between truncated and full-length version of Myc and Max [29].

Since Myc:Max acts as transcription factor dimer, its primary localization is the nucleus. Therefore it was necessary to determine the localization of the newly cloned PCA-tagged Myc- and Max-constructs. To visualize the Myc:Max PPI a different PCA reporter was used, the Venus YFP reporter. In principle, it would be possible to use the *Renilla* luciferase reporter for visualization of Myc:Max complexes but this is technically challenging and provides images with low resolution.

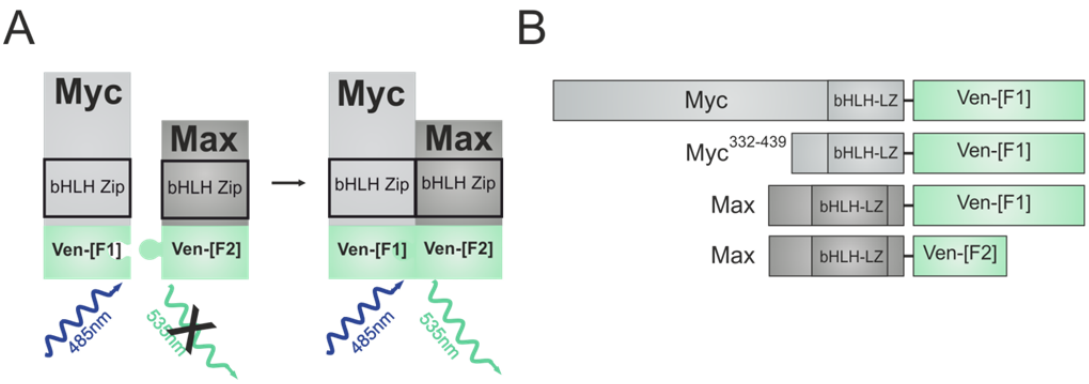


Figure 13. Venus YFP PCA principle and PCA-tagged Myc and Max PCA constructs. (A) Schematic depiction of the principle behind Venus YFP PCA. **(B)** Different PCA constructs used for subcellular localization experiments.

The general principle of Venus-YFP PCA based on PPI of Myc:Max is shown in **Figure 13A**. Binding of Myc to its binding partner Max brings the two unfolded Venus-YFP reporter fragments into close proximity. This allows folding and reconstitution of the Venus-YFP protein which results in measurable fluorescent activity. Venus-YFP has an excitation peak at 485 nm and emission peak at 535 nm. We generated different constructs for PPI localization studies in living cells (**Figure 13B**). As control, PKA regulatory type II (RIIβ) subunits fused to either Ven[F1] or Ven[F2] were used. RIIβ is known to form homodimers and is located in the cytoplasm [40].

5.3.1 Cloning of Venus YFP tagged Myc and Max constructs

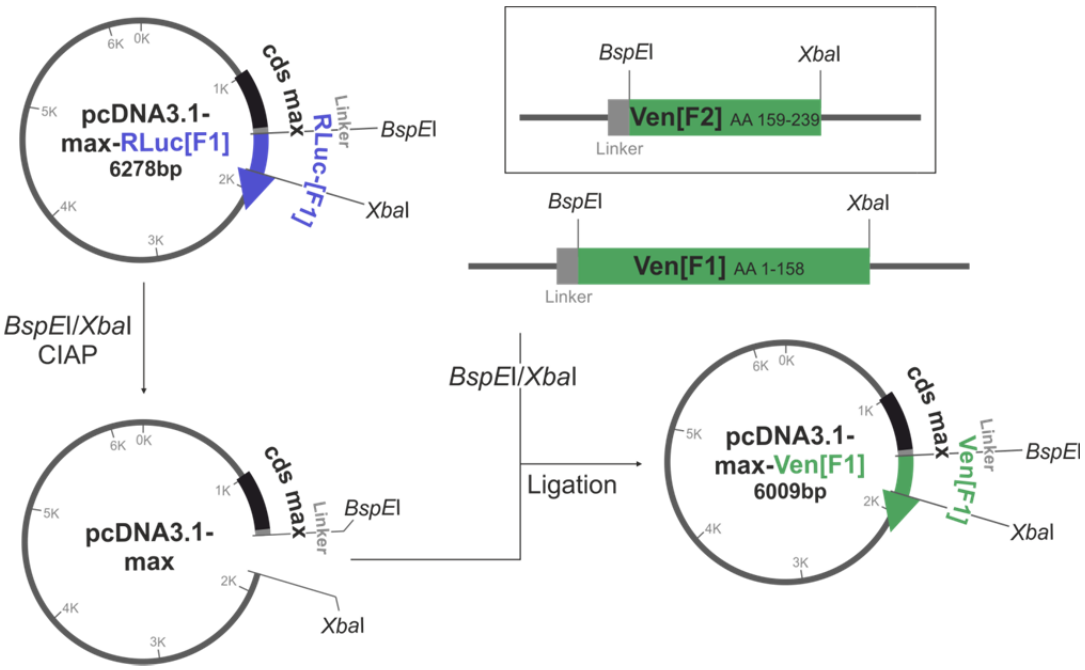


Figure 14. Cloning strategy of the Venus YFP PCA constructs. Venus YFP PCA fragment 1 or fragment 2 cut with *BspE*I and *Xba*I were ligated into pcDNA3.1-max, -myc or pcDNA3.1-myc$^{332-439}$vector. The pcDNA3.1 vector backbone was cut with *BspE*I and *Xba*I followed by phosphorylation. CIAP, calf intestine alkaline phosphatase; *BspE*I and *Xba*I, restriction endonucleases.

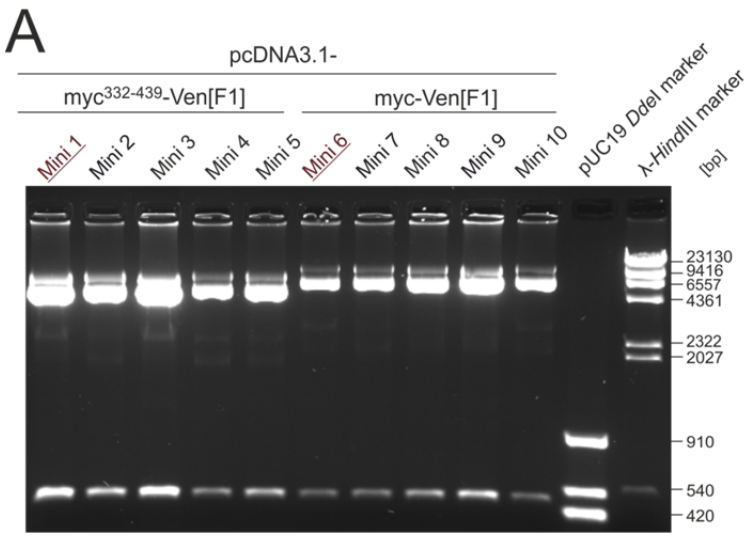
Construction of Myc-, Max- Venus-PCA was performed by a simple exchange of the *R*Luc-PCA DNA fragments with Ven-PCA DNA fragment using restriction enzymes (**Figure 14**).

The Venus YFP PCA reporter was available in a pcDNA3.1 vector. The coding sequence of either YFP PCA fragment 1 (aa 1-158) or YFP PCA fragment 2 (aa 159-239) was fused at the 5´-end separated by a linker (aa sequence: GGGGSGGGGS) [68]. Both fragments are flanked by a 5´-*BspE*I and a 3´-*Xba*I restriction site which simplified the exchange of the PCA fragments. A digest of the Venus YFP PCA fragments using *BspE*I/*Xba*I restriction endonucleases resulted into 480 bp long Ven[F1] and 252 bp long Ven[F2] inserts.

The *R*Luc-PCA-tagged Myc, Max pcDNA3.1 plasmids were used as vector backbone. *R*Luc PCA fragments were removed in double digests with *BspE*I/*Xba*I in order to insert Ven-[F1] or -[F2]. To prevent re-ligation of the vector a CIAP reaction was performed.

The next step was the ligation of the two inserts Ven[F1] and Ven[F2] with the pcDNA3.1 vector backbone, containing either the full-length or the truncated version of Myc.

The ligation mixture was then transformed into the competent *E.coli* strain XL10-Gold using a heat shock protocol. To sort out bacteria which did not contain the plasmid of interest (pcDNA3.1-myc-Ven[F1], pcDNA3.1-myc$^{332-439}$-Ven-[F1], pcDNA3.1-max-Ven[F1]/[F2]) the cells were plated onto ampicillin containing LB-Agar plates. Bacteria that took up the right plasmid provide the ampicillin resistance and form colonies on the selection plate.



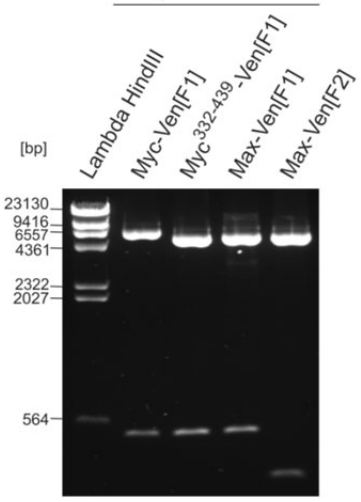
B

pcDNA3.1-	
myc$^{332-439}$-Ven[F1]	myc-Ven[F1]
5378	6369
480	480

Figure 15. **Restriction mapping of pcDNA3.1-myc$^{332-439}$-Ven[F1] and pcDNA3.1–myc-Ven[F1]. (A)** Restriction digest of indicated DNA preparations using *BspE*I/*Xba*I restriction endonuclease. Lambda *Hind*III and pUC19 *Dde*I markers were used as size reference. All clones expressed the right plasmid DNA, Mini 1 and Mini 6 and were used for further DNA preparation steps. **(B)** Theoretical sizes in base pairs (bp) of the fragments resulting from *BspE*I/*Xba*I digest.

Next plasmid DNA was isolated from bacteria colonies using an alkaline lysis protocol. The isolated DNA was digested using *BspE*I/*Xba*I restriction endonuclease to identify positive clones (**Figure 15A**). Clones that display the correct amount and size of fragments (**Figure 15B**) after the restriction digest were used for large scale DNA preparation.

A



B

pcDNA3.1-			
Myc-Ven[F1]	Myc$^{332-439}$-Ven[F1]	Max-Ven[F1]	Max-Ven[F2]
6369	5378	5529	5529
480	480	480	252

Figure 16. Restriction mapping of different Venus constructs with *BspE*I/*Xba*I. (A) Restriction digest of indicated DNA preparations using *BspE*I/*Xba*I endonucleases. Lambda *Hind*III marker was used as size reference. **(B)** Theoretical sizes in base pairs (bp) of the fragments resulting from the *BspE*I/*Xba*I digest.

Following large scale DNA preparation we confirmed the integrity of pcDNA3.1-myc-Ven[F1], pcDNA3.1-myc$^{332-439}$-Ven-[F1], and pcDNA3.1-max-Ven[F1]/[F2] constructs: Restriction mapping showed that all constructs displayed the right amount and size of fragments after a *BspE*I/*Xba*I digest (**Figure 16A**). DNA-cycle sequencing was performed to confirm the sequence integrity of the different Myc constructs. According to the sequencing result the constructs displayed 100% coverage with the theoretical DNA sequence.

5.3.2 Fluorescence microscopy

For the localization experiments, QEF cells were transfected with the pcDNA3.1-myc-Ven[F1], pcDNA3.1-myc$^{332-439}$-Ven-[F1], and pcDNA3.1-max-Ven[F1]/[F2] to analyse the subcellular localization of the transcription factor dimers.

One day before transfection, 100,000 QEF cells were seeded per well on a 4-well IBIDI dish (µ-slide 4 –well, IBIDI, # 80426). The cells were co-transfected with Venus-YFP PCA expression vectors coding for full length and truncated version of Myc, Max, and PKA RIIβ subunits tagged to the indicated fragments of Venus PCA as shown in **Figure 13B**. 24 h after calcium phosphate transfection the medium was changed. 48 h after transfection the cells were subjected to fluorescence imaging.

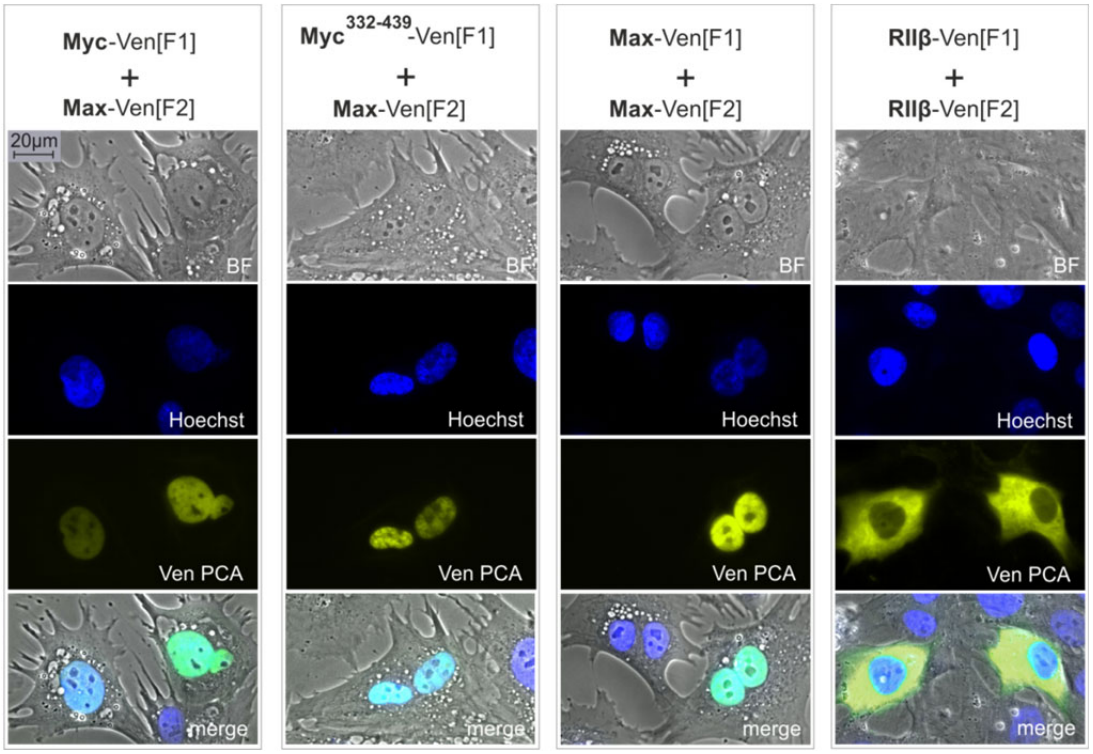


Figure 17. Localization experiment of Myc:Max using Venus YFP PCA. Localization of transiently transfected QEF expressing indicated constructs tagged with either Venus YFP fragment 1 [F1] or fragment 2 [F2]. bHLH Zip, basic-helix-loop-helix-Leucine-zipper; BF, bright field; Hoechst, Hoechst33342 nuclear staining; Ven PCA, Venus YFP Protein fragment complementation assay.

For visualization of the Venus YFP-based PCA, we used an Axiovert 200M microscope with the Axiovision 4.6 software (Carl Zeiss). Imaging was performed in the presence of Hoechst33342, a cell-permeant nuclear staining that emits blue fluorescence when bound to dsDNA. The images entitled with “merge” represent double staining of the cells (**Figure 17**).

QEF cells expressing Myc-Ven[F1] and Max-Ven[F2] showed nuclear localization of the transcription factor dimer. The same was true for Max and the truncated version Myc$^{332-432}$. These data confirm that the C-terminus of Myc is sufficient for binding to Max. Also, homodimers of Max were localized to the nucleus. In contrast, we observed exclusively cytoplasmic localization of co-transfected PKA RII subunits [40].

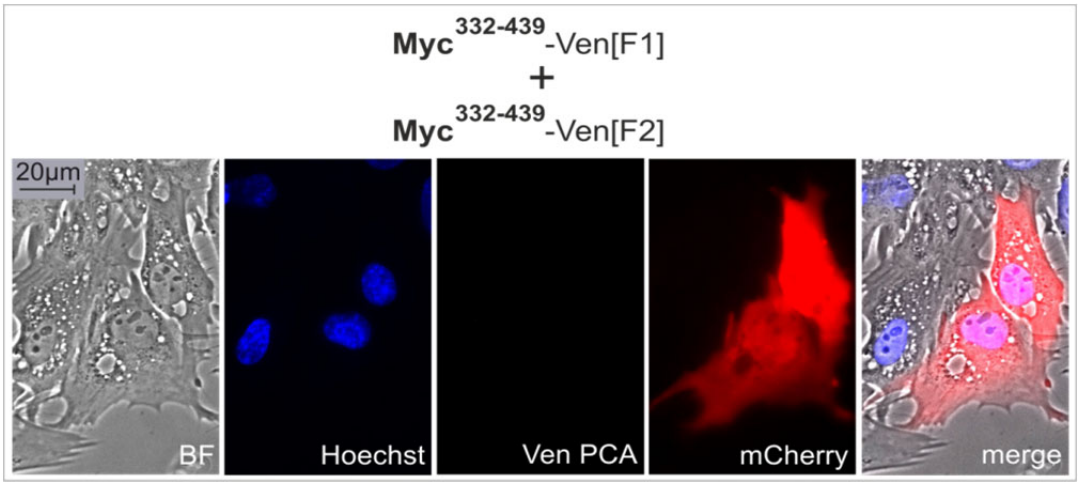


Figure 18. Control experiment using RFP mCherry as a transfection marker. QEF cells grown on a transparent microscope slide were cotransfected with Venus PCA Myc$^{332-439}$-Ven[F1]/[F2] expression vectors and mCherry. RFP, red fluorescence protein; BF, bright field; Hoechst, Hoechst33342 nuclear staining; Ven PCA, Venus YFP Protein fragment complementation assay.

Since Myc does not form homodimers under physiological conditions, another control was done by co-transfection of cells with Myc$^{332-439}$-Ven[F1] and Myc$^{332-439}$-Ven[F2] (**Figure 18**). To confirm that transfected cells were chosen for imaging

we co-transfected RFP mCherry (red fluorescent protein which has an excitation/emission peak at 587nm and 610nm). As predicted we could not detect homodimer formation of Myc which implicated a non-reconstituted reporter protein and thereby no measurable fluorescence activity. These data underline the specificity of the Venus YFP reporter to study PPI of nuclear transcription factors.

5.3.3 Quantification of Venus PCA signals

The same Ven PCA constructs were transiently co-expressed in HEK293 cells. Additional combinations of Venus PCA constructs were used to quantify PPI using the fluorescence reporter as readout. The quantification of Venus PCA signals gave information about the extent of protein-protein binding affinity, about their favourable binding partner, and about the amount of background signal.

HEK293 cells were transfected with combinations of Myc, Myc$^{332-439}$, Max, and RIIβ fused to either Ven-[F1] or Ven-[F2]. To monitor the overall transfection efficiency, full length YFP was transfected in parallel. The empty pcDNA3.1 vector served as a control. DNA transfection was carried out using TransFectin (Biorad). 48h after transfection the cells were washed with PBS and transferred into a 96 black-welled plate for measuring the fluorescence intensities. The VICTOR™ X3 Multilabel Plate Reader was used for these measurements. The fluorescence intensity was measured by 0.1s bottom reading using an emission of 485nm that results in an excitation peak at 535nm.

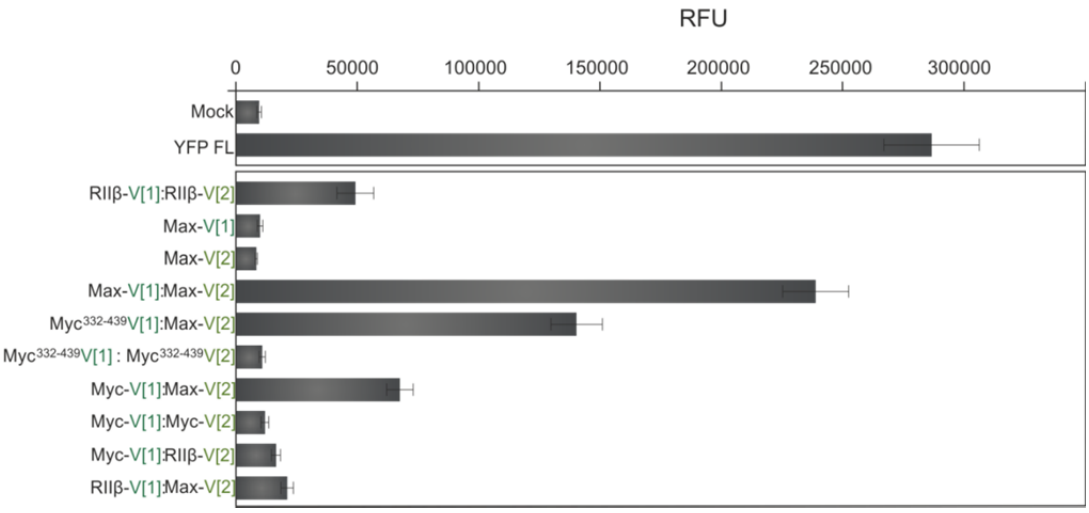


Figure 19. Quantification of PPI using the Venus YFP reporter. HEK293 cells were transiently transfected with different combinations of Venus YFP tagged constructs. After 48h of expression fluorescence was measured using VICTOR™ X3 Multilabel Plate Reader 485nm/535nm (exposure time 0.1s). Mock refers to cells transfected with pcDNA3.1 empty vector (representative of n=6; ± SD from sextuplicates). RFU, relative fluorescence unit.

We observed the highest PPI signal for the Max:Max protein complex, which reached almost the level of full length YFP representing the overall amount of transfected cells. Also, the truncated versions of Myc and Max showed complex formation. Full length Myc bound to Max showed similar relative fluorescence unit (RFU) intensities as we have observed with RIIβ dimers. Background fluorescence signals were obtained with mock transfected cells and cells transfected with Max-Ven[F1], Max-Ven[F2] alone, most likely due to auto-fluorescence of the cells. The overall background signal was determined in coexpression experiments of RIIβ:Max. We assume that it represents the rate of Venus PCA fragments forming a functional fluorophore by itself but without direct PPI (**Figure 19**).

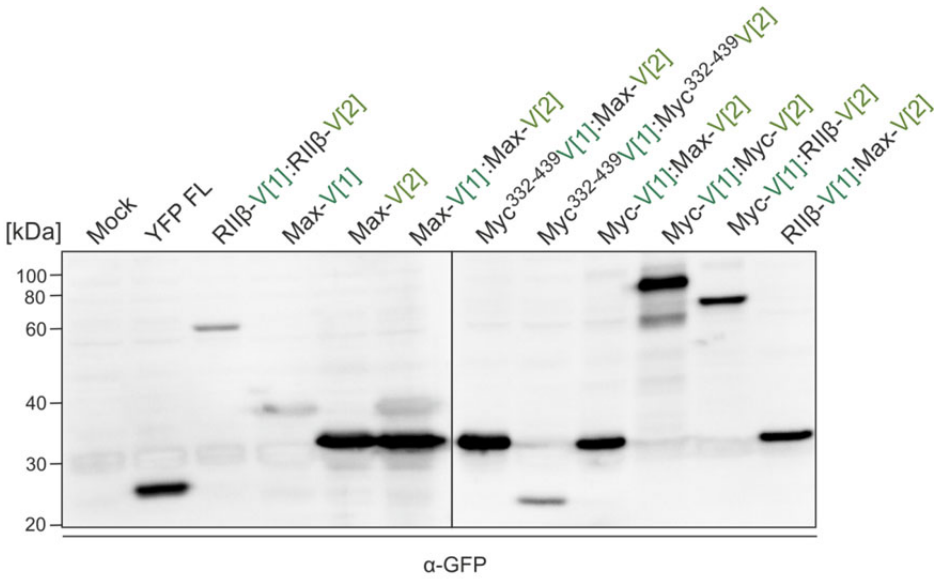


Figure 20. Analyses of protein expression of different combinations of Venus YFP tagged constructs. HEK293 cells were transiently transfected with indicated combinations of Venus YFP tagged constructs. After 48h of expression cell lysates were made and subjected to immunoblotting analyses. Detection of proteins was performed with monoclonal GFP antibody which detected Ven[F2]. kDa, kilo Dalton; GFP, green fluorescence protein.

Protein expression of all used PCA constructs was monitored by immunoblotting (**Figure 20**). Using a monoclonal antibody directed against GFP which detects

exclusively Ven[F2] tagged proteins. Immunoblotting showed similar expression levels, with the exception of RIIβ and Myc$^{332-439}$ which showed lower protein expression

5.3.3.1 Localization studies of bioluminescence reporter

To visualize bioluminescence signals from the full length *R*Luc, live cell imaging was performed of transiently transfected HEK293 cells. The cells were grown over night and imaged on an Axiovert 200M microscope (Carl Zeiss). Addition of the substrate Benzyl-coelenterazine enables the catalyses of the bioluminescent reaction and the emitted light could be visualized.

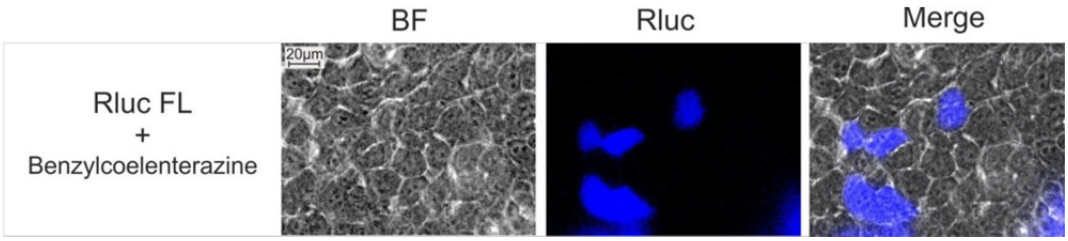


Figure 21. Subcellular imaging of *R*Luc emitted bioluminescence. Transiently transfected HEK293 cells expressing full-length *R*Luc (integration time 120 sec). BF, bright field; *R*Luc FL, *Renilla* luciferase full-length.

In principle, when transfecting the cells with full-length *R*Luc reporter, the imaging worked well to visualize a whole cell (**Figure 21**). For analyses of subcellular localizations of *R*Luc PCA reporter, a more powerful microscope setting would be necessary.

5.4 Dynamics of the Myc:Max *R*Luc PCA reporter

Recently we have developed in our laboratory a Myc:Max *R*Luc PCA reporter to quantify and dissect complex formation of Myc:Max. Disruption of the Myc:Max complex results in reduction of DNA binding, of transcriptional activity, and of oncogenic potential [29]. The Myc inhibitors KJ-Pyr-9 and KJ-Pyr-10 were tested using the *R*Luc PCA reporter as readout. Treatment of *myc*$^{332\text{-}439}$:*max* expressing HEK293 cells with the two inhibitors resulted in reduction of Myc$^{332\text{-}439}$:Max complex formation by approximately 35-40% [29, 38]

It raised the question, if a second messenger molecule like cyclic adenosine monophosphate (cAMP) can also affect this nuclear PPI reporter by upstream signaling. The diterpene derivative Forskolin was best suited to raise physiological cAMP levels in the cell. Forskolin was found in the Indian plant *Coleus forskohli* [69] and is known to activate adenylyl cyclase and thereby raising intracellular cAMP levels [70]. Increased cAMP levels lead to activation of PKA by binding of cAMP to the R subunits of PKA and dissociation of the activated, catalytic subunit (PKAc) [71, 72].

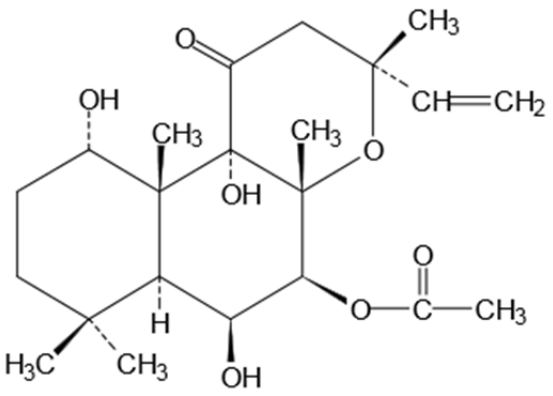


Figure 22. Chemical structure of Forskolin.

Since Forskolin has such a dramatic effect on PPI of *R*Luc PCA tagged PKA subunits we wanted to know if Forskolin also effects PPI using the Myc:Max *R*Luc PCA reporter [39]. Therefore, SW480 cells (human epithelial cells derived from grade 3-4 colon adenocarcinoma) were split one day before transfection (12-well cell culture plate). The cells were grown over night following transfections with full-length *R*Luc, RIIβ-[F1]:PKAc-[F2], and Myc-[F1]:Max-[F2] combinations using TransFectin (Biorad). 48h after transfection half of the cells were stimulated with 10µM Forskolin for 1 hour. For the measurement the cells were washed with PBS, re-suspended and transferred to a 96-well white-walled plate. Then, the cells were subjected immediately to bioluminescence analysis using a LMaxTM^{II}384 luminometer.

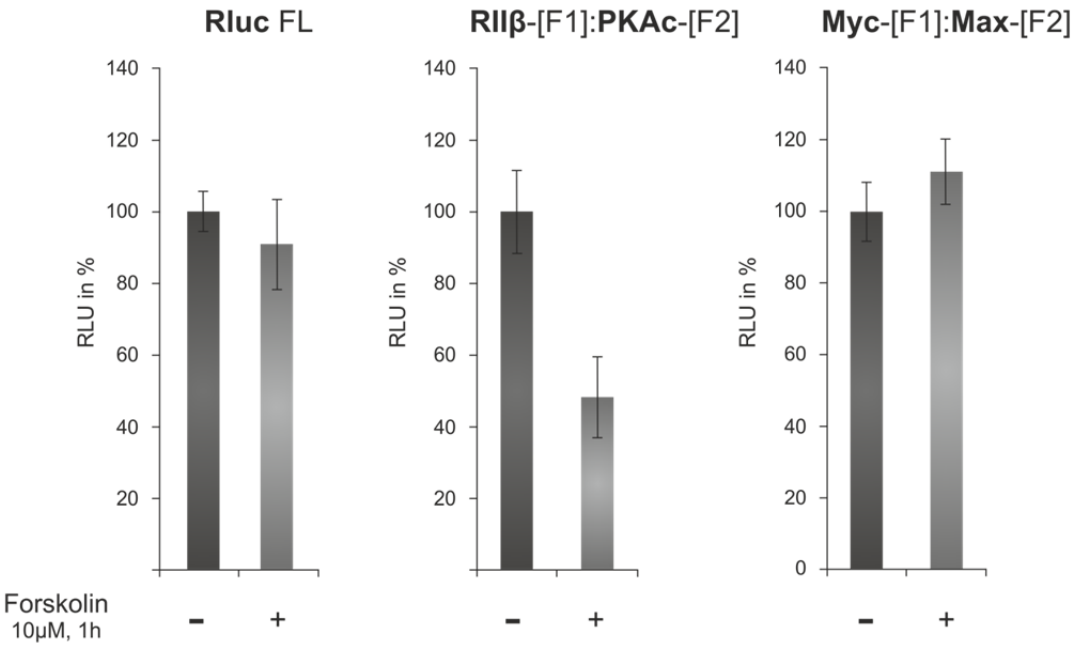


Figure 23. **Impact of Forskolin on defined PPI.** SW480 cells were transfected with different expression vectors for *R*Luc PCA. 48h after transfection cells were stimulated with 10µM Forskolin for 1h and subsequently measured using the LMaxTM^{II}384 luminometer; representative of n=4, ± SD from triplicates; RLU, Relative light unit.

Cells expressing full-length *R*Luc did not show any significant change upon treatment with Forskolin, suggesting that Forskolin did not have any effects on the *R*Luc bioluminescence signal. In contrast, cells co-expressing RIIβ and PKAc displayed a reduction of about 50% in bioluminescence assay after treatment with 10 µM Forskolin. Forskolin triggered activation of adenylyl cyclase and consequentially catalysed the conversion of ATP to cAMP. cAMP bound to the PKA RIIβ subunit which resulted in dissociation of the catalytic PKA subunit. These data confirmed previous observations that the *R*Luc PCA reporter is a reversible reporter which can be used to analyse dynamic PPI interactions [41, 67, 72]. However, in cells co-expressing Myc and Max PCA constructs we observed no significant change of PPI upon treatment with Forskolin. This suggests that the elevation of cAMP elevation has no impact on the Myc:Max interaction (**Figure 23**).

5.5 Interaction between Myc and CaM

Cell transformation driven by Myc involves transcriptional activation or inhibition of target genes that act either oncogenic or tumor-suppressive. BASP1 (brain acid-soluble protein 1) was identified as a target that is supressed by Myc [48]. A known binding partner of BASP1 is the calcium (Ca^{2+}) binding protein calmodulin (CaM). CaM is a small, highly conserved protein that transduces signals by binding Ca^{2+} and thereby modifies interactions with target proteins. The CaM structure is featured by four EF-hand motifs which undergo conformational changes upon binding to Ca^{2+} [54, 55].

An interaction between the transcription factor complex vMyc:Max and CaM was identified recently in our laboratory (unpublished data). Consequently, this observation raised the question whether dimerization of Myc and Max is required for CaM binding. To answer this question, it was necessary to overexpress and to purify the proteins of interest. Therefore the coding sequences of v-Myc and Max were cloned into a pET3d vector for recombinant protein expression. To test possible PPI, GST-pull-down experiments were performed using immobilized GST-CaM.

Cloning of pET3d-p15-v-myc was as described [8]. The expression plasmid p15-v-myc encodes the C-terminal v-Myc amino acids 314-416 and two N-terminal residues (Met, Val). The amino acids 314-416 encompass the basic region of v-Myc as well as the HLH-LZ domain (**Figure 24**).

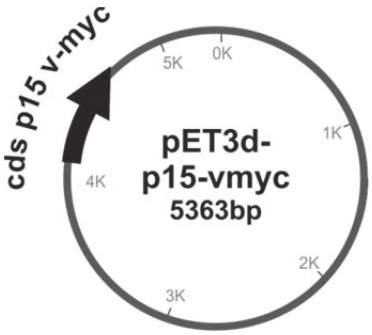


Figure 24. pET3d expression vector containing p15-vmyc insert. p15-vmyc refers to the C-terminal part of Myc, encompassing basic region, helix-loop-helix, and leucine zipper domain.

The pET3d-p15-vmyc plasmid was transformed into Rosetta^{TM}-2(DE3)pLysS-Singles, an *E.coli* bacteria strain, which is recommended for using the pET-System. The cells were plated onto an Ampicillin-containing agar plate and incubated over night at 37°C.

RosettaTM-2(DE3)pLysS-Singles bacteria derived from BL21 strain were designed to enhance the expression of eukaryotic proteins. DE3 refers to the host which is a lysogen of λDE3 phage that carries T7 RNA polymerase gene which is under control of the lacUV5 promoter. By addition of isopropyl-β-D-thiogalactoside (IPTG), the expression of inserted genes is induced in this strain. Another advantage of this bacterial strain is the expression of T7 lysozyme encoded by the pLysS plasmid, which suppresses basal expression of T7 RNA polymerase prior to induction with IPTG.

Bacterial colonies containing the pET3d-p15-vmyc plasmid were picked and inoculated in LB medium containing an appropriate amount of ampicillin and cultured over night at 37°C. The next day the overnight culture was diluted 1:50 in M9 medium to yield a 200ml culture. Every 30 min the OD_{600} was measured until an optical density of 0.55 at 600nm was reached. At this time point a sample was taken, centrifuged, re-suspended in 1x SDS-sample buffer and stored at -20°C. Protein synthesis was induced by addition of IPTG and incubation was continued for 12 hours.

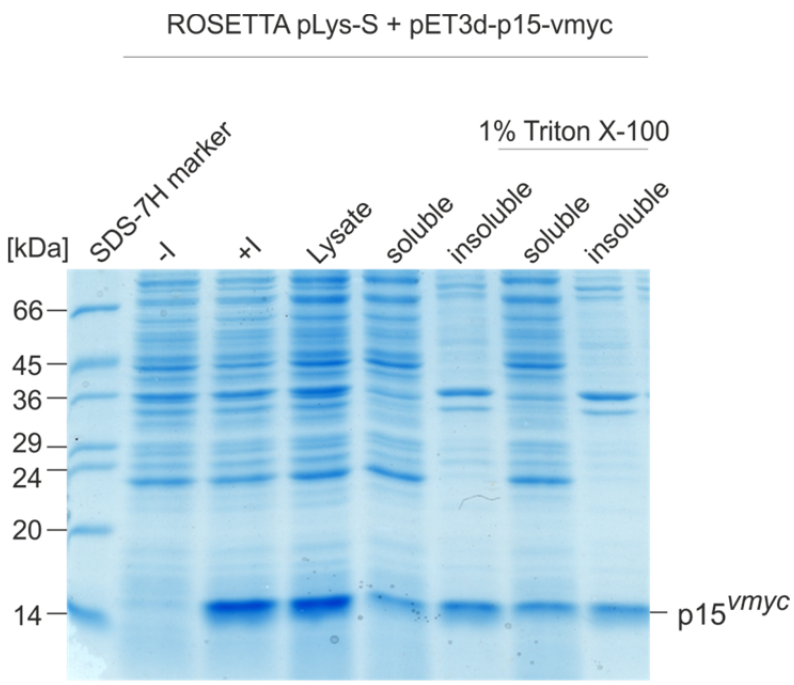


Figure 25. **Protein expression of p15-v-myc upon IPTG induction**. Expression of p15-vmyc protein using the pET-system. Samples were taken before and after induction with IPTG, monitored by SDS-PAGE and Coomassie brilliant blue staining. kDa, kilo Dalton; -I, before induction; +I, after induction.

Samples, taken before and after induction with IPTG were loaded on a protein gel, which was then stained with Coomassie brilliant blue (CBB). After induction with IPTG we observed expression of the p15-vmyc protein with a predicted size of 15 kDa (**Figure 25**).

Approximately 50% of the Myc protein remained in the insoluble fraction. 1% Triton X-100 was added to the bacterial lysates to enhance the solubility of the Myc protein. Triton X-100 is a non-ionic comparatively mild detergent that is used to solubilize proteins. Addition of 1% Triton resulted in a slight increase in solubility of the expressed Myc-protein.

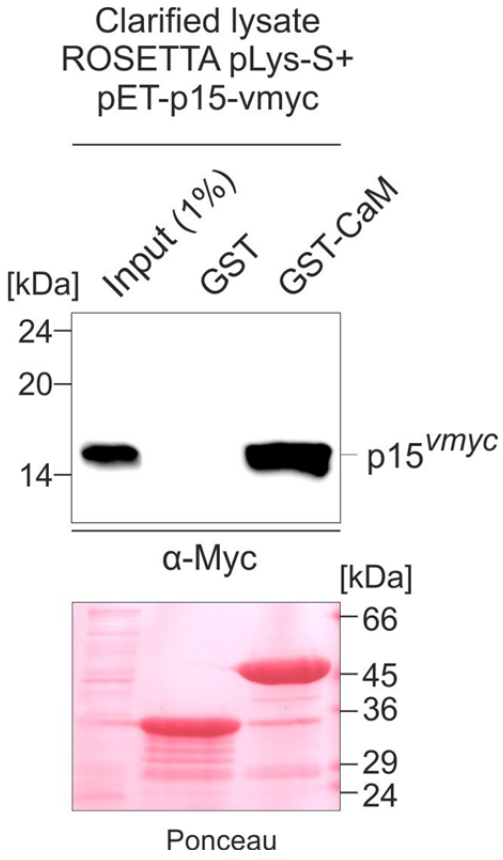


Figure 26. Interaction of p15-vmyc and CaM. GST pull down assay of p15-v-myc using GST and GST-CaM. Samples were subjected to SDS-PAGE. The separated proteins were blotted onto a nitrocellulose membrane and detected by $p15^{vmyc}$ antibody. GST, Glutathione S-Transferase; CaM, calmodulin; kDa, kilo Dalton.

Clarified bacterial lysates containing p15-vmyc were subjected to GST pull-down analysis using the fusion protein GST-CaM (**Figure 26**). 1% of the clarified lysates from ROSETTA pLys-S+pET-p15-vmyc was loaded on the gel as input-sample. Bacterial GST-CaM proteins were immobilized on beads and incubated with clarified bacterial lysates containing p15-vmyc. GST pull down assays were carried out in the presence of 0.5mM $CaCl_2$.

Non-specifically bound proteins could be washed away using lysis buffer. Before detection of the proteins by an anti-Myc antibody, the nitrocellulose membrane containing the transferred proteins was stained with Ponceau S. Ponceau S is a diazo dye which is used for rapid reversible staining of proteins transferred to a

Polyvinylidene fluoride (PVDF) or nitrocellulose membrane and functions as a loading control.

The input sample shows the expressed Myc protein after induction with IPTG. Analysis of the clarified bacterial lysates pulled-down by GST and GST-CaM revealed a clear PPI between p15-vmyc and CaM. In contrast, GST alone was not able to pull down Myc proteins.

To answer the question if heterodimer formation of Myc and Max is necessary for binding of CaM, the same pull down strategy was done using recombinant Max protein. The Max pull-down experiments were performed in our laborytory (data not shown). In this control experiment no interaction between Max and CaM was observed. We conclude that CaM binds to Myc, but not to Max, which suggests that heterodimer formation of Myc and Max is not necessary for the binding of CaM.

5.6 Mapping the CaM interaction domain of Myc

Myc contains a bHLH domain at its C-terminus which is common to many transcription factors, like Max, MyoD, TCF4 and HIF [73–76]. This bHLH domain plays crucial roles in dimerization and binding to DNA. We observed that this interaction side of Myc is involved in CaM binding. To narrow down the region of potential binding, a deletion mutant of Myc was generated.

Figure 27. C-terminal part of the amino acid sequence of full length Myc. The red box indicates basic region of the Myc protein. H1, helix 1; L, loop; H2, helix 2; L-Zip, Leucine-zipper.

In **Figure 27** the C-terminal part of the amino acid sequence of the full-length Myc protein is shown. The basic region was deleted, indicated by a red box, using an overlapping PCR protocol. This results in a Myc protein that lacks the basic region (MycΔBR). Next, the MycΔBR protein was subjected to a GST-pull down assay in order to test the binding of CaM.

5.6.1 Cloning of MycΔBR expression construct

An overview of the cloning strategy used for the generation of pET3d-p15-*vmyc*-ΔBR construct is given in **Figure 28**.

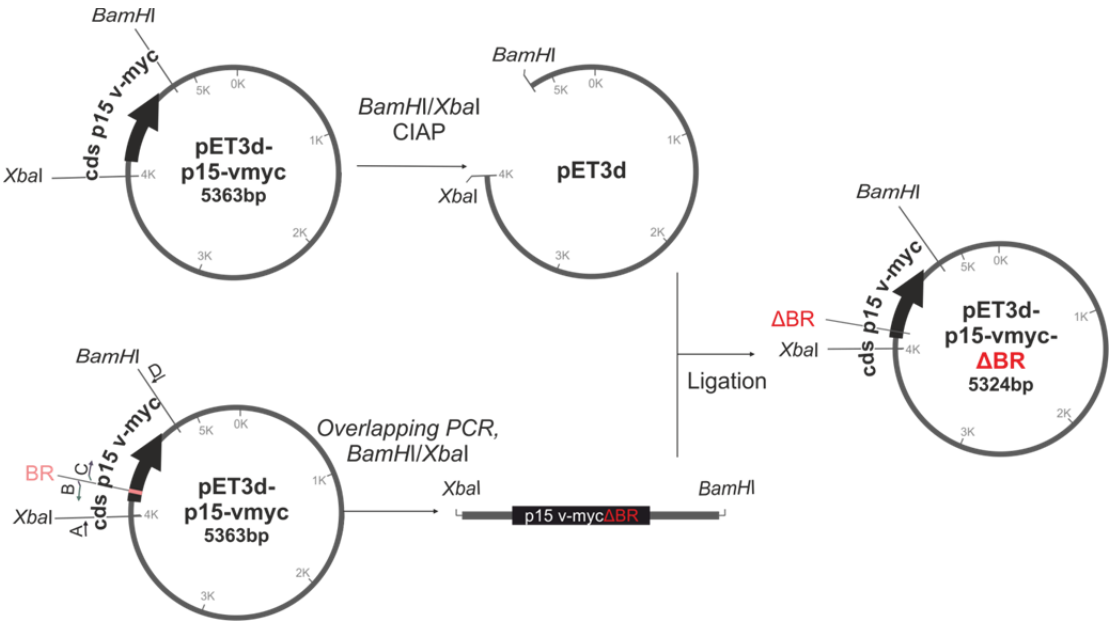


Figure 28. Construction of pET3d-p15-v*myc*-ΔBR. pET3d vector backbone preparation was done by *BamH*I/*Xba*I restriction digest followed by CIAP reaction. For deletion of the basic region out of the *myc* coding sequence an overlapping PCR was performed followed by *BamH*I and *Xba*I restriction digest. An insert containing the mutant myc coding sequence in which the entire basic region is deleted, was ligated in the opened pET3d vector. PCR, polymerase chain reaction; *BamH*I and *Xba*I, restriction endonucleases; CIAP, calf intestine alkaline phosphatase; ΔBR, deleted basic region.

To generate a double-stranded DNA fragment encoding for a v-Myc p15 protein without basic region, 4 different primers had to be designed. Primer B and C possess overhangs of 25 nucleotides and are complementary to each other. This allows the elimination of the region between these two primers. In the first round two PCRs had to be performed, one involves the primers A and B, the second one PCR primers C and D (**Figure 29**). This resulted in two linearized PCR fragments that were fused together in an overlaping PCR. By using the two outer

primers A and D and a mixture of the first PCR product as a template, a DNA fragment was generated containing the desired deletion thereby creating a coding sequence of Myc that lacks the basic region (**Figure 29**).

Table 9. Synthetic oligodeoxynucleotides for construction of MycΔBR deletion mutant by PCA mutagenesis. Sequences are written in 5´ to 3´ direction. Nucleotides indicated in blue represent overhangs. fwd, forward; rev, reverse; nt, nucleotids.

Primer		Sequence	[nt]
pET3d-p15-vmyc-ΔBR (Overlapping PCR)	A fwd	TAATACGACTCACTATAGGG	20mer
	B rev	GCAAAGAAACGCAGCTTCAGCTCATTGTCGTTCTCCTCTGAGTCTAACG	49mer
	C fwd	CGTTAGACTCAGAGGAGAACGACAATGAGCTGAAGCTGCGTTTCTTTGC	49mer
	D rev	GCTAGTTATTGCTCAGCGG	19mer

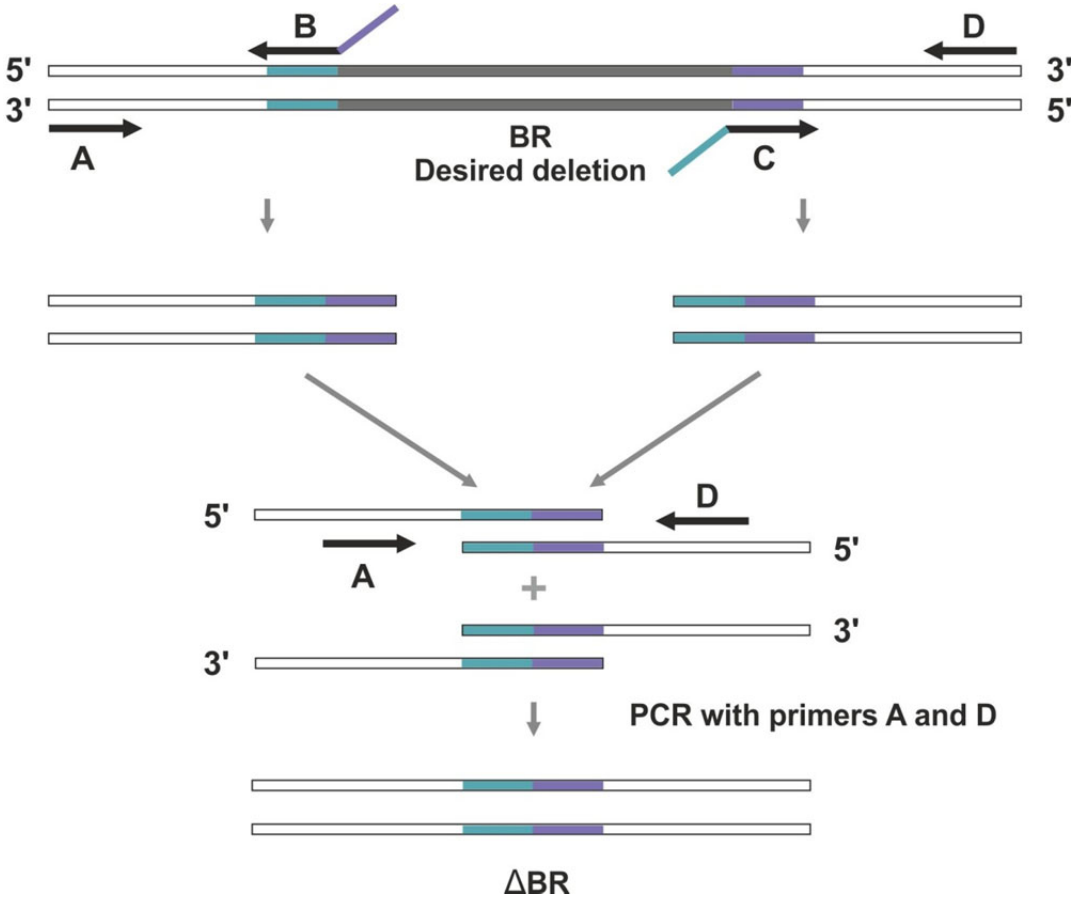


Figure 29. Schematic representation of the cloning strategy of v-*myc*ΔBR. Four different primers indicated as arrows (A, B, C, and D) were used for an overlapping PCR in order to delete the basic region of *myc*. Primers B and C possess an overhang, that is complementary to the opposing DNA template. By using the primer pairs AB and CD two PCR products were generated. The whole basic region to be deleted was not amplified during this first PCR. Primer A and D were used again in the second PCR in which the two fragments from the first PCRs were joined together.

In order to generate sticky ends the purified PCR fragment was digested using the two restriction endonucleases *BamH*I (5´-G/GATCC-3´) and *Xba*I (5´-C/TCGAG-3´). The resulting *v-myc*-ΔBR fragment was ligated into the BamHI/XbaI/CIAP processed pET3d vector backbone.

The ligation mixture was then transformed into the chemical competent *E.coli* strain XL10-Gold using a heat shock protocol. To sort out bacteria which did not contain the plasmid of interest (pET3d-p15-*vmyc*-ΔBR) the cells were plated onto

ampicillin containing LB-Agar plates. Bacterial colonies that contain the correct plasmid also provide the ampicillin resistance and grow on the selection plates.

Next, we isolated plasmid DNA from bacteria using an alkaline lysis protocol. The isolated DNA was digested using *BamH*I/*Bgl*II restriction endonucleases to identify positive clones. Clones that display the correct amount and size of fragments after the restriction digest were used for large scale DNA preparation.

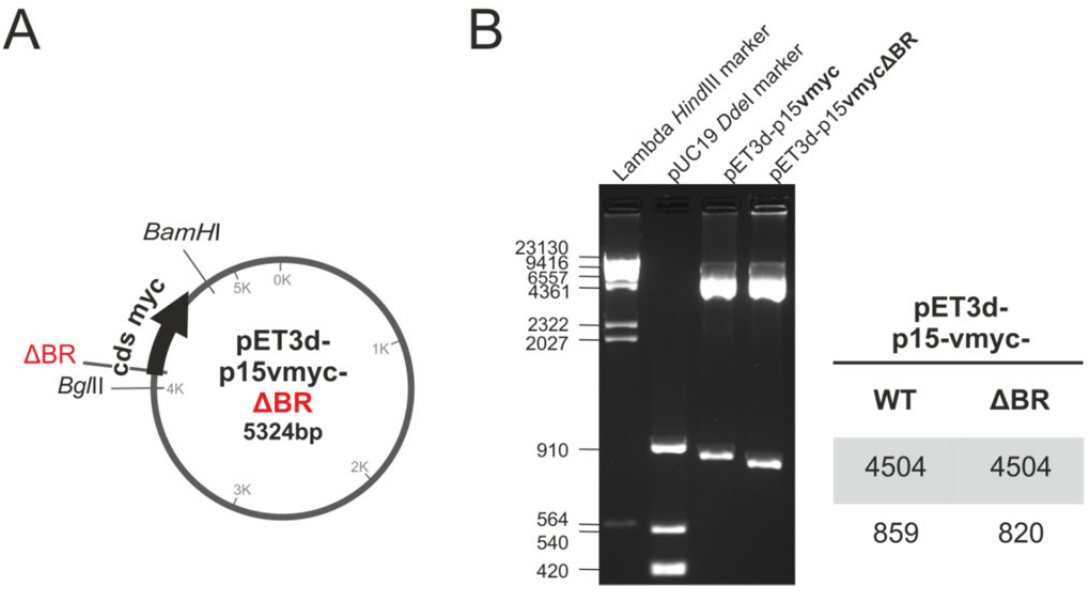


Figure 30. Mapping of pET3d-p15-vmyc-ΔBR after large scale DNA preparation (A) Schematic illustration of pET3d-p15vmyc-ΔBR plasmid with indicated *BamH*I and *Bgl*II restriction sites used for control digest. **(B)** Digested DNA and expected fragments in bp using BamHI/BglII restriction endonucleases. The pET3d-p15-vmyc construct was used as a control. WT, wild type.

Following large scale DNA preparation we confirmed the integrity of the pET3d-p15-*vmyc*-ΔBR construct. By defined restriction mapping all constructs displayed the right amount and size of fragments after *BamH*I/*Bgl*II restriction digest (**Figure 30A,B**). DNA-cycle sequencing was performed to confirm the sequence integrity of the pET3d-p15-*vmyc*-ΔBR construct. According to the sequencing

result, the construct displayed 100% coverage with the theoretical DNA sequence.

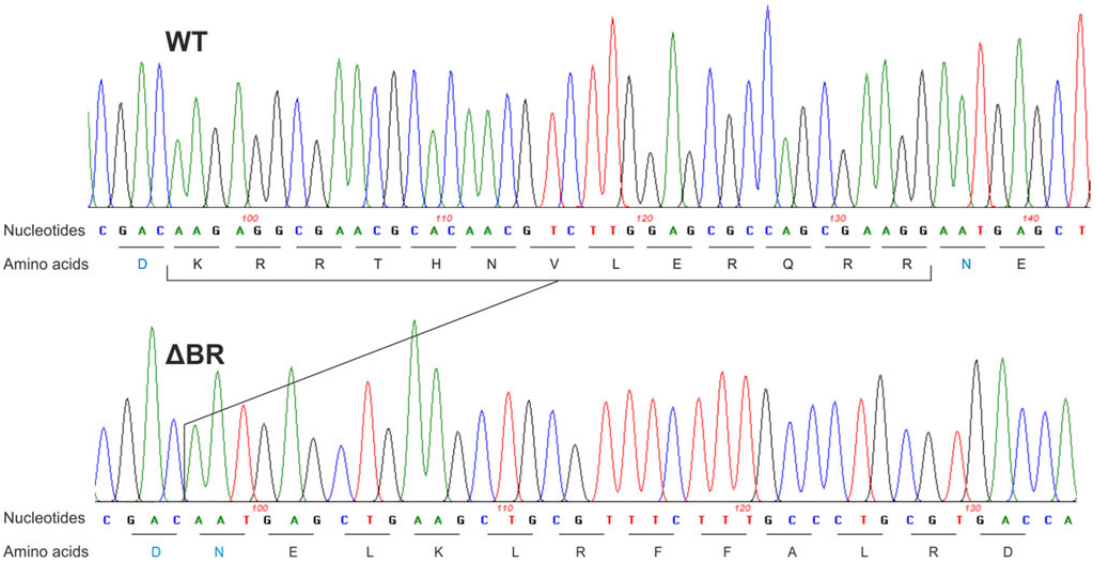


Figure 31. Sequencing result of pET3d-p15-v-*myc*ΔBR. Comparison of v-*myc* deletion mutant with the wild type form of v-*myc*. Deleted amino acids of the basic region are indicated in the wild type sequence. WT, wilde type; ΔBR, deleted basic region.

The sequenced section around the basic region of v-*myc* is shown in **Figure 31**. Electropherograms, nucleotide- and amino acid-sequences of the wild type v-*myc* and the basic region deleted v-*myc* were aligned using ClustalW Alignment. In the sequence of pET3d-p15-v-*myc*ΔBR, the two flanking amino acids in the basic region (D, aspartic acid; N, asparagine), indicated in blue clearly show the deletion of the basic.

Next step was the transformation of the pET3d-p15-*vmyc* plasmid into Rosetta^{TM}-2(DE3)pLysS-Singles, an *E.coli* bacteria strain, which is recommended using the pET-System. The cells were plated onto an Ampicillin-containing agar plate and incubated over night at 37°C.

Bacterial colonies containing the pET3d-p15-*vmyc* plasmid were picked and inoculated in LB medium containing an appropriate amount of ampicillin and cultured over night at 37°C. The next day the overnight culture was diluted 1:50 in M9 medium to yield a 200 ml culture. Every 30 min the OD_{600} was measured until an optical density of 0.55 at 600 nm was reached. At this time point a sample was taken, centrifuged, re-suspended in 1x SDS-sample buffer and stored at -20°C. Protein synthesis was induced by addition of IPTG and incubation was continued for 12 hours. After 12 hours of induction a sample was taken as described previously.

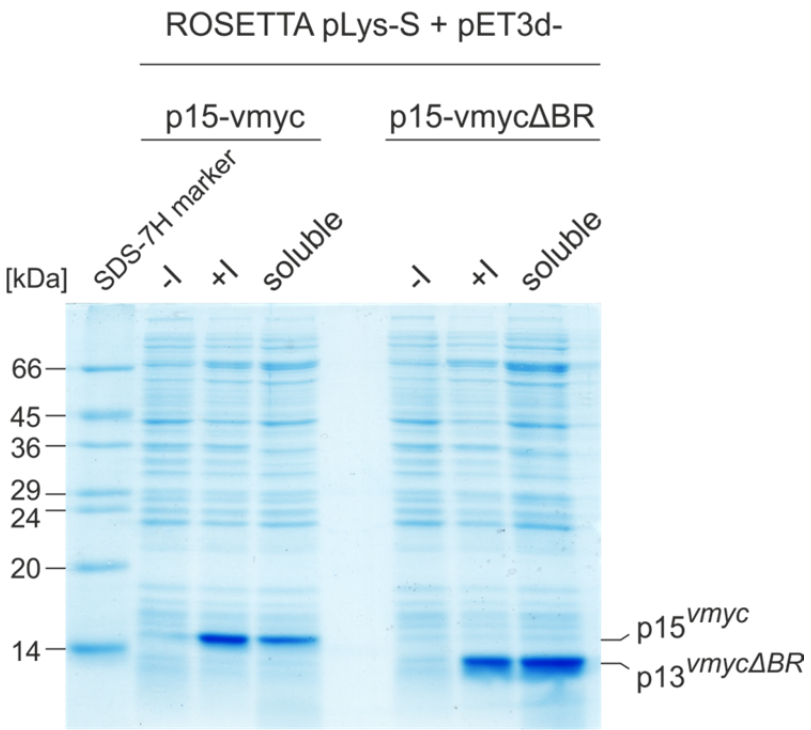


Figure 32. **Protein expression of p15-*vmyc*-ΔBR upon IPTG induction using pET system**. Samples were taken before and after induction with IPTG, monitored by SDS-PAGE and CBB staining. kDa, kilo Dalton; -I, before induction; +I, after induction.

For comparison both the wild type p15-*vmyc*- and p15-*vmyc*-ΔBR-samples were loaded onto a SDS-PAGE and stained with CBB. Both proteins were expressed upon induction with IPTG. However the amount of protein found in the soluble

fraction was higher in the mutant v-Myc recombinant protein when compared to the wild type (wt) (**Figure 32**).

5.6.2 Localization of the CaM binding domain of Myc

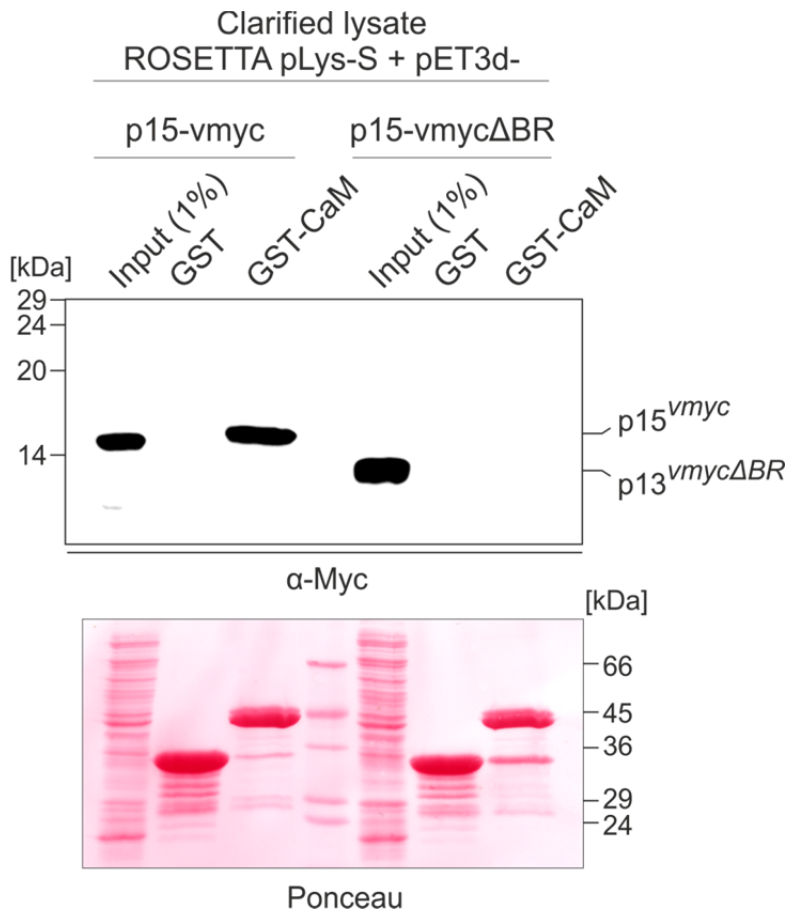


Figure 33. **Pull down analyses of p15-vmyc-ΔBR with GST-CaM.** Samples were subjected to SDS-PAGE. The separated proteins were blotted onto a nitrocellulose membrane and detected using $p15^{vmyc}$ antibody. GST, Glutathione S-transferase; CaM, calmodulin; kDa, kilo Dalton.

One percent of clarified *E. coli* lysates expressing wt and mutant v-Myc proteins were loaded onto SDS-PAGE which served as input sample (**Figure 33**). For the pull down of Myc by GST or GST-CaM, proteins were immobilized on beads and incubated with clarified bacterial lysates containing either p15-vmyc wt or p15-

vmyc-ΔBR. GST pull down assays were carried out in the presence of 0.5mM $CaCl_2$. We detected the protein by using an anti-Myc antibody. Binding of wt v-Myc to GST-CaM was reproduced in this experiment, the deletion mutant of Myc, however, failed to bind GST-CaM. This suggests that the basic region near the C-terminal end of v-Myc plays a crucial role in v-Myc:CaM PPI. Both recombinant proteins did not bind to GST. This eliminates the assumption of possible non-specific bindings.

In summary, we confirmed PPI of Myc and CaM in GST pull down analyses. In addition, the obtained data suggest that CaM does binds to the bHLH-LZ domain of vMyc but not to the Max bHLH-LZ domain. By creating a deletion mutant of Myc we identified the basic region of Myc as an interface for CaM binding. We conclude that the basic region is essential for binding of Myc to CaM. Currently we analyse the functional relevance of Myc:CaM interaction.

6 Discussion

Deregulation or overexpression of Myc leads to tumorigenesis [2, 4, 10, 16, 26]. We observed significant variations in the oncogenic potencies when comparing all members of the chicken Myc family (c-Myc, N-Myc, L-Myc, and the c-Myc derived v-Myc). v-Myc displayed the highest oncogenic potential, followed by N-Myc, c-Myc, and L-Myc in descending order. The obtained results correlate with differences of the binding affinities between members of the Myc family and Max. N-Myc and v-Myc showed the strongest interaction with Max, followed by c-Myc, and L-Myc [29]. These data underline that the oncogenic potential of Myc variants correlate with their binding affinities to Max. Now, cell lines expressing different Myc variants can be subjected to further experiments to gain a deeper insight into the mechanism of Myc-driven cell proliferation. The established stable cell lines provide the basis for high throughput screenings, to test approved drugs or to screen for new lead compounds targeting deregulated Myc activities [77].

Another strategy to identify or to test Myc-specific inhibitors in primary cells is the *R*Luc based PCA. Using this approach, we have tested the direct effect of the second messenger molecule cAMP on Myc:Max PPI. The obtained results revealed that general cAMP elevation has no impact on the stability of Myc:Max complexes. It has been proven that PCA reporters can be applied to study PPI dynamics. Besides the possibility to identify lead molecules, the PCA is also an easy adaptable PPI reporter platform for the testing of Myc inhibitors in a dose dependent and variant specific manner.

Recently, a small-molecule inhibitor of Myc (KJ-Pyr-9) was identified in a Kröhnke pyridine library. KJ-Pyr-9 has been reported to specifically interfere with Myc:Max complex formation [29, 38]. By disturbing Myc:Max complex formation, the small molecule inhibits Myc-induced oncogenic transformation in cell culture and also stopped tumor growth in mouse xenografts.

In order to determine a possible dislocation of PCA tagged Myc complexes, we visualized PPI directly in living cells. Using Venus YFP PCA reporters, we analyzed the subcellular localization of Myc:Max, $Myc^{332-439}$:Max, and Max:Max in primary QEFs. Both, the truncated version of Myc and full length Myc formed complexes with Max and were detected in the nucleus. In contrast, exclusively cytoplasmic localization of PKA RIIβ subunit dimers was observed. These data confirmed the nuclear localization of the transcription factor complex Myc:Max. Furthermore, we showed that tagging of Myc and Max with PCA reporters has no effect on the original localization of these proteins. In addition, we have shown that the C-terminus of Myc is sufficient for binding to Max and does not affect its correct localization. To prove the specificity of the Ven PCA reporter, cells were co-transfected with $Myc^{332-439}$-Ven[F1]:$Myc^{332-439}$-Ven[F2]. As predicted, no measurable fluorescence activity was detected, since Myc does not form homodimers under physiological conditions. In general, the Ven PCA reporter represents a versatile tool to determine the subcellular localization of interacting proteins. Deregulations of the subcellular localization, caused by mutations or fusions with other proteins, can be analysed using the PCA as readout.

In this context, a potential Myc dominant negative called Omomyc was recently described [78]. Omomyc consists of the Myc bHLH-LZ domain containing four amino acid substitutions which lead to efficient homodimerization and heterodimerization with members of the *myc* gene family. Omomyc interferes with Myc:Max complex formation and thereby affects Myc-binding to E-boxes [78, 79]. Since Omomyc lacks the NLS, cytoplasmic localization would be expected. However, by immunofluorescence, almost exclusive nuclear localization (about 85%) of Omomyc was shown. Probably this is due to its small size or to dimerization with endogenous Myc or Max [80]. The Ven PCA approach offers the possibility to visualize the exact subcellular localization of PPI emanating from Omomyc. We assume that Ven PCAs would allow the visualization of nuclear trans-localized PPI complexes involving wild type Myc or Max.

Another naturally occurring but cytoplasmically located version of Myc is Myc-nick. Myc-nick was reported as a cytoplasmic cleavage product of Myc that lacks the NLS and the bHLH-LZ domain [78, 81]. Hence, Myc-nick is unable to enter the nucleus, to dimerize with Max and to bind DNA. However, the levels of Myc-nick have been reported to increase during muscle cell differentiation, while the levels of full-length Myc are diminished. In this study the authors have shown that Myc-nick forms a complex with microtubules and the histone acetyl transferase GCN5 via its MBII. Thereby acetylation of α-tubulin is mediated [82]. Again, application of PCA may lead to a deeper insight in function and localization of Myc-nick and its PPIs.

Another interaction partner of Myc, which was identified in our laboratory, is CaM. The analysis of a possible connection between Myc and CaM was mainly initiated by the discovery of the Myc target BASP1 [48]. Myc regulates BASP1 in a suppressive manner and BASP1 by itself has the ability to inhibit Myc-induced cell transformation. BASP1 is a binding partner of CaM [51, 83, 84], however the exact function and mode of action of BASP1 on Myc is still unknown. We speculate that CaM could be the crucial factor to identify the mode of action of BASP1 upon Myc-induced cell transformation. We identified Myc:CaM complex formation. It is the basic region of Myc which provides the interaction site for CaM. Furthermore we found that CaM does not bind to Max, even though the basic region of Max shows high similarities with the basic region of Myc. Currently we are screening for critical residues in the basic region which mediate Myc:CaM PPI. Therefore, we exchanged amino acids in Myc which differ in the amino acid composition of Max. We plan to subject the collection of point-mutated Myc proteins to PPI and functional assays.

Apart from targeting Myc, CaM by itself represents an interesting target in cancer therapies. CaM regulates a broad range of cellular processes in a Ca^{2+}-dependent manner, such as mitosis, metabolism, and muscle contraction. It has been reported that multi-drug resistant cancer cells have a greater intercellular

Ca^{2+} level when compared to non-resistant cells. This contributes to a higher sensitivity on CaM antagonism of multi-drug resistant cells [54, 55, 85]. For further characterization studies of CaM and the identified interaction with Myc, the cellular Ca^{2+} levels need to be taken into account.

In conclusion, we have shown that the different transforming potentials of the Myc variants correlate with Max binding affinities. We introduced CaM as a new interaction partner of Myc and characterized the requirements for binding. Finally, we presented various application possibilities for PCAs, such as quantification of PPI, testing of interfering molecules, and subcellular visualization of PPI.

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10 Abbreviations

aa	amino acids
amp	ampicillin
ATP	adenosine triphosphate
BASP1	brain acid-soluble protein 1
bHLH-LZ	basic region helix-loop-helix-leucine zipper
bp	base pair
BR	basic region
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CBB	Coomassie Brilliant Blue
CIAP	calf intestine alkaline phosphatase
ck	chicken
DNA	deoxyribonucleic acid
DTT	Dithiothreitol
E-box	Enhancer box
FL	full length
fwd	forward
GST	glutathion S-transferase
GTP	guanosine triphosphate
HEK	human embryonic kidney cells
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilo dalton
MB I-IV	myc box I-IV
NLS	nuclear localization signal
o/n	over night
PCA	protein fragment complementaion assay
PCR	polymerase chain reaction

PKA	protein kinase A
PKAc	catalytic subunit of PKA
PKC	protein kinase C
PNK	T4 polynucleotide kinase
POD	peroxidase
PPI	protein protein interaction
PVDF	polyvinylidene fluoride
QEF	quail embryonic fibroblasts
rev	reverse
RFP	red fluorescence protein
RIIβ	regulatory subunit IIβ of PKA
*R*Luc	*Renilla* luciferase
rpm	rounds per minute
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TF	transcription factor
Ven	Venus YFP
YFP	yellow fluorescence protein
wt	wild type

11 Curriculum vitae

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