How to overcome the ATRA resistance with the 9aaTAD activation domains in retinoic acid receptors

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Abstract (200 words)

In higher metazoa, the nuclear hormone receptors activate transcription through their specific adaptors, nuclear hormone receptor cofactors NCoA, which are surprisingly absent in lower metazoa. In this study, we demonstrated that the 9aaTAD from NHR-49 receptor activates transcription as a small peptide. We showed, that the 9aaTAD domains are conserved in the human nuclear hormone receptors including HNF4, RARa, VDR and PPAR γ . The small 9aaTAD peptides derived from these nuclear hormone receptors also effectively activated transcription and that in absence of the NCoA adaptors. We identified adjacent inhibitory domains in the human HNF4 and RARa, which hindered their activation function.

In acute promyelocytic leukaemia (PML-RARa), the receptor mutations often caused all-trans retinoic acid (ATRA) resistance. The fact that almost the entire receptor is needed for ATRA mediated receptor activation, this activation pathway is highly susceptible for loss of function when mutated. Nevertheless in the most of the reported mutants, the activation domains 9aaTAD are still intact. The release of activation 9aaTAD from its dormancy by a new drug might be the sound strategy in combat the ATRA resistance in PML leukaemia.

Introduction

The 9aaTAD activation domains are universal activators of transcription in numerous transcription factors, which are recognized throughout the eukaryotes¹⁻³. The 9aaTAD domains are recognized by multiple mediators of transcription including TAF9, MED15, CBP and p300⁴⁻⁹. We have also demonstrated that the activation domains 9aaTAD from p53, MLL, E2A, NFkB, Sp1, CTF and SOX transcription factors activated transcription as small peptides^{1,2,10,11}.

The direct interaction of transcription factors and general mediators of transcription is the canonical activation of transcription, which was lost in nuclear hormone receptors in higher eukaryotes and have been replaced by specific hormone adaptors, NCoAs (SRC-1/NCoA-1, TIF-2/NCoA-2 or RAC-3/NCoA-3)¹²⁻¹⁴.

The nematode *C.elegans* has a complex physiology including neuronal network, reproduction organs with ovary in female and testis in male¹⁵. Most of nuclear hormone receptors in *C.elegans* have a common HNF4 ancestor and share high degree of homology¹⁶. The nuclear hormone receptor NHR-49 is the key regulator of lipid metabolism, dietary response and life span in *C.elegans*¹⁷⁻¹⁹. Both NHR-49 and NHR-64 nuclear hormone receptors are recognized directly by the transcriptional mediator MED15 (MDT-15)¹⁷.

Recently from the Taubert lab²⁰, the 9aaTAD domain was identified in the nuclear hormone receptor NHR-49 by our 9aaTAD prediction algorithm (www.piskacek.org). The gain-of-function mutation NHR with V411E substitution was shown to cause significant reduction in MED15 binding²⁰.

In this study, we investigated the 9aaTAD function in *C.elegans* nuclear hormone receptor NHR-49 in parallel to its human homolog Hepatocyte Nuclear Factor 4 (HNF4) and paralogs including Retinoic acid receptor alpha (RARa), Vitamin D receptor (VDR) and Peroxisome proliferator-activated receptor gamma (PPARg).

Results

Activation and Inhibitory domains in the nuclear hormone receptor NHR-49

The nuclear hormone receptor NHR-49 in *C.elegans* interacts with transcriptional mediator MED15 (MDT-15) ¹⁷. The mutation V411E in NHR-49 caused significantly reduced MED15 binding.

We generated hybrid constructs with LexA (prokaryotic DNA binding domain) and activation domain of the NHR-49 (**Figure 1A**). The construct NHR-49 (H11+H12+F, 391-438 aa) included the activation domain 9aaTAD (located in the H12 domain) and adjacent domains H11 and F. The construct (H11+H12, 391-424 aa) included the 9aaTAD activation domain and the adjacent domain H11.

The construct NHR-49 (H12, 409-424 aa) included only the activation domain 9aaTAD. The constructs NHR-49 (H11+H12+F, 391-438 aa, V411E) and NHR-49 (H11+H12, 391-424 aa, V411E) included the mutation V411E.

