Rsp5 promotes Gene Activation mediated by **9aaTAD Transcription Factors** Oaf1 and Gal4

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Nine-amino-acid transactivation domain, 9aaTAD, defines a large superfamily of yeast and mammals transcription factors. The transactivation of the 9aaTAD has been addressed to multiple general co-activators TAF9, MED15, CBP and p300. We demonstrate for the 9aaTAD transcription factors Oaf1 and Gal4 functional and physical interaction with E3-Ubiquitin Ligase Rsp5. The Rsp5-associations with RNA polymerase II and TFIID were reported previously.

Nine-**a**mino-**a**cid **T**rans-**A**ctivation **D**omain, **9aaTAD**, defines a novel domain common to a large super-family of eukaryotic transcription factors represented in yeast by Gal4/Oaf1/Pip2/Leu3/Pdr1/Pdr3/Rtg3/Pho4/Gln3/Gcn4 and in mammals by p53/E2A/NFAT/NFkB/HSF1/NF-IL6/KLF2/MLL-ALL1/EBNA2/VP16. The prediction and online annotated results (including Pdr1 and Oaf1) are available on www.expasy.ch/tools.

Several transcription factors of the 9aaTAD superfamily have been found to interact with the general transcriptional factors of the initiation complex TFIID . The 9aaTAD of transcription factors p53, VP16, MLL, NF-κB, E2A, HSF1, NF-IL6, and NFAT1 interact directly with the general transcriptional factor TAF9, component of TFIID and SAGA complexes . Interactions KIX domain of cofactors MED15, CBP and p300 with a number of the 9aaTAD transcription factors were reported (p53, VP16, MLL and NF-κB*, for review see). KIX-domain of Gal11/MED15 was shown to interact with short transactivation domain of Pdr1 and Oaf1 (12 and 9 amino acids respectively, NMR data), which were annotated to the 9aaTAD family previously (online annotated results 2007 on www.expasy.ch/tools).

Ubiquitin ligase Rsp5 and the human homolog Nedd4 have mainly been assigned to protein degradation and more recently to other cellular events such as DNA repair or RNA transport . Interestingly, Rsp5 was originally isolated from a genetic screen for suppressors of Spt3 mutation, a component of the SAGA complex (Spt/Ada/Gcn5 acetyltransferase complex) suggesting a role for Rsp5 in initiation of transcription. The histone acetylase complex SAGA is involved in RNA polymerase II dependent transcription and functionally overlaps with the TFIID initiation complex . The Rsp5 associates with the TFIID complex and the C-terminal domain (CTD) of RNA polymerase II . In addition to physical interaction of Rsp5 with

transcriptional machinery, it was reported, that overdose of either Rsp5 or Spt3 could stimulate the transcription of progesterone and glucocorticoid nuclear receptors in mammalian cell lines . Very recent reports from Rotin and del Olmo labs place Rsp5 also into pathways responsible for the regulation of chromatin function and transcription (*"Ubiquitin ligase Rsp5p is involved in the gene expression changes during nutrient limitation - Rsp5p controls gene expression at the entry of stationary phase").*

We show in this study that Rsp5 interacts physically with 9aaTAD and that Rsp5 is essential for efficient transcription mediated by 9aaTAD transcription factors. The loss of Ubiquitin Ligase activity of Rsp5 has no impact on transcription mediated by 9aaTAD unlike to loss of entire Rsp5 protein. Therefore the three interaction domains of Rsp5 predisposing them for scaffold protein are supposed to promote initialization of 9aaTAD transcription by physically protein-protein interaction (initialization complex stabilizing) and not by its ubiquitination.

Results and Discussion

Rsp5 effects transactivation in ubiquitin ligase independent way

Regulated induction of peroxisomal genes by fatty acids was described previously by us and others . The promoter response element common to these genes was isolated from the Catalase A (CTA1) promoter region . This response element is recognized in yeast by native hetero-dimer of transcription factors Oaf1 and Pip2 , where the Oaf1 governs transcription of *CTA1* gene in signal dependent manner . From a screen for effectors of CTA1 response element it turned out that a strain having an allelic mutation in the Rsp5 gene strikingly decreased CTA1 expression (P. Pavlik personal communication and). We employed *rsp5*-knockdown *npiI* strain generated by Ty1 transposon insertion into *RSP5* promoter region to determinate a correlation of Rsp5 expression with activation of CTA1 (Rsp5 expression is 10-fold down regulated in the *rsp5*-knockdown strain *npiI*).

Decrease of transcription was observed for peroxisomal gene *CTA1* in the *rsp5*-knockdown strain *npiI* (Fig. 1). Both ectopic expressed full length (1-809 amino acids) and truncated Rsp5 (1-424 amino acids) missing E3 Ubiquitin ligase activity donated by Huibregtse and reported previously are effective suppressor of *CTA1* gene activation in *npiI* strain. Thus we supposed that Rsp5 takes a part on Oaf1 mediated transactivation, but E3 Ubiquitin ligase activity is not involved in this process.

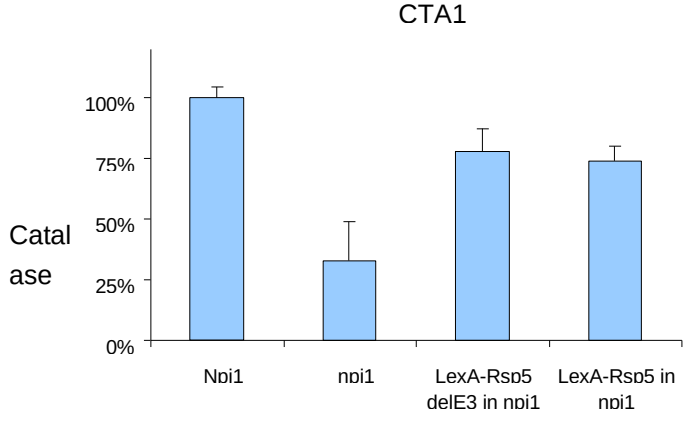
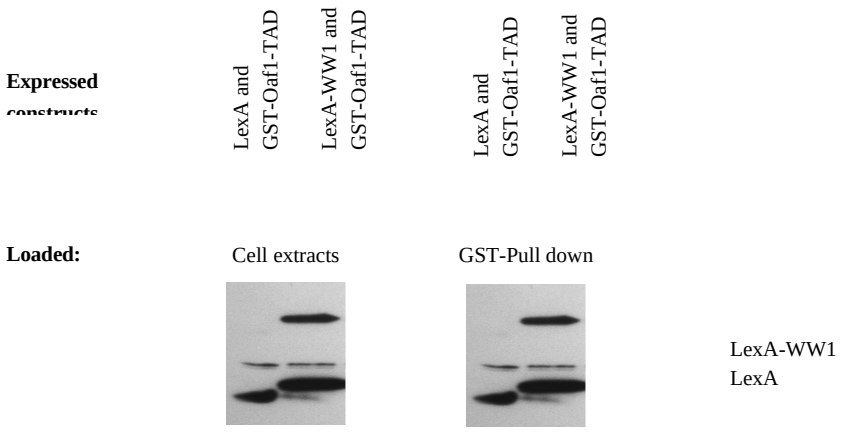


Fig.1. Decrease of activation of CTA1 gene in *rsp5*-knockdown strain *npiI*. The transcriptional efficiency of peroxisomal promoters *CTA1* was monitored in *rsp5*-knockdown strain *npiI* transformed with constructs for expression of either Rsp5 or Rsp5 delta E3-ubiquitin ligase domain (constructs A and K donated by J. M.Huibregtse) and parental wild type strains *NpiI* , growing on oleic acid based medium (YPO). Activation of *CTA1* promoter was monitored by measurement allelic expressed of catalase A. Values represent catalase activity as a percentage of parental wild type strain (values of wild-type strains were set to 100%, means and plusmn; SD; n=3).

Physical interaction of Rsp5 with transcription factor Oaf1

The decreased activation of *CTA1* gene in *rsp5*-knockdown strain *npiI* could be explained by disturbing the initiation of transcription. The transcription factor Oaf1 responsible for activation of *CTA1* gene might be a potential candidate that interacts with Rsp5. Thus we tested the physical interaction between Oaf1 and Rsp5 in pull-down experiments. To elucidate, if Rsp5 is directly involved in Oaf1 dependent activation, we tested Rsp5 WW domain, reported previously to mediate protein interaction for interaction with minimal transactivation domain of Oaf1. The critical region for Oaf1 transactivation described recently is the nine amino acid motif, 9aaTAD, located in the C-terminal 27 amino acids of Oaf1, Oaf1-TAD (1021-1047aa) (Fig. 2).

It was conceivable that the WW region of Rsp5 could be involved since it already was reported that this region mediates binding directly to other Rsp5 substrates. By GST pull-down we were able to show that the WW1 domain is sufficient to bind the C-terminal 27 aa region including 9aaTAD (Fig. 2). These data indicate that Rsp5 interacts with Oaf1 transactivation domain 9aaTAD and take physically a part on initialization of the transcription mediated by 9aaTAD.



Read out: LexA and LexA-WW1 (domain of Rsp5)
WB: anti-LexA antibody

Fig.2. The interaction of Oaf1 with Rsp5. WW1 domain of Rsp5 was tested for interaction with TAD of Oaf1. *GST*-Oaf1-TAD (1021-47 aa) fusion protein was pulled down with Glutathione-beads from cell extracts of yeast strains expressing either *LexA* or *LexA*-WW1 fusion protein (single WW domain of Rsp5). The cell extracts (input, the loading control) and read out of the pull-down experiments were detected on Western blot with anti-*LexA* antibody.

Rsp5 effects transactivation of other 9aaTAD transcription factors

We supposed that Rsp5 interaction with 9aaTAD is not restricted only to Oaf1, but rather to be relevant for other transcription factors of the 9aaTAD superfamily. We chose other two representing members of the 9aaTAD transcription factor superfamily and employed them in reporter assay to monitor their transactivation dependency on Rsp5. The transactivation regions of Oaf1, Gal4 and E2A transcription factors were fused to LexA. The transactivation activity was measured for LexA dependent reporter.

We observed dramatic Rsp5 effect on the reporter activity driven by either 9aaTADs of Oaf1, Gal4 or E2A (Fig. 3). We concluded that Rsp5 has impact on initialization of transcription mediated by diverse 9aaTAD transcription factors.

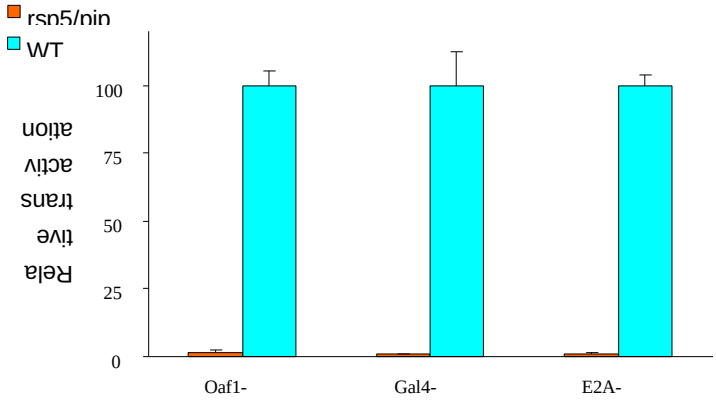
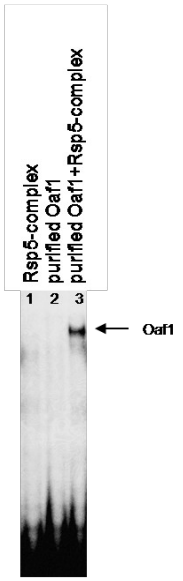


Fig.3. Diminished transactivation in *rsp5/pip1 strain* of various 9aaTADs. Each reporter construct was generated by fusion of *LexA* and 9aaTAD of transcription factor as reported previously: Oaf1-TAD construct comprises nine amino acids long region of Oaf1 (1035-1043 aa), Gal4-TAD construct comprises twelve amino acids long region of Gal4 (860-871 aa) and E2A-TAD construct comprises 99 amino acids long region of E2A transactivation domain (1-99 aa). The transactivation activity was tested in *rsp5/pip1* mutant and parental wild-

type strain GA1-8C, both co-transformed with *LacZ*-reporter driven by 2x*LexA* operator (construct pSH18-34, kindly provided by R. Brent). Values represent - galactosidase activity of the constructs as percentage of transactivation in the wild type strains (values in wild-type strains were set to 100%, means and plusmn; SD; n=3).

Rsp5 binds cofactors necessary to activate Oaf1 DNA binding

Oaf1 9aaTAD is responsible for physical interaction with Rsp5. We supposed that Rsp5 interplays with initiation coactivators of 9aaTADs by physical interaction and stimulates the transactivation on this way.



It was previously demonstrated that Rsp5 associates with coactivators of initiation complex . Therefore we postulated an existence of Rsp5-coactivator complex (further Rsp5 protein complex) and expected to co-purify those factors performing Rsp5 pull-down from cell extracts (Δoaf1/pip2 strain expressing His6x-HA-Rsp5).

We have investigated Oaf1 binding in EMSA experiment with and without Rsp5 associated proteins. To our surprise the purified Oaf1 is unable to bind DNA unless the probe is supplemented with the Rsp5 protein complex (Fig. 4, lane 3).

Fig.4. Rsp5 protein complex is essential for Oaf1 DNA binding. Rsp5 with associated proteins was pulled down and Oaf1 was purified with Ni-beads from cell extracts of Δoaf1/pip2 strains expressing either *His6x-myc3x*-Oaf1 or *His6x-myc3x*-Rsp5 (for details see materials and methods). Both samples were tested separately (lines 1 and 2) and together (pre-incubated, line 3) in EMSA for Oaf1 specific DNA binding.

Rsp5 is recruited to the Gal4 dependent promoter

General histone acetyltransferase complex SAGA/STAGA (SPT3-TAF9-GCN5-acetylase) was implicated in recruitment of TATA-binding protein (TBP) to the *GAL1* promoter , for loading of initialization complex TFIID . A Spt3 mutant could be suppressed by Rsp5 overdose and TAF9 was implicated for direct physical interaction with 9aaTAD . Therefore we tested whether Rsp5 is also physically involved in initialization of transcription from *GAL1* promoter. The initialization complex is recruited to promoter via Gal4 transcription factors in response to galactose . If the initiation complex recruited by Gal4 9aaTAD would include Rsp5, we should observe galactose dependent recruitment of Rsp5 to the *Gal1* promoter. To monitor Rsp5 promoter recruitment in response to galactose, we used chromatin immuno-precipitation PCR assays (CHIP). The cell extracts of *myc3x*-Rsp5 expressing strain growing on either YP-ethanol representing non-inducing condition or YP-galactose medium representing inducing

condition for Gal4 were used in CHIP assay. The read out of this experiment is the amplification of chromosomal DNA of the specific *Gal1* promoter region by PCR.

The *Gal1* promoter region could be specifically amplified when compared to control chromosomal targets (YCL049C, FUS1 and HIS3 are used as negative controls) from cells growing on galactose (Fig. 5). This result demonstrates that Rsp5 was recruited to the *GAL1* promoter with transcriptional machinery as expected.

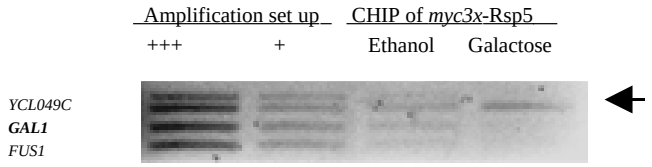


Fig.5. Signal dependent Rsp5 recruitment to the promoter *GAL1*. Chromatin immuno-precipitation PCR assays were carried out with mouse anti-myc antibody and ProteinG-beads. Cell extracts of strain expressing *myc3x*-Rsp5 were grown on either YP-ethanol (non-inducting condition) or YP-galactose medium (inducting condition for *GAL1-10* promoter). Arrow indicates *GAL1* specific PCR product from cells stimulated with galactose compare to non-induced cells grown on ethanol based medium. The Gal4 unrelated templates YCL049C, FUS1 and HIS3 were used as negative control. The number of plus indicates the amount of template chromosomal DNA used for set up.

Conclusion

The functional and physical interaction of Rsp5 with Oaf1 9aaTAD can be addressed to initiation of transcription. Rsp5 has three interaction domains (WW), which predispose Rsp5 for multiple protein-protein interactions. Since Rsp5 interacts with both, CTD of RNA polymerase II and TFIID , we suppose that Rsp5 scaffolding of those promotes the initialization transcription mediated by 9aaTAD transcriptional factors Oaf1 and Gal4.

Materials and methods

Yeast strains and plasmids. W303 (MATa leu2 his3 ura3-1 trp1 ade2 can1), L40 (MATa leu2 his3 trp1 ade2 LYS2::(lexAop)$_4$-HIS3 URA3::(lexAop)$_8$-*lacZ* GAL4 gal80) , *pip1-MF24-6x* strain with a mutant allele of *RSP5* was derived from *MF24-6x* (MATa, leu2 his3 trpl cttl-l gal2 URA3::(CTAI-184/-198)6-CYCI-lacZ) respective from GA1-8C (MATa, leu2 his3 trpl cttl-l gal2 ura3) ; *rsp5*/*pip1*strain (MATa, leu2 his3 trpl cttl-l gal2 ura3 rsp5) was derived from *pip1-MF24-6x* strain crossed with relative strain GA1-10B (MATalfa, leu2 his3 cttl-l gal2 ura3) (diploid was sporulated, haploids were segregated and tested) ; *NpiI* strain (*MATa, ura3, npi1/rsp5*) is parental strain to *npi1* strain with Ty1 transposome insertion in *RSP5* promoter (#27038a) . BJ (MATa, leu2 trpl ura3-52 pep4-3 prbl-1122) and BJ1991 oaf1/pip2 (MATa, leu2 trp1 ura3-52 pep4-3 prb1-1122 pip2D::kanMX4 oaf1D::LEU2) strains were described previously . Yeast transformants were selected and grown on minimal medium containing 2% glucose and 0.67% Yeast Nitrogen Base without amino acids (Difco). YP with Oleate (YPO) contained 1% yeast extract, 2% peptone, 100 mM phosphate buffer pH 7.0, 0.2% oleic acid. The N-terminal Rsp5 and Oaf1 His6x-*myc*3x tag were introduced in YIPlac211 , LexA one hybrid constructs of Rsp5 and Oaf1 are derived from pBTM116 , (WW1 domains of Rsp5 corresponds to amino acids 214-272) .

EMSA. Cells were harvested by centrifugation and lysed by shaking with glass beads for 30 minutes at 4°C in a the buffer containing 200 mM Tris-HCl pH 8, 10% glycerol and proteinase inhibitor cocktail (Boehringer). Extracts were centrifuged for 5 min at 4.000 rpm at 4°C. DNA probe (*FOX3* Oaf1 response element) was described by Einerhand et al. . DNA binding experiments were done in 4 mM Tris-HCl pH 8, 100 mM NaCl, 4 mM $MgCl_2$, 25 ng/µl poly(dI-dC), 10% glycerol, and 0,2 ng of the ^{32}P-labeled DNA probe in a final volume of 20 µl containing 1 µl cell extract at room temperature for 25 minutes. Free and bound DNA were separated on 4% polyacrylamide 0.5xTBE gels for 1,5 h at 200V. Protein expression was confirmed by immunoblotting with anti-GST-antibody.

Protein purification, Pull-down and CHIP. The proteins were pulled down with ProteinG-, Glutathione- or Ni-beads from cell extracts of Δoaf1/pip2 strains expressing GST- or His- fusion proteins according manufacturers protocol (Pharmacia). The purification of His6x-Oaf1 was achieved by modification of pull-down protocol by including 0,5% tween20 in washing buffers to eliminate co-precipitation of interacting proteins. Rabbit anti-Nedd4 ab (kindly provided by M.Noda, N.A.Jenkins lab), rabbit anti-LexA ab (kindly provided by J.Little, University of Tucson), mouse anti-GST ab (Serotec), monoclonal mouse anti-c-myc 9E10, secondary donkey anti-mouse and anti-rabbit-HRP (Amersham, UK) were used in standard methods for Western blots, immunoprecipitation and pull-down (Pharmacia). Chromatin immunoprecipition PCR assays were performed as described . Yeast cells were crosslinked with 1% formaldehyde for 20 min at 30°C. The cell extracts were sonicated for 30, 30 and 20 seconds on ice using a Virsonic 100 cell disrupter.

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References

Supplementary Figure S1