The 9aaTAD activation domains in the four Yamanaka Oct4, Sox2, Myc, and Klf4 transcription factors essential during the stem cell development

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Abstract

Somatic cells can be reprogrammed by the Yamanaka factors Oct4, Sox2, Myc and Klf4 activators into induced pluripotent stem cells. Throughout their genome, the Oct4, Sox2 and Klf4 cooperate with mediators of transcription, where the DNA binding sites serve as scaffolds for the phase-separated transcriptional condensates at distinct genome loci. In this study, we identified the 9aaTAD activation domains as the common interaction interface of the Yamanaka factors for transcription machinery. All four activation domains were identified by our online 9aaTAD prediction service and experimentally confirmed as strong activators of transcription. We considered the mediator interactions granted by 9aaTADs as part of the Yamanaka factors ability to reprogram cell fate.

Introduction

Our longstanding effort has been to determine the Nine-amino-acid TransActivation Domains (9aaTADs) in eukaryotic activators. Previously, we identified the 9aaTAD in a large set of transcription activators that universally recruit multiple mediators of transcription (1–11). The 9aaTAD family is represented by Gal4, p53, MLL, TCF3/E2A and SREBP1. The 9aaTADs were found in the SP/KLF family (Sp1, Sp2, Sp3, Sp4, Klf1, Klf2, Klf3, Klf4, Klf5, Klf6, Klf7, Klf8, Klf12, Klf15, WT1), the SOX family (Sox18 and SoxE), in hormone receptors (RARA, HNF4, PPAR, VDR, NHR49), in yeast transcription factors (Oaf1, Pip2, Pdr1, Pdr3, Rtg3, Gln3, Gcn4, Pho4, Msn2, Msn4, Met4), and in artificial activators of transcription (P201, B42, p53-ECapLL, KBP 2.20, pRJR200, G80BP-A, G80BP-B (12–19).

Although the activation domains have enormous variability, they are universally recognized by transcriptional machinery throughout eukaryotes (20). The 9aaTADs have the competence to activate transcription as small peptides (9 to 14 amino acids long). Currently, all tested human 9aaTADs have been shown to be functional in yeast. Therefore, we considered the 9aaTADs universal function in all eukaryotes as a further ground property of the 9aaTAD family (15, 17, 18, 20, 21). Besides the amino acid pattern, a specific distribution of amino acids in the 9aaTAD is essential. Among the characteristic features is a tandem of hydrophobic clusters surrounded by hydrophilic regions (15, 18). These hydrophobic clusters are often accompanied or are fully substituted by hydrophilic amino acids with aromatic side chains as are tryptophan (W) or tyrosine (Y), e.g. in p53. Accumulation of valines or isoleucines can compromise or fully inactivate the 9aaTAD function (22).

The 9aaTADs are well balanced with hydrophilic amino acids, which may be either positively or negatively charged. Some of them are acidic and negatively charged *e.g.* Gal4 others are positively charged *e.g.* Sp1. The general acidic character of activation domains is old and sadly fixed error, which still persists for Gal4 and also other activators (23). The Gal4 activation domain (DDVY N YLFD) is conserved only in the 9aaTAD region especially in ancestral orthologs as are *Hansenula fabianii* or *Wickerhamomyces ciferrii*, whose activation domain is hardly acidic (NDFY S LIFN)(24). Moreover, we recently demonstrated that the exchange of all negatively charged residues for positively charged ones and *vice versa* in Gal4 and respectively in Sp1 activation domains did not interfere with their function as strong activators of transcription (22).

The 9aaTADs bind to one or more of the mediator's domains on MED15 or CBP/p300 (1, 23). From structural data for the E2A and MLL activation domains in complex with the KIX

domain of CBP mediator, the 9aaTADs form short helices whose lengths vary from 9 to 12 aa (14). Online 9aaTAD prediction (using a residue position matrix search and amino acid clustering) is available on www.piskacek.org. The curated 9aaTADs have been annotated on the protein database UniProt, which now accounts for 145 annotations with 39 human activators (<https://www.uniprot.org/uniprot/?query=9aatad&sort=score>) (Including the current study, the list of 9aaTAD annotations will be further extended on 165 annotations with 52 human activators).

The Yamanaka transcription factors including the Oct4, Sox2, Myc and Klf4 activators, are all essential for chromatin remodelling and gene activation during the cell reprogramming (25). These activators can reprogram somatic cells into induced pluripotent stem cells (iPSCs) (26). In pluripotent cells, Oct4 / Pou5f from POU family associates with Sox2 to maintain pluripotency or with Sox17 to induce primitive endoderm commitment. The direct interaction between Oct4 and Sox2 is DNA dependent and involves the POU helix A1 from Oct4 and the HMG helix A3 from Sox2 (27). Klf4 cooperates with Oct4 and Sox2 (28, 29) to establish embryonic stem cells (ESCs). Oct4 and Sox2 can form a complex and cooperate during cell reprogramming (30, 31). The activation domain and associated SUMO-interacting motif (SIM, amino acid pattern K/RxE) are required for KLF4 protein stability and are essential for cell pluripotency (32–34).

The SOX family are close relatives of transcription factor SRY (SOX for SRY-related HMG box and SRY for sex-determining region Y) (35–37). The SOX transcription factors are divided into groups A to H and are involved in cell fate determination, development and cancer (38–41).

The most prominent member of the SOX family is Sox2, which supports cellular reprogramming and stem cell pluripotency (40). The Sox2 transactivation is associated with the general TFIID activator complex (42) and with stem cell XPC activator complex (38, 43, 44), with Oct4 (45), with Pax6 (46), with Trim24 (47) and with PARP-1 (48). Sox2 post-translational modifications have been reported to modulate transactivation activity (49–51). Sox2-mediated cell reprogramming could be further enhanced by Sox2 fusion with a strong viral activator VP16 (52). Sox1, Sox3, and Sox15 can replace the function of Sox2 in mouse ES (53, 54).

The gene expression programs, which are controlled by master transcription factors, define the identity of each cell. Recent studies have revealed that master activators form phase-separated condensates with the mediators of transcription at specific genomic loci containing their binding sites (55). The phase separation is a universal cooperative mechanism for

transcription, focusing the transcriptional machinery onto DNA enhancer sites, including Oct4, Sox2 and Klf4 binding sites (56, 57). Moreover, activation domains drive nucleosome eviction after activators binding to specific genomic loci (58) and after Oct4 binding, the chromatin accessibility is facilitated by chromatin remodelling factors (59, 60).

In our study, we have focused on the of transcription factors Oct4, Sox2, Myc and Klf4 to determinate their activation interface for the interaction with mediators of transcription, which is a part of cell reprogramming.

Results

POU family including Oct4

We have performed our online 9aaTAD prediction for Pou5f / Oct4 (9aaTAD prediction service online, www.piskacek.org) (18) and revealed a single perfect hit to amino acid sequence GHLASDFAF (**Figure 1**). By sequence alignment, we have identified the activation domains in other members of the POU family (**Figure 1**).

We generated LexA constructs, which included the prokaryotic DNA binding domain LexA and POU's region coding for the 9aaTAD from selected members of clade 1, 3, and 5. The constructs were tested for their ability to activate transcription (**Figure 2**). Both tested human POU activation domains of Pit1 and Pou5f1 have the capacity to activate transcription. Less activity was observed for Pou5 orthologs in *Callorhinus ursinus* (cur Pou5, Northern fur seal, gene ID: XP_025704146) and *Sarcophilus harrisii* (shr Pou5, Tasmanian devil, gene ID: G3VJ82), but none in *Lingula unguis* (lak Pou, brachiopod, gene ID: XP_013392366) or *Callorhynchus milii* (cmk Pou5, Ghost shark, gene ID: XP_007894073).

SOX family

We have performed online 9aaTAD prediction for Sox2 and revealed two hits above 80% match (**Figure 1**). The first hit was positioned within the DNA binding domain and was therefore excluded. The second hit was located in the Sox2 region 272-280, which corresponds to reported 9aaTAD activation domains in Sox18 (61) and SoxE (12). By sequence alignment, we have identified activation domains in other human members of the SOX family and their distal orthologs from early metazoans, such as *Mnemiopsis leidyi* (Ctenophora), *Amphimedon queenslandica* (Porifera), *Strongylocentrotus purpuratus* (Echinodermata) and *Saccoglossus kowalevskii* (Hemichordata), which shared significant similarity with Sox2 and other members

of the family (**Figure 3**). Importantly, members of the groups B and F have supported the common origin of their activation domains. In contrast, the well-studied paralogs SRY, Sox15 and Sox30 (62–66) have largely diverged and do not share a clear 9aaTAD motif or significant activation domain homology with the aforementioned members of SOX family.

We generated LexA constructs, which included the prokaryotic DNA binding domain LexA and SOX's region coding for the 9aaTAD from selected members of clade B, C, E and F. The constructs were tested for their ability to activate transcription (**Figure 2**). All tested SOX activation domains have the capacity to activate transcription.

MYC family

We have analysed the 9aaTAD activation domains in MYC activators. The MYC family has multiple conserved regions including MYC boxes MB0 to MBIV and the DNA binding domain (67) (**Figure 4**). From previous studies, the c-Myc region 105-143 was essential for transforming activity (68, 69). Insertion at codon 105 or removal of region 106-143 diminished c-Myc transcriptional activity (70). Based on this, we predicted the activation domain to be localised between MYC boxes MBI and MBII (**Figure 4**).

To identify the 9aaTAD activation domain, we ran our online 9aaTAD prediction service for trans-activating region 55-120 (localised between MYC boxes MBI and MBII) and revealed a 92% hit (sequence EMVT E LLGG, region 100-120) (**Figure 1**). Similarly, we revealed a 100% hit for the c-Myc mutant, which has an intact activation potential despite of the internal deletion (fusion result for modified trans-activation region with modified sequence EMVT E LLVS, fusion of regions 100-105 to 144-146).

We have analysed several previously reported mutants with surprising phenotypes. Reported insertion of glutamic acid into c-Myc (EMVT EL/ E /LVS, region 100-105 / inserted E / 144-146) (70) diminished the 9aaTAD pattern and its function. The activation domain 9aaTAD was also destroyed in mutants with deletion 3-103 and 106-143. Nevertheless, partial activity was observed in mutants with deleted regions 41-103, 56-103, 93-103 (including also small inserts due to DNA manipulation), which seem to have a partial repair of their 9aaTAD motif (QSEL / E LLGG, ELLD / E LLGG and GSSI / E LLGG).

The longest reported construct with full activity included the c-Myc region 1-262 (69). Despite of the presence of the activation region, a longer construct including region 1-336 has lost abruptly the capacity to activate transcription. Similarly, the mutant with deleted region 7-91 lost the capacity to activate transcription (70). Additionally, mutants with deleted regions 3-53 or 145-304 have no capacity to activate transcription regardless of their activation domains

(regions 55-100). Together, we concluded that in these mutants with intact activation domains, the local structural aberrations hindered function of their activation domains.

The 9aaTAD activation domains are well conserved in the active human Myc paralog N-Myc, but completely absent in human paralog L-Myc, what well corresponds with its poor activation potential (71). Interestingly, we found conservation of the activation domains in all three Myc paralogs in lobe-finned bony fish coelacanth (*Latimeria chalumnae*, fin-to-limb transition, related to lungfishes and tetrapods) but not in reptiles and other higher metazoans, which suggests that loss of L-Myc activation capacity have occurred already in early tetrapod evolution (**Suppl. Figure S1**). Besides the L-Myc clade, we found conservation of the activation domains in the entire Myc family from human to the last unicellular ancestor of animals, the *Monosiga brevicollis* (72).

The regions coding for predicted activation domains were fused with the DNA binding domain of LexA in construct i) mbr Myc for flagellate filozoans *Monosiga brevicollis* (gene ID: A9V5B4), ii) construct aqu Myc for poriferans *Amphimedon queenslandica* (gene ID: XP003390966), iii) constructs lch c-Myc, lch N-Myc, lch L-Myc for lobe-finned fish *Latimeria chalumnae* (gene IDs: XP005992710, XP005993959, XP006009573), and iv) constructs hsa c-Myc and hsa N-Myc for humans. No construct was created for the human L-Myc, which has deleted activation domain and was reported as a poor activator (71). All tested Myc activation domains from human and coelacanth have the capacity to activate transcription (**Figure 2**). Previously, the accumulations of valines and isoleucines in activation domains of SP and KLF transcription factors were linked with their full or partial inhibition, which corresponds well to lower activities of mbr and aqu Myc constructs in our transactivation assay (22).

Discussion

The activation domains have been reported as intrinsically disordered regions, which facilitate fuzzy binding with mediators of transcription. Their binding mechanism should be sequence independent because mutation of nearly every residue within is tolerated (23). In spite of enormous activation domain variability observed within the natural and also artificial activation domains, we generated a 9aaTAD pattern and prediction algorithm based on the amino acid distribution and clustering (18). In this and our other studies, the 9aaTAD online prediction (<https://www.med.muni.cz/9aaTAD/index.php>) generated reliable predictions, which led to the identification of over hundred activation domains, which have been experimentally confirmed as powerful activators of transcription. Moreover, the 9aaTAD structures seem to be helical after binding to mediators and their interaction with mediators were found not fussy but rather well fixed after binding e.g. MLL, E2A, Myb, p53 (1, 14, 73, 74). We also observed high similarity and partial identity in some of the 9aaTADs e.g. MLL and E2A (SDIM D FVLK - SDLL D FSAM) or human Myc and rat p53 (EMVT E LLGG - QDVA E LLEG) or human / Crassostrea gigas KLF4 and Oaf2 (LLDL D FIL / LLDY D FIL - LFDY D FLF)(14, 22). All that supports that 9aaTAD domains have a natural prevalence for some variants despite their tentatively huge variability.

In our study, we have identified activation domains in all four Yamanaka factors, Oct4, Sox2, Myc, and Klf4. All of them fulfil the criteria for the 9aaTAD domains, which activated transcription as short peptides. The loss of 9aaTAD in human L-Myc (as well as in reptiles and higher animals) explained its poor activator function and pointed to the functional diversification of MYC family during vertebrates' evolution. The members of SOX clades B, F, C, E possess functional 9aaTADs, could partially substitute for each other and their function also largely diversified first in vertebrates. Similarly, the KLF family and their successor SP family (Klf1, Klf2, Klf3, Klf4, Klf5, Klf6, Klf7, Klf8, Klf12, Klf15, WT1, respectively Sp1, Sp2, Sp3, and Sp4), all possess a functional 9aaTAD and have versatile functions in cell fate determination.

We found the 9aaTAD pattern deterioration in *Callorhynchus milii* (Ghost shark) cmk Pit1 (Pou1 clade) indicating that the Pit1 9aaTAD activation domain is the latter evolution event in vertebrates. Similarly, Pou5 orthologs in *Callorhinus ursinus* (cur Pou5, Northern fur seal) and *Sarcophilus harrisii* (Tasmanian devil) have less transcriptional activity and even the loss

of activity was found in *Lingula unguis* (brachiopod), which has also a deteriorated 9aaTAD motif. Moreover, we found a similar trend in Myc orthologs from flagellate filozoans *Monosiga brevicollis* and poriferans *Amphimedon queenslandica*, where the activation domains have substantially compromised function (disadvantageous accumulation of valines and isoleucines in their 9aaTADs, e.g. inactivated 9aaTAD in Sp2).

By cooperative interactions, active enhancers interact with mediators of transcription to enable assembly of the transcriptional machinery. The phase separation contributes to stabilization of transcription machinery at the specific genomic loci even at very low protein concentrations. Throughout the genome, the Oct4, Sox2 and Klf4 binding sites and mediators of transcription distribution have an exponential occurrence correlation, whereby the DNA binding sites serve as scaffolds for the phase-separated transcriptional condensate and cooperate during cell reprogramming. In summary, we consider the mediator interaction interface facilitated by the 9aaTADs in higher vertebrates as a part of Yamanaka factors ability to reprogram cell fate.

Methods

Constructs. The construct pBTM116-HA was generated by insertion of an HA cassette into the EcoRI site of the vector pBTM116. All constructs were generated by PCR and subcloned into the pBTM116 EcoRI and BamHI sites. All constructs had a spacer of three amino acids inserted into the EcoRI site: the peptide GSG. All constructs were sequenced by Eurofins Genomics. Further detailed information about constructs and primer sequences is available on request.

Assessment of enzyme activities. β -galactosidase activity was determined in the yeast strain L40 [58, 59]. The strain L40 has a chromosomally integrated lacZ reporter driven by the lexA operator. In all hybrid assays, we used the 2 μ vector pBTM116 for generation of LexA hybrids. The yeast strain L40, *Saccharomyces cerevisiae* genotype: MATa ade2 his3 leu2 trp1 LYS::lexA-HIS3 URA3::lexA-LacZ, is deposited at the ATCC (#MYA-3332). The average value of the β -galactosidase activities from three independent transformants is presented as a percentage of the reference with the standard deviations (means plus and minus SDs; n = 3). We standardized all results to the previously reported Gal4 construct HaY including the 9aaTAD with the activity set to 100% [14].

Databases used in the study. UniProt, ExPASy, NCBI, Ensembl Metazoa, KEGG <http://www.genome.jp>, Japanese Lamprey Genome Project <http://jlampreygenome.imcb.a-star.edu.sg/blast/>, Sun Yat-Sen University Lancelet Genome Project <http://genome.bucm.edu.cn/lancelet/blast.php>, Compagen Genomics Platform <http://www.compagen.org/blast.html>, Florida University Neurobase <https://neurobase.rc.ufl.edu/>, UCSC genomic annotation <https://genome.ucsc.edu>, NIH <https://research.nhgri.nih.gov/mnemiopsis/blast/>.

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Author contributions

MP, KJ, MR, and MH performed the experiments. MP conceived the project. MP and AK wrote the manuscript. All authors have contributed critical intellectual content and have approved the final manuscript.

Conflict of interest

The authors declare no potential conflicts of interest.

Figure Legends

Figure 1. Activation domain prediction. The online 9aaTAD prediction service for activation domain was applied on Pou5F / Oct4, Sox2, Myc (prediction for trans-activation region 50-120 localized experimentally) and Klf4. Algorithm for the 9aaTAD amino acid pattern was applied in the search, and region clustering conformity was assessed by percentage.

Figure 2. Transactivation Assay. The regions with the identified 9aaTAD activation domains were tested in a reporter assay with hybrid LexA DNA binding domain for the capacity to activate transcription. The average value of the β -galactosidase activities from two independent transformants is presented as a percentage of the reference with standard deviation (means and plusmn; SD; n = 3). We standardized the results to positive control p53 construct 6p53, which was set to 100%. The 9aaTADs activation domains are colored for faster orientation. Deuterated 9aaTADs or their mismatch are in grey. Abbreviations: Homo sapiens (hsa), *Callorhinchus milii* (cmk, Ghost shark, gene ID: XP_007894073), *Callorhinus ursinus* (cur Pou5, Northern fur seal, gene ID: XP_025704146), *Sarcophilus harrisii* (shr Pou5, Tasmanian devil, gene ID: G3VJ82), *Lingula unguis* (lak Pou, brachiopod, gene ID: XP_013392366), *Mnemiopsis leidyi* (mle SoxB, comb jelly, gene ID: A0A059XHC3), *Amphimedon queenslandica* (aqu SoxB, gene ID: B1A9Y6), *Strongylocentrotus purpuratus* (spu SoxB, purple sea urchin, gene ID: Q9Y0D7 and spu SoxF, gene ID: W4YEI9), *Saccoglossus kowalevskii* (sko SoxB, hemichordate, common ancestor of chordata, gene ID: Q7YTD4 and sko SoxF, gene ID: B5THP2), *Monosiga brevicollis* (mbe Myc, filozoans, last unicellular ancestor of animals, gene ID: A9V5B4), *Amphimedon queenslandica* (aqu Myc, poriferans, gene ID: XP003390966), *Latimeria chalumnae* (lch c-Myc, lch N-Myc, and lch L-Myc, lobe-finned fish, related to lungfishes and tetrapods, gene IDs: XP005992710, XP005993959, XP006009573).

Figure 3. Alignment of SOX family. The C-terminal regions of SOX proteins were aligned by sequence similarities to Sox2 and predicted 9aaTAD activation domain (9aaTAD prediction service online, www.Piskacek.org). The Sox orthologs from early diverged eukaryotes *Mnemiopsis leidyi* (mle SoxB, gene ID: A0A059XHC3), *Amphimedon queenslandica* (aqu SoxB, gene ID: B1A9Y6), *Strongylocentrotus purpuratus* (spu SoxB, gene ID: Q9Y0D7 and spu SoxF, gene ID: W4YEI9) and *Saccoglossus kowalevskii* (sko SoxB, gene ID: Q7YTD4 and sko SoxF, gene ID: B5THP2) have strong conservation of 9aaTAD. Diversification of the

9aaTAD motif, especially in position p5 (in red) could be seen in human SoxF but not in spu SoxF and sko SoxF. The members of SoxE clade contain two activation domains; the 9aaTADs-I have more conserved sequence but the 9aaTADs-II have more conserved position in the protein and are associated with the SOX C-terminal domains, similarly to the 9aaTADs in all other SOX clades. The 9aaTADs activation domains are colored for faster orientation. Dots in sequences represent stop codon.

Figure 4. Alignment of the MYC family. The N-terminal regions of MYC proteins were aligned by sequence similarities and their predicted 9aaTAD activation domains are shown. The 9aaTADs activation domains are colored for faster orientation. A) Contrary to coelacanth (*Latimeria chalumnae*, fin-to-limb transition, related to lungfishes and tetrapods), the L-Myc from humans, reptiles and higher animals have no 9aaTAD motif and the corresponding region is deleted. B) The MYC clades with conservation MYB box O, I and II are shown. The deletion of activation domain in L clade are in grey box. Conservation of threonine in position 55 is highlighted in red. Pseudo repeat of the 9aaTAD motif with similarity to Sp2 9aaTAD is shown.

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Online prediction for activation domains

The **9aaTAD prediction** result for the **Pou5F / Oct4** activator
less stringent pattern resulted in a single perfect match

online 9aaTAD prediction service
www.med.muni.cz/9aaTAD

[illegible]

The **9aaTAD prediction** result for the **Sox2** activator
less stringent pattern, cut off 80% match

Sequence	Start	End	RC1	RC2	RC3	RC4	RC5	RC6	RC7	RC8	RC9	RC10	RC11	RC12	= Rules for amino acid clustering
MNAFMVWSR	45	53	+	+	-	+	+	-	+	+	+	+	+	+	83% match
DMISMYLPG	272	280	+	+	+	+	-	-	+	+	+	+	+	+	83% match

The **9aaTAD prediction** result for the **c-Myc** trans-activating region 1-120 less stringent pattern, cut off 80% match

Sequence	Start	End	RC1	RC2	RC3	RC4	RC5	RC6	RC7	RC8	RC9	RC10	RC11	RC12	= Rules for amino acid clustering
MPLNVSF FTN	1	9	+	-	+	+	+	+	+	+	+	+	+	-	83% match
EMVTE LLGG	100	108	+	+	+	+	+	-	+	+	+	+	+	+	92% match

The **9aaTAD prediction** result for the **KLF4** activator most stringent pattern resulted in a single perfect match

[illegible]

Transactivation assay		peptides used in assay		ID	Position	Reporter activation	
Pou1 clade	hsa Pit1	MSCQ	9aaTAD AF TS A DT Fi	PLN	po1	1-16	30 ±04
	cmk Pit1	MHL	vS Ve E LR At	VK	po2	1-14	1 ±01
Pou5 clade	hsa Pou5f1/Oct4	MAG	HL AS D FA Fs		po6	1-12	21 ±04
	cur Pou5	MAG	HL Ss D LA Fs		po8	1-12	12 ±02
	shr Pou5	MAG	HL Ap E YF Sp		po7	1-12	9 ±01
Pou predecessor	lak Pou	D	HL Sd M KY Ms	AHN	po11	37-49	0 ±01
SoxB clade	hsa Sox1-3	DLR	DM Is M YL PP		hS3	396-407	217 ±10
SoxC clade	hsa Sox4		SA LD R DL DF	NFE	hS4	426-437	114 ±11
SoxE/F clade (TADI)	hsa Sox17	VDR	TE FE Q YL HF		hS17	358-371	111 ±02
SoxE clade (TADII)	hsa Sox9		TG Ly S TF TY	MNP	hS9	461-471	46 ±04
	hsa Sox8		PG Ly Q YP CF	HSP	hS8	401-411	83 ±06
c-Myc clade	hsa c-Myc	QL	EM VT E LL GG		hsa-C	98-108	62 ±09
	lch c-Myc	QL	EM Vs E FL GD		lch-C	79-89	60 ±08
N-Myc clade	hsa N-Myc		SW VT E ML LE		hsa-N	76-84	128 ±14
	lch N-Myc	L	DW AS E LL LL	P	lch-N	94-104	125 ±20
L-Myc clade	lch L-Myc	KL	EW Vs E FL GA		lch-L	59-69	174 ±44
Myc predecessors	aqu Myc	D	SL Is N IM EY	E	aqu	99-109	12 ± 1
	mbe Myc	LN	SV MD E IL GV	S	mbr	74-85	10 ± 1
Control constructs	p53 - 9aaTAD	LSP	DD IE Q WF TE	DP	6p53		set to 100 ±11
	empty vector	TM	DD		Hdd		0 ±01

SOX clade B, F, C

Activation domain
9aaTAD - I

SOX clade E

Activation domain
9aaTAD - I

Activation domain
9aaTAD - II

SoxB

hsa SOX1

hsa SOX2

hsa SOX3

hle SoxB

aqu SoxB

spu SoxB

sko SoxB

SoxF

spu SoxF

sko SoxF

hsa Sox7

hsa Sox17

hsa Sox18

SoxC

hsa Sox4

hsa Sox11

SoxE

hsa Sox9

hsa Sox8

hsa Sox10

GD LR

EM IS M YL PA

GEGGDP AAAAAAAQSS

RLHS L PQ HY QGA

GAGVN GTV PL THI.

GD LR

DM IS M YL PG

AEVP EPA

APS

RLHM SQ HY QSGPVP

GTAIN GTL PL SHM.

GD LR

DM IS M YL PP

GG DAADAASPLPGG

RLHG V HQ HY QGA

GTAVN GTV PL THI.

P RDLT

DM IN M YL TA

QDP T

HY GMMHPDSTLMN

GGAMQ PL THL.

GAESDLR

NM IS T YL EE

SNSXP GPTETPPPSGSSRPPTAEFKLLNASAQCTDFIASNSSNXTNSAESLLDGAG GTL PL QHL M.

GD SSLR

DM IN M YL PG

DANDP NVQRQHAMQQAAAAAYHGTSVTSTS

GVGVN GTV PL THM.

GD LR

EM IS M YL PG

DANDP NA RQHSNAMVAHQEQYSQNPS

GVGVN G V SHY DHELK.

LD FAR

ED FD M YL PQ

ETNQF CDSNGSFLAALTD

ASNMSNAQS CLY.

GD VDR

DE FD M YL NT

QQF NFESTLI SVLADA

SAS MYM MN.

GD MDR

NE FD Q YL NT

PGHP DSATGAMAL

SG HV PVSQVTPTGPTETSL ISVLADATA TYY NSYSVS.

GE VDR

TE FE Q YL HF

VCKP EMGLPY

QG HD SGVNLPSHGA ISSVSDASSA VYY CNYPDV.

AD VDL

TE FD Q YL NC

SRTRP DAPGL

PY HV ALAKLGPRAMSCPEESSLISALSDASSA VYY SACISG.

GS FSSS

SA LD R DL DF

NFEP GS

GS HF EFP DYCT PEV SEMI S GDW LESSISNLVFTY.

GN LSL

SL VD K DL DS

FSE GSL GS HF EFP DYCT PEL SEMI A GDW LEANFSDLVFTY.

ETFDV

NE FD Q YL PP

NGHP GVPA...GQ

G

TG LY S TF TY

MNP AQREPM YTPADTS

GVPSIP QT HS

P QHW EQPVYTQLTRP.

DAFDV

HE FD Q YL PL

GGP APPE...GY

A

PG LY Q YP CF

HSP RRPY ASPLLN

GLALP PA HS

PT SHW DQPVYTTLTRP.

ETFDV

AE LD Q YL PP

NGHP GHVS...GQ

A

SG LY S AF SY

MGP SQRPL YTAISD

PSPSGP QS HS

P THW EQPVYTTLSRP.

MYC family			Activation domain 9aaTAD										MYC box II MBII		
Human															
L-Myc	(with 9aaTAD deletion)	GIG PPE	PW	P			GG			CT	GDEAESR		GHSKGWGRNY	ASIIIRRDCMWSG	
N-Myc		HSS EPP	SW	VT	E	ML	LE		NELW	G	SP	AEDDAF	GLGGLGGLTP	NPVILQDCMWSG	
C-Myc		STA TQL	EM	VT	E	LL	GG		DMVN	QSFI	CD	PDETFI		KNIIIQDCMWSG	
Latimeria chalumnae															
L-Myc		SSG DKL	EW	VS	E	FL	GA		DEE	QFKI	NP		GEIWGNL	SSIIIHDCMWSG	
N-Myc		WSG DPL	DW	AS	E	LL	LL		PE	GDLW	SG	CE	GEEDQF	ELGSGSRLEQGNL	NAIILQDCMWSG
C-Myc		STA DQL	SV	MD	E	IL	GV		SPEE				KTKP	SSVTLHDDGFGSG	

Human and viral Myc group

hsa L-Myc	inactive myc paralog
hsa N-Myc	active myc paralog
hsa C-Myc	active c-myc
Myc T17 Q67004	inactive viral v-myc
MYC_AVIM2 P10395*	active viral v-myc
Myc deletion 106-143	active mutant c-myc

C-Myc group

hsa-C	
1ch-C XP005592710	lobe-finned bony fish Latimeria chalumnae
cmi-C XP007901659	jawed cartilaginous fish Callorhynchus milii
rti-C XP020371961	jawed cartilaginous fish Rhinodon typus
bbe-C BAD93381	Cephalochordata Branchiostoma belcheri
bfl-C C3Y235	Cephalochordata Branchiostoma floridae
aca-C XP003219450	Reptilia Anolis carolinensis
pbi-C XP007438325	Reptilia Python bivittatus

L-Myc group

hsa-L	inactive myc paralog
1ch-N XP005593959	lobe-finned bony fish Latimeria chalumnae
rti-L XP020379445	jawed cartilaginous fish Rhinodon typus
xtr-L NP001011144	Reptilia Xenopus tropicalis
cmi-L M7B8F5	Reptilia Chelonia mydas
psi-L XP006139201	Reptilia Pelodiscus sinensis
asi-L A0A1U7RG17	Reptilia Alligator sinensis
gja-L XP015273252	Reptilia Gekko japonicus
afo-L XP009274523	Aves Aptenodytes forsteri
gga-L F1NXY9	Aves Gallus gallus
sha-L G3X282	Marsupialia Sarcophilus harrisii

N-Myc group

hsa-N	
1ch-N XP006009573	lobe-finned bony fish Latimeria chalumnae
rti-N XP020371801	jawed cartilaginous fish Rhinodon typus
psi-N K7G066	Reptilia Pelodiscus sinensis
gga-N E1C3E1	Reptilia Gekko japonicus

Distal Myc orthologs in early branched metazoans

hsa-C	
mbr A9V5B4	unicelar Choanoflagellate Monosiga brevicollis
aqu XP003390966	Porifera Amphimedon queenslandica
tad B3S238	Placozoa Trichoplax adhaerens
nve A7RIE4	Cnidaria Nematostella vectensis
hec AEG66931	Cnidaria Hydraactinia echinata
hvu D2KB98	Cnidaria Hydra attenuata
lak A0A1S3JL92	Protostomia brachiopoda Lingula unguis
cgi XP011441199	Protostomia mollusca Crassostrea gigas
pfu AGS18763	Protostomia mollusca Pinctada fucata
mye XP021367795	Protostomia mollusca Mizuhopecten y.
cte ELT88315	Protostomia annelida Capitella teleta
pdu AGS55451	Protostomia annelida Platynereis dumerilii

MYC box MB0				MYC box MB0				MYC box MBI + T58				Linker		9aaTAD		+ repeat		MYC box MBII				
														Deletion								
M	DYD	SYQHY	FY	DYD	CGED	FY		R	STAPSEDI	WKKFELVPSPTSPPW		GLGPGAGDPAPGIGP	PE	FW		P	GG	C	TGDEAESR	GHSKGWGRN	YASIIRRD	CMWSG
MPSCSTSTMPGMICKNP	DL	EFD	SLQPC	FY	PDEDDFYFGGPD				STPPGEDI	WKKFELLPTPLSPSR			GFAEHSSE	PP	SWVT EMLL		E	NELWG	SPAEDAFGLGLGLT	PNPVILQDCMWSG		
MPLN	VSF	TNRNY	DL	DYD	SVQPY	FY	CDEEENFYQQQQQ	SELQ	PPAPSEDI	WKKFELLPTPLSPSR	SGLCSPSYVAVTFPSLRGNDGGGGS	FSTAD	QL	EMVT ELLG		G	DMVN	QSFIC	DPDDET		IKNIIQDCMWSG	
MPLN	VSF	ANRNY	DL	DYD	SVQPY	FY	CDEEENFYQQQQQ	SELQ	PPAPSED												AN	HKNIIQDCMWSG
MPLT	ASL	PSKNY	DY	DYD	SVQPY	FY	FEDEEENFYLAQQRGSELQ		PPAPSEDI	WKKFELLPTPLSPSR		SSLAASAC	FPSTAD	QL	EMVT ELLG		G	DMVN	QSFIC	DPDDES	F	VKSIIQDCMWSG
MPLN	VSF	TNRNY	DL	DYD	SVQPY	FY	CDEEENFYQQQQQ	SELQ	PPAPSEDI	WKKFELLPTPLSPSR	SGLCSPSYVAVTFPSLRGNDGGGGS	FSTAD	QL	EMVT ELLV	S	EKLASYQAARKDSGSPN...						
MPLN	VSF	TNRNY	DL	DYD	SVQPY	FY	CD EENFYQQQQQSELQ		PPAPSEDI	WKKFELLPTPLSPSR	SGLCSPSYVAVTFPSLRGNDGGGGS	FSTA D	QL	EMVT ELLG		G	DMVN	QSFIC	DPDDET		IKNIIQDCMWSG	
MPLS	SSF	PSKNY	DY	DYD	SVQPY	FY	CDYEDEHFYHHQQNLQ		PSAPSEDI	WKKFELLPTPLSPSRSS		FSSIYPSTAD	QL	EMVS EFLG	D	DVVN	QTFIC	DPEDDS	F	LKSIIHDCMWSG		
MGRG...	NFLC	KNYNY	DY	DYD	SFQPV	FY	DEDENFYQQQL		PXAPSEDI	WKKFELLPTPLSPSRSPS		FSLFPSNAD	QL	EMVT EFLG	D	DLVN	QSFIC	DSDES	V	LKSIIQDCMWSG		
MPLTASATFLG	KSYNY	DY	DYD	SFQPV	FY		DEEENFYQQQL		PAPSEDI	WKKFELLPTPLSPSRSPFS		LFPSNAD	QL	EMVT EFLG	D	DLVN	QSFIC	DADSE	V	LKSIIQDCMWSG		
MPGINAHAVS	VPSK	HY	DYD	SLQPY	FY	EETDDPAREDDFYST	PSPSVPPSEDI	WKKFELLPTPPSPSHPAK				SFIPTVAE	KL	EMVS ELLD	E	DVVN	QSFIF	PLDTQS		LKSKLIQDCMWSG		
MPGINAHAVT	VPSK	HY	DYD	SLQPY	FY	EETDDPAREDDFYST	PSPSVPPSEDI	WKKFELLPTPPSPSHPAK				SFIPTVAE	KL	EMVS ELLD	E	DAVN	QSFIF	PLDTQS		LKSKLIQDCMWSG		
MPLT	GPSF	PSRTY	DY	DYD	SVQPY	FY	FEDEEENFYLSAHNRGCELQ	PPAPSEDI	WKKFELLPTPLSPSRGGC		YYPSSGAD	QL	EMVT ELLG	S	DAVN	QSFIC	DPDDAF		VKSIIQDCMWSG			
MPLT	VPSY	PSRAY	DY	DYD	SVQPY	FY	YEDEEENFYLSAQRGCELQ	PPAPSEDI	WKKFELLPTPLSPSRRS	SGS		FPSSVD	QL	EMVT ELLG	S	EVVN	QSFIC	DPDDAF		VKSIIQDCMWSG		
														Deletion								
M	DYD	SYQHY	FY	DYD	CGED	FY	RST		APSEDI	WKKFELVPSPTSPPW		GLGPGAGDPAPGIGP	PE	FW		PGGCTG	DEAESRGH	SKGW	GRN	YASIIRRD	CMWSG	
M	EYD	TSQHY	FY	DDD	NEED	FY	RSI		APSEDI	WKKFELVPTPSPAPWITGDK		NFYPPSGD	KL	EMVS EFLG	A	DEEQFKINP	GEIW		GN	LSSIIHDCMWSG		
MYEIATKANPAAHVTGGILMQILTSEGGFATNNS...								LLEEDFYQTCEE	LKTVEMLPASQSPLEPKSN		ICDSLSSLSPKSD	QL	ELMS EFLD			DDEEFISQS	LI	CDLE	ASLKMEQDCMWSN			
M	DFGSCNNHY	FY	DVD	MKED	FY	RCI		APSEDI	WKKFELVPGFPLSPGGCP		GGG GT	DWGA ELMQ			L GWESPMKLT	GL			SSVLLRDCMWSG			
M	EFD	SYQHY	FY	DHD	SEED	FY	RST		APSEDI	WKKFELVPTPPTSPCLSAAGK		ACCPGAAE	RS	DWLS		HCCLA	GEEPEYLIGT	GEIF		GN	LSAFVLQDCMWSG	
M	EFD	SHQHY	FY	DHD	SEED	FY	RST		APSEDI	WKKFELVATPRKAPCPAGGK		ACCPGAGE	RS	DRLS		HCCLP	GEEPEYLLGP	GAIL		GN	LSAFMLQDCMWSG	
M	ELD	SYQHY	FY	DYD	SEED	FY	RST		APSEDI	WKKFELVPTPPTSPACGPADK		ACCSGAAGE	LG	DWL		PSCCLA	GEEP	GEIF		AN	LSAFILQDCMWSG	
M	ELD	PPHQPYFF	EAD	PQAED	FH	PSS		APSEDI	WKKFELVPTPLLGAGPADK		A	D			YLL EP	GGLL			GN	LSAFVLQDCMWSG		
M	EFD	SYQHY	FY	DHD	PQAED	FH	RST		APSEDI	WKKFELVPTPLSPGLTPEK		ACCSGAED	RS	GWLS		RYCLA	GEEPEYLIGT	GEIF		GN	LSAFILQDCMWSG	
M	ERD	AYQHY	LY	DYD	AGED	FH	RST		APSEDI	WKKFELVPTPLSPGLGERP		GCSAAEE	RG	CPP		RCCLP	DEPEYLIGT	QQLF		GN	LSAFILQDCMWSG	
M	EFD	SYQHY	FY	DCD	YEED	FY	RST		APSEDI	WKKFELVPSPPMSPPW		SGSGSPGACCSAANGPPE	PWPGG			CGV	DEAEGRGY	SKAL	ARN	YASIIIRSD	CMWSG	
MPSC...	TMFGM	ICKNP	DL	EFD	SLQPC	FY	PDEDDFYFGGPD		TPPGEDI	WKKFELLPTPLSPSR		GFAEHSSE	PP	SWVT EMLL	ENELWGS	PAEE	DAFGL	GGLGGLT	PNPVILQDCMWSG			
MPGI	ISKNS	DL	EFD	SLQPC	FY	PDEDDFYFCGPDS		APPGEDI	WKKFELLPTPLSPSPRA		GLQGDPAAGSPTESGGGALLGWYGRGGRWSGD	PL	DWAS ELLL	LPE	GDLSGCEG	EEDQFEL	GSGSRLEQGN	LNAIILQDCMWSG				
MPGV...	V	GS	SKCDY	DL	EYD	SYQPY	FY	PDDYGPEADF	YPPSEDI	WKKFELLPTPLSPSPRA		XEMPS	PX	CWVS EMLL	LEEDGDGEEP...		GAGI	RSQSGVSRSQ	ANPVLQDCMWSG			
MPGM	VSKNP	DL	EFD	SLQPC	FY	PDEDDFYLCGPDS		APPGEDI	WKKFELLPTPLSPSPRA		GLQENPPGGA	PL	PWGGVALGGFTRD	PL	DWAS ELLL	LPPEADLWGSTDGG	DLFET	GFEERGN	LNSIIQDCMWSG			
MPGM	ICKNP	DL	EFD	SLQPC	FY	PDEDDFYLCGPDS		APPGEDI	WKKFELLPTPLSPSPRA		GLQEHPPGGA	PL	PWGGALGSCRPAD	PL	DWAS ELLL	LPPEAELWGSTDGA	DFET	GLGASNN	LNSIIQDCMWSG			
MPLN	VSF	TNRNY	DL	DYD	SVQPY	FY	CD EENFYQQQQQSELQ		PPAPSEDI	WKKFELLPTPLSPSR	SGLCSPSYVAVTFPSLRGNDGGGGS	FSTA D	QL	EMVT ELLG		GDMVN	QSFIC	DPDDET		IKNIIQDCMWSG		
M	SSF	CAMS	SLD	HLS	PGMKN	SF	E		SLSPGAQH	FLRSLETTTNSHTDRDLR	PSPVDEDDDFEFGARISASLRE	LN	SVMD EILG		VS	PEE		KTG	PSSVTLHDDGFSG			
MASL	VES	GAE	LLP	HLQ	EERQM	YM	AEFTAISFAEQFE	SPLYDLHEET	FRRLYLRSPPSPDPDCEPSCSQSPVEETPTNSQFNQEQARVQDNI	STDM AD	SLIS NIME	YEQIMDALQ	EAEF		ALSSGSSQ	PAEDLIQDCMWSA						
MA	VHA	EAFSN	KL	DYD	PYGSY	YF	GED	SEDDNI	WSCLDLPTPLSPSPARQ	QYITDTSNYLA	DKLLQT EN	LDPD NALI	DMVGDT	NSIF	NGGSK	LRSLLIQDCMWSA						
MTL	VAE	HLLMDTFGS	DFD	SLPPS	LF	KDF		PEDGFN	MKKSMTSEEDMSDYSFPPTTPI	SPGCSIAS	EIGDPE RI	QPVC DELE	DDFNFAA	EKSLYF		QEND	FKDILIKDCMWSG					
MI	ITS	ANRDLSPWS	NID	ESDNEEIM	NLAKPMMDA			SNPSDDI	WKKFALPTPLSPSPSR		TYGETSDEDRTDIAE RL	HDVC ESLD	STFDLT	SYNVS	KLAVLN	LRSKLISDCMWSG						
MT		GS	NWC	THD	ILPTDEIL	LHKSILD		TSSPSDDV	WKKF LTPSSPSQSGESS		ESEETDSFDTCAL RL	QYVC DNLD	IGLELT		TRCTNSFN	LRSKLISDCMWSG						
MPR	VQMKKMAHSLVKD	DYE	TFQPI	YF	QDNDEHLLHA			GPAPSEDI	WKKFELLPTPLSPSPRR		DREPPNITIPSDINTTE CL	YKVS ELLD	DDFL	TQTQILF	PGST	EQGSESGHCQCPPP						
MLIGRKNE	KTA	IM	DYD	MFQPC	FY	ENEPETI		SPSSLPNEDM	WKKFELLPTPLSPSR		DDDYDM	NS	LDLE DFNL	IFDEFFE	KESAI	PKIMTLSETFPN	LNSNLIQDCMWSG					
			M	DYD	TFQPC	FY	ENETEL	SPVGSYPNEDM	WKKFELLPTPLSPSR		DDQFD	ST	LDLE NLTL	PLTDYIL	NDDDDASLF	EKMTFFESPSPS	LHSLKLIQDCMWSG					
MV	VRT	AKAHSGH	ENE	MHRQ	T	CDN		GTTLCMNI	WKKFGLGPTPLSPSPPH		ETMS	DF	FLD	GFNL	SDNDVEFF	DSREEDV	LATMKHIFAEPTPLN	LQSKLIQDCMWSG				
MNR...	SMTKHSLSQNTPLM	EFD	EVREGASAW		SLSEVEFYGDM			SSPNMNI	WKKFEMQPTPTSPASPER		DSSTDGDDFS	SQ	SMWD SLLD	QNLNF	AHNEP	LNTKLIQDCMWSA						
MDEK	MCS	LHQR	PVSE	DYD	SFNPY	FF	EVGTEDEFYDV	PAPSEDI	WKKFELLPTPLSPSPH		ETSYPSSTASTGLMNSTLE	KL	QWVS DILE	PVSLTSDFSMF	PEDCALCSRDITLFS...	ELNQS	LKSNLIQDCMWSG					

Graphical abstract