The reporter activation was monitored by the β -galactosidase assay (LexA dependent reporter for transcription activation assay). We standardized all results in **Figure 1** to the construct NHR-49 (H11+H12+F, 391-438 aa, V411E) and set the activity to 100%. The expression of the proteins were confirmed for each constructs by western blotting (**Figure S1**). The constructs with the mutation V411E showed significantly higher reporter activation than the corresponding wild type construct. Therefore, we concluded that the mutation V411E interfered with inhibitory domain activity.

The construct NHR-49 (H12) including only the 9aaTAD domain showed the most activity in contrast to construct NHR-49 (H11), which included both the activation domain 9aaTAD and the adjacent domain H11. These results suggested the presence of an inhibitory domain within the H11 region.

Activation and Inhibitory domains in human nuclear hormone receptor NHR4

Following the observations in nuclear hormone receptor NHR-49 in *C.elegans*, we next investigated the human HNF4 receptor activation domain and its adjacent domain H11. Several HNF4 constructs were reported previously²¹ (**Figure S2**), some that included the 9aaTAD domain and activated transcription and other constructs that have not.

Similarly as above, we predicted the presence of inhibitory domain, which could inhibit the 9aaTAD activation domain in HNF4. Accordingly, we generated LexA constructs with and without the predicted inhibitory H11 domain. The construct HNF4 (H11+H12, 346-379 aa) included the activation domain and the adjacent H11 domain. The construct HNF4 (H12, 365-379aa) included only the activation domain 9aaTAD located in H12 region (**Figure 1B**).

We then compared the construct HNF4 (H12) to a very strong activator Gal4 and found that the activity of the construct HNF4 (H12) reached about 28% of the Gal4 activity (**Figure 2**). The activation domain I and II of the human and rat p53, which all share sequence similarity with hormone receptors, was added to experiment. We concluded that the 9aaTAD activation domain is a powerful activator in HNF4. The HNF4 inhibitory domain inhibited about 10-fold the 9aaTAD function (**Figure 2**). Our observations and conclusions are in a good agreement with previously reported HNF4 constructs²¹ (**Supplementary Figure S2**).

Activation domains in the human nuclear hormone receptors

Next, we generated sequence alignments (UniProt DB) of selected nuclear hormone receptors including RARa, PPARg and VDR and focused on C-terminal ends with the H10, H11 and H12 domains (described previously for HNF4 and RARa^{22,23})(**Figure 3**).

We have predicted putative 9aaTAD activation domains in several nuclear hormone receptors including RARa, PPARg and VDR. We then generated LexA constructs with the predicted 9aaTAD activation domains and tested their ability to activate transcription. We then confirmed that all 9aaTADs in RARa, PPARg and VDR hormone receptors are strong activators of transcription as small peptides (**Figure 2**) and that without their hormone adaptors NCoAs.

Activation and Inhibitory domains in the nuclear hormone receptor RARa

In parallel to NHR-49, we also identified inhibitory domains in RARa, which could cause the inhibition of the predicted activation domain 9aaTAD. Accordingly, we generated LexA constructs with and without the H11 predicted inhibitory domain. The construct RARa (H12) included only the activation domain 9aaTAD located in H12 region. The construct RARa (H11+12) included the H12 region with activation domain 9aaTAD plus the adjacent H11 domain (**Figure 4**). As expected, only the construct RARa (H12) without the inhibitory domain H11 powerfully activated transcription.

Discussion

The nuclear hormone receptors in vertebrates are evolutionary distinguished group of unconventional transcription factors using specific adaptors NCoAs, including SRC-1/NCoA-1, TIF-2/NCoA-2 or RAC-3/NCoA-3, to activate transcription^{22,24,2512–14}.

The NCoA adaptors are essential for nuclear hormone receptor activation in higher invertebrates and all vertebrates, but are completely absent in *C.elegans* genome and all other early branched invertebrata including nematoda, annelida, cnidaria, placozoa, porifera and ctenophora (database Kyoto Encyclopedia of Genes and Genomes, KEGG, Mnemiopsis Genome Project Portal at NHGRI/NIH, genome assembly Ensembl EMBL-EBI)(graphical abstract)²⁶. The absence of the NCoA adaptors in *C.elegans* therefore requires direct interaction of the nuclear hormone receptors with the general mediators of transcription.

In this study, we demonstrated that small 9aaTAD peptides derived from nuclear hormone receptors effectively activate transcription in absence of the NCoA adaptors. We showed that adjacent domains in these hormone receptors have inhibitory effect on their 9aaTAD activation domains.

In the higher eukaryotes, the interface of the nuclear hormone receptor HNF4 with the NCoA adaptors involve multiple regions H3, H4 and H12 domains²². The conserved residua Lys194 (from helix H3) and Glu363 (from helix H12) (**Figure 5 and 6**) together with the hydrophobic cleft created by hydrophobic residua Val190 and Phe199 (from H3 and H4 domains) facilitated NCoA1 adaptor binding.

In the *C.elegans* NHR-49, where NCoA adaptors are absent, these residua are also conserved (Lys194/240, Glu363/417, Val190/236 and Phe199/245) and might be an evolution prone for the modern nuclear hormone NCoA adaptors, which merged already in mollusca (graphical abstract).

Previously, the bivalent NHR4 character with AF1 and AF2 activation domains were reported²². Strikingly, only a single residue (Glutamic acid Glu363) from the AF2 domain was involved in the interaction with NCoA. The other residua of the HNF4 involved in interaction with NCoA are located far away in the HNF4 protein from the activation domains AF1 and AF2 (Lys194, Val190 and Phe199)(**Figure 5**). The Glu363 residue is in the position p5 of the activation domains 9aaTAD, which generally face to solvent and did not contribute to binding with mediators as are CBP, p300, MED15²⁷. For the activation domain 9aaTAD function, only the region of the H12 domain is needed (this study). Differently, almost the entire receptor structural integrity is needed for the NCoA mediated activation of transcription.

Following the above, the modern hormone nuclear receptors posses two activation pathways, which one is primordial (canonical, found already in *C.elegans* NHR-49, independent on NCoAs and almost dormant in modern receptors) and another is gained (dependent on NCoAs and required structural integrity of the whole ligand binding domain of the receptor).

The position, structure and motif of the 9aaTAD domain in the nuclear hormone receptor HNF4 matches to human RARa^{22,23} (**Figure 5**). The H12 helix is in well agreement with other helical activation domains 9aaTAD^{27–29}. The RARa interacts directly with the general mediator ADA3 of transcription also without NCoAs involvement, suggesting an early ancestry^{23,30}. The interaction was restricted by mutants analysis to the H12 region and therefore identical to the 9aaTAD activation domain.

Another interaction, which is outside of the major NCoA scheme for vertebrates nuclear hormone receptors, was reported for human nuclear hormone receptor PPAR γ . In a ligand-dependent manner, the PPAR γ interacted directly with mediators p300 and CBP and that without attendance of the NCoA adaptors^{31–34}. The last six amino acids of the PPAR γ , which are part of the activation domain 9aaTAD, were essential for the interaction. The interaction of CBP or p300 was reported also for other nuclear hormone receptors including PPAR α , RAR α , RXR, TR and ER^{34–38}. These observations did not oppose the function of molecular adaptors, rather show an alternative for the transcriptional activation in modern nuclear hormone receptors.

The conventional activation of transcription facilitated by direct interaction of activation domains with the general mediators of transcription were found in the most of transcription factors inclusive ancient nuclear hormone receptors. The modern nuclear hormone adaptors NCoA are evolutionary gained and broad out the diversification of the nuclear hormone network. The activation of transcription found in ancient and modern nuclear hormone receptors showed intriguing origin, which differentiated during evolution from simple to complex.

The conservation of the ancestral 9aaTAD activation domains in hormone receptors offered new strategy against cancer. The PML-RAR α fusion protein cause about 95% acute promyelocytic leukaemia (APL) and could be well treated in most cases by all-trans retinoic acid (ATRA). However, the ATRA resistance is observed already during initial treatments and is more pronounced during relapses^{39,40}. The mutations of the RAR α causing ATRA resistance spread over the ligand binding region in PML-RAR α fusions. Therefore, the nuclear hormone adaptors NCoAs could no longer bind to mutated PML-RAR α . In the most reported cases, the dormant 9aaTAD activation domain stayed intact (UniProt DB), but is still naturally inactivated by RAR α inhibitory domain H11. Thus, a small molecule, which would released the 9aaTAD from the inhibition might provide the cure.

Materials and Methods

Constructs

The construct pBTM116-HA was generated by insertion of the HA cassette into the EcoRI site of the vector pBTM116 (HA cassette nucleotide sequence: TGG CTG - GAATTA - GCC ACC ATG GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT GTC GAG ATA - GAATTC, which render in amino acids sequence: W L - E L - A T M A Y P Y D V P D Y A V E I - E F). The constructs were generated by PCR and sub-cloned into the multi-cloning site of pBTM116 vector by EcoRI and PstI sites. All constructs were sequenced by Eurofins Genomics. Further detailed information about constructs, primer sequences are available on the request.

Assessment of enzyme activities

The β -galactosidase activity was determined in the yeast strain L40 41,42. The strain L40 has integrated the lacZ reporter driven by the lexA operator. In all hybrid assays, we used 2 μ vector pBTM116 for generation of the LexA hybrids. The yeast strain L40, the *Saccharomyces cerevisiae* Genotype: MATa ade2 his3 leu2 trp1 LYS::lexA-HIS3 URA3::lexA-LacZ, is deposited at ATCC (#MYA-3332). For β -galactosidase assays on paper stick, the cells were dropped on Watman paper, lysed by two freeze/thaw cycle (-20°C), soaked in about 400 μ l of 100 mM phosphate buffer pH7 with 10 mM KCl, 1 mM MgSO₄ and 0.4% X-gal, incubated at 37°C (in a plastic well) until the positive control turned in blue and dried on tissue paper. For standard β -galactosidase assays, overnight cultures propagated in YPD medium (1% yeast extract, 2% bactopectone, 2% glucose) were diluted to an A600 of 0.3 and further cultivated for two hours and collected by centrifugation. The crude extracts were prepared by vortexing with glass beads for 3 minutes. The assay was done with 10 μ l crude extract in 1ml of 100 mM phosphate buffer pH7 with 10 mM KCl, 1 mM MgSO₄ and 0.2% 2-Mercaptoethanol; reaction was started by 200 μ l 0.4% ONPG and stopped by 500 μ l 1 M CaCO₃. The average value of the β -galactosidase activities from three independent experiments is presented as a percentage of the reference with the standard deviation (means and plusmn; SD; n = 3). We standardized all results to previously reported Gal4 construct HaY including Gal4 activation domain 9aaTAD with the activity set to 100% 1.

Western Blot Analysis

The crude cell extracts were prepared in a buffer containing 200 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10% glycerol (v/v), separated by SDS-PAGE, and blotted to nitrocellulose. The immuno-detection of proteins was carried out using mouse anti-HA antibody (#26183, ThermoFisher Sci) or mouse anti-LexA (#306-719, EMD Millipore Corp). The secondary antibodies used were anti-mouse IgG antibodies conjugated with horseradish peroxidase (#A9044, Sigma Aldrich). The proteins were visualized using Pierce ECL (#32106, ThermoFisher Sci) according to the manufacturer's instructions.

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Figure Legends

Figure 1

Predicted activation domains 9aaTAD in the *C.elegans* NHR-49 and human HNF4a nuclear hormone receptors are activators of transcription

The presence inhibitory domains and interference of NHR-49 V411E with the inhibition was monitored. The DNA binding domain of LexA were used with parts of NHR-49 for generation of hybrid constructs (BTM116 backbone, standard LexA hybrid assay with β -galactosidase reporter). The average values of the β -galactosidase activities from duplicates experiments are presented as a percentage of the reference with standard deviation (substrate ONPG, means and plusmn; SD; n = 3). We standardized all results to the construct NHR-49 F, including activation domain 9aaTAD and both adjacent domains H11 and F, which activity was set to 100%. The construct DD with deleted Gal4 activation domain served as negative control (sequence shown in Figure 3).

Figure 2

Predicted activation domains 9aaTAD in the human nuclear hormone receptor are universal activators of transcription

The DNA binding domain of LexA were used with putative activation domains for generation of hybrid constructs (BTM116 backbone, standard LexA hybrid assay with β -galactosidase reporter). The average values of the β -galactosidase activities from duplicates experiments are presented as a percentage of the reference with standard deviation (substrate ONPG, means and plusmn; SD; n = 3). The β -galactosidase was previewed by using 0,5 % X-gal on filter paper (no value are available) and show for demonstration. We standardized all results to the previously reported strong Gal4 activator of transcription, construct HaY including LexA and Gal4 activation domain 9aaTAD, which activity was set to 100%. The construct DD with deleted Gal4 activation domain served as negative control. The activity of the LexA-p53 constructs 1p53, 6p53 and 8p53 are shown as extended positive controls and comparable activation domain sequences.

Figure 3

Sequence alignment of the nuclear hormone receptor Family

The C-terminal sequences of selected nuclear hormone receptors from *C.elegans* and humans were aligned. Those activation domains were cloned and tested for activation of transcription (shown in Figure 3) are blue labelled. The structural domains H10, H11 and H12 are clustered. The amino acids in H11, which are like to provide interaction with H12 are bolded. The predicted activation domains 9aaTAD are coloured for fast orientation. The end of sequences are marked with single dots.

Figure 4

RARa activation and inhibitory domain - Screening strategy for small molecule.

The DNA binding domain of LexA were used with RARa activation domains with and without inhibitory domains for generation of hybrid constructs (BTM116 backbone, standard LexA hybrid assay with β -galactosidase reporter). The average values of the β -galactosidase activities from duplicates experiments are presented as a percentage of the reference with standard deviation (substrate ONPG, means and plusmn; SD; n = 3). The Screening strategy for small molecule are schematically shown.

Figure 5

Structures of the human nuclear hormone receptors HNF4a and RARa

The activation domains 9aaTAD are shown and selected residua are coloured. The residue I₃₅₇ in HNF4a, which corresponds to V₄₁₁ in NHR-49, is labelled purple. The HNF4a residue critical for interaction with NCoA E₃₆₃ and K₁₉₄ are labelled red and purple.

Figure 6

Evolution in the nuclear hormone receptor family

Schema of the conventional (canonical) activation of transcription in ancient nuclear hormone receptor NHR-49, which is strait forward and is provided by general mediator of transcription MED15, but highly complex and diversified in the modern hormone system with specific NCoA adaptors (in yellow). The activation domains 9aaTAD are coloured for fast orientation. For details see text.

V411E mediates release from H11 inhibition

Figure 6

Short peptides corresponding to activation domains 9aaTAD located in the helixes H12 (AF2) of the nuclear hormone receptors were tested for their competence to activate transcription in the yeast LexA hybrid assay

Construct	Region	ID	Remarks	Hybrid+Spacer	Proximal residues	9aaTAD		Reporter activation
LexA-HA-HNF4a (H12)	(365-378 aa)	HNF4 H12	Helix H12 (AF2)	LexA-HA tag	KID	NL LQ E ML LG	GSP	28% ±
LexA-HA-NHR-49 (H12)	(365-378 aa)	HNR49 H12	Helix H12 (AF2)	LexA-HA tag	AEVD	SL LS E FI LD	DIN	173% ±
LexA-HA-RARa (H12)	(406-418 aa)	RARa H12	Helix H12 (AF2)	LexA-HA tag	MP	PL IQ E ML EN	SE	34% ±
LexA-HA-PPARg (H12)	(493-505 aa)	PPARg H12	Helix H12 (AF2)	LexA-HA tag	LH	PL LQ E IY KD	LY	57% ±
LexA-HA-VDR (H12)	(414-427 aa)	VDR H12	Helix H12 (AF2)	LexA-HA tag	LT	PL VL E VF GN	EIS	25% ±
LexA-HA-p53 (TAD-I)	(14-27 aa)	1p53	positive control	LexA-HA tag	LSQ	ET FS D LW KL	LP	109% ±
LexA-HA-p53 (TAD-II)	(45-58 aa)	6p53	positive control	LexA-HA tag	LSP	DD IE Q WF TE	D P	108% ±
LexA-HA-p53 (RAT)	(46-59 aa)	8p53	positive control	LexA-HA tag	LF LP	QD VA E LL EG	D P	67% ±
LexA-HA-Gal4	(860-871 aa)	HaY	positive control (+)	LexA-HA tag	TM	DD VY N YL FD	D	set to 100% ±1
LexA-HA-Gal4 (del)	(860-863 aa)	HDD	negative control (-)	LexA-HA tag	TM	DD.		3% ±

Figure 6

Nuclear hormone receptors

C.elegans

NHR49

NHR69

NHR175

NHR1

NHR274

NHR3

NHR88

NHR64

Human

HNF4A

NR2E1

NR2E3

NR1H2

RARA

ESR2

PPAR γ

VDR

Domain H10	Domain H11	Domain H12 activation domain 9aaTAD	
YGN ILLLA PAL KALTQ	LL I EN M TLT K F FGLA	E V D SL L S E F IL D	DIND
SGK LLLLL PSL QAIAQ	QL V ED V QLA R L FGLV	N V D SL M E E L IL N	DMKP
YGQ LILLL GNI RCAVK	LV Y NQ T KVS D L FNAY	K F D LY V R S F ILT.	
YGS LLMMT ASI QNILA	QNE EN M QVM E L FKNW	E V D PF V K E L CM K	RA.
FGT IILLT SSI RCAIN	AL Y NQ T RVS D v FDL M	K F D PL V R D V LS.	
FAH LLLLI ASA TRVAY	SL S SF F QLS R D VNY	E I D YV L E E L L F L	DRI.
FGN LLLLF PPL AKLSS	Li G EN V QLA K M FGI	P I D SL L V E L Y V D	ADSP
FAN LLLLL PPM LAISR	DL V ED V QLA K L FGLA	S I D NL M L E L ML P	NEGK
FGE LLLLL PTL QSITW	QM I EQ I QFI K L FGMA	K I D NL L Q E M L L G	GSPS
FGK LLLLL PAL RSISP	ST I EE V FFK K T IG N V	P I T RL L S D M Y K S	SDI.
FGK LLLLL PSL RFITA	ER I EL L FFR K T IG N T	P M E KL L C D M F K N.	
FPR MLMKL VSL RTLSS	VH S EQ V FAL R L QDK	K L P PL L S E I W D V	HE.
FPK MLMKI TDL RSISA	KG A ER V ITL K M EIPG	S M P PL I Q E M L E N	SEGL
LAN LLMLL SHV RHASN	KG M EH L LNM K C KNVV	P V Y DL L L E M L N A	HVLR
FAK LLQKM TDL RQIVT	EH V QL L QVI K K T ETDM	S L H PL L Q E I Y K D	LY.
YAK MIQKL ADL RSLNE	EH S KQ Y RCL SFQ P EC S M	K L T PL V L E V F G N	EIS.

Construct	Region	ID	Hybrid+Spacer	Inhibitory Domain H11	9aaTAD	Reporter activation
LexA-HA- RARa (H11 + H12+F')	(383-424 aa)	Ra+Inh	LexA-HA tag	DLRSISAKGAERVITLKMEIPGS M_P	PLI_Q E ML_{EN} SEGLDTLS	2% ±
LexA-HA- RARa (H12)	(406-418 aa)	RARa H12	LexA-HA tag	M_P	PLI_Q E ML_{EN} SE	100% ±

Screening strategy for small molecule
 Implication: ATRA resistance in acute promyelocytic leukaemia with PML-RARa fusion proteins

Figure 6

Lower Invertebrates, up to Nematoda

Ctenophore

Sea Anemone

C. elegans

ancient nuclear hormone receptors

with activation domain **9aaTAD**

without **NCoA**

+ **MED15**

→ **Transcription**

Higher Invertebrates, from Mollusca

Octopus

Daphnia

Beatles

modern nuclear hormone receptors

with dormant activation domain **9aaTAD**

with **NCoA**

+ **CBP**

→ **Transcription**

Suppl. Figure S1

Suppl. Figure S1. Expression of the constructs. The protein level produced from the constructs were monitored by Western blotting. The proteins comprise of LexA DNA binding domain. The peptides shown were tested in the reporter assay with hybrid Lex DNA binding domain for the capacity to activate transcription.

Hadzopoulou-Cladaras et al.

This study

Suppl. Figure S2. Activation of transcription by HNF4 constructs. The schema of human HNF4 constructs from Hadzopoulou-Cladaras et al 1997 and our constructs from Figure 1 are aligned and their functional domains are graphically organised according structural domain H11 and H12, originally domain A to F and activation of transcription are shown in percent. Our HNF4 construct 9aaTAD+ID correspond to reported construct CD7 and therefore correspond to activity of 10%. The activation domains 9aaTAD are coloured for fast orientation. The proximal amino acid I357 corresponding to V411 in C.elegans NHR-49 is in purple. The arrows indicated two lost of functional domain between two constructs.