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RESEARCH ARTICLE

Mutational analysis of the C-terminal FATC domain of *Saccharomyces cerevisiae* Tra1

Stephen M. T. Hoke · A. Irina Mutiu · Julie Genereaux · Stephanie Kvas · Michael Buck · Michael Yu · Gregory B. Gloor · Christopher J. Brandl

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Abstract Tra1 is a component of the *Saccharomyces cerevisiae* SAGA and NuA4 complexes and a member of the PIKK family, which contain a C-terminal phosphatidylinositol 3-kinase-like (PI3K) domain followed by a 35-residue FATC domain. Single residue changes of L3733A and F3744A, within the FATC domain, resulted in transcriptional changes and phenotypes that were similar but not identical to those caused by mutations in the PI3K domain or deletions of other SAGA or NuA4 components. The distinct nature of the FATC mutations was also apparent from the additive effect of *tra1-L3733A* with SAGA, NuA4, and *tra1* PI3K domain mutations. Tra1-L3733A associates with SAGA and NuA4 components and with the Gal4 activation domain, to the same extent as wild-type Tra1; however, steady-state levels of Tra1-L3733A were reduced. We suggest that decreased stability of Tra1-L3733A accounts for the phenotypes since intragenic suppressors of *tra1-L3733A* restored Tra1 levels, and reducing wild-type Tra1 led to comparable growth defects. Also supporting a key role for the FATC domain in the structure/function of Tra1, addition of a C-terminal glycine residue resulted in decreased association with Spt7 and Esa1, and loss of cellular viability. These findings demonstrate the regulatory potential of mechanisms targeting the FATC domains of PIKK proteins.

Keywords Gene expression · Tra1 · PIKK protein family · FATC domain · Yeast · SAGA complex

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S. M. T. Hoke · A. Irina Mutiu · J. Genereaux · S. Kvas · G. B. Gloor · C. J. Brandl (✉)
Department of Biochemistry,
Schulich School of Medicine and Dentistry,
University of Western Ontario, London N6A 5C1, Canada
e-mail: cbrandl@uwo.ca

M. Buck
Department of Biochemistry,
School of Medicine and Biomedical Sciences,
State University of New York, New York 14124, USA

M. Buck
Center of Excellence in Bioinformatics and Life Sciences,
Buffalo, NY 1420, USA

M. Yu
Department of Biological Sciences,
State University of New York, Buffalo, NY 14260, USA

Present Address:
A. Irina Mutiu
Southern Crop Protection and Food Research Centre,
Agriculture and Agri-Food Canada,
1391 Sandford St., London N5V 4T3, Canada

Introduction

Tra1 is a component of the yeast SAGA and NuA4 complexes, being the principal component that interacts with transcription activators (Bhaumik et al. 2004; Brown et al. 2001; Fishburn et al. 2005; Reeves and Hahn 2005). Tra1 is essential for viability in *Saccharomyces cerevisiae* (Saleh et al. 1998). Its mammalian homolog TRRAP is required for early embryonic development (Herceg et al. 2001) and the function of key cellular regulators such as c-Myc, p53, E2F1, β-catenin, and BRCA1 (reviewed by Murr et al. 2007). Tra1 and TRRAP are members of the phosphatidylinositol 3-kinase (PI3K) related kinase (PIKK) family,

which also includes ATM, ATR, DNA-PKcs, TOR, and SMG-1. All of these molecules are important players in stress response, particularly related to DNA damage, cell growth, and proliferation (Abraham 2004). Tra1/TRRAP retains the PI3K domain, but the protein kinase activity demonstrated for many members of the family has not been found (McMahon et al. 1998; Saleh et al. 1998; Vassilev et al. 1998).

The SAGA complex is engaged in a number of nuclear processes. Its roles include facilitating recruitment of the transcriptional preinitiation complex (Bhaumik and Green 2001, 2002; Larschan and Winston 2005), promoting nucleosome eviction (Govind et al. 2007) and replication-coupled nucleosome assembly (Burgess et al. 2010). These regulatory functions occur through the acetylation of nucleosomal histones H2B, H3, and Htz1 by the component protein Gcn5 (Grant et al. 1997; Millar et al. 2006; Ruiz-Garcia et al. 1997; Suka et al. 2001), the deubiquitylation of histone H2B by Ubp8 (Henry et al. 2003), and interaction with the basal transcriptional machinery (Dudley et al. 1999; Mohibullah and Hahn 2008; Saleh et al. 1997). The presence of the nuclear pore component Sus1 within SAGA also links the complex with mRNA export (Kohler et al. 2006, 2008).

The catalytic subunit of the NuA4 complex, Esa1, is essential for viability in *S. cerevisiae* and acetylates histones H2A, H4, and Htz1 (Allard et al. 1999; Millar et al. 2006). Acetylation by Esa1 is required for transcriptional regulation (Allard et al. 1999) and the DNA-damage response (Bird et al. 2002; Choy and Kron 2002; Downs et al. 2004). A subset of the other NuA4 component proteins, Eaf2, Act3/Arp4, Act1, and Yaf9, are shared with the Swr1 complex that introduces Htz1 into chromatin (Bird et al. 2002; Choy and Kron 2002; Downs et al. 2004; Krogan et al. 2003, 2004).

We previously characterized a class of *tra1* alleles having mutations within the PI3K domain (Mutiu et al. 2007a). The most severe allele, *tra1-SRR3413* is a triple alanine scanning mutation that alters the serine-arginine-arginine residues found at positions 3413 to 3415. The changes in gene expression in the *tra1-SRR3413* strain partially overlap those seen in strains with deletions of SAGA or NuA4 components and result in phenotypes consistent with the involvement of Tra1 in cell wall stability and stress response. Synthetic genetic array analysis identified genetic interactions of *tra1-SRR3413* with genes involved in gene expression, mitochondrial function, and membrane sorting/protein trafficking (Hoke et al. 2008b). In addition, *tra1-SRR3413* shows generation-dependent telomere shortening, a phenotype not seen with deletions of SAGA or NuA4 components (Mutiu et al. 2007a).

The extreme C-terminus of the PIKK proteins contains a 35-amino acid residue FATC domain (FRAP-ATM-TRRAP C-terminus; Bosotti et al. 2000). For ATM, DNA-PKcs, mTOR, and SMG-1, the FATC domain is necessary for the kinase activity of the adjacent PI3K domain (Beamish et al. 2000; Morita et al. 2007; Priestley et al. 1998; Sun et al. 2007; Takahashi et al. 2000). In addition, the FATC domain of ATM is required for interaction with Tip60, the mammalian homolog of Esa1 (Sun et al. 2005). ATR, TRRAP, and DNA-PKcs FATC domains can substitute for the native domain of ATM, restoring kinase activity and interaction with Tip60 (Jiang et al. 2006); however, functional equivalency across the family is not absolute since the ATM FATC domain cannot replace that of mTOR (Takahashi et al. 2000). A solution structure for the isolated FATC sequence of *S. cerevisiae* Tor1 consists of an α-helix with a C-terminal disulfide bonded loop (Dames et al. 2005). The generality of this structure is unclear given that the cysteine residues that form the disulfide bond are not present in other PIKK family members.

The goal of this study was to identify features of the FATC domain that are important for the function of the Tra1/TRRAP molecules. By analyzing mutations within the FATC domain of Tra1, we show that the FATC domain and precise positioning of the C-terminal carboxyl group are required for function. Addition of a C-terminal glycine resulted in loss of viability and altered association with NuA4 and SAGA components. Alanine substitutions at L3733 or F3744 resulted in growth phenotypes and transcriptional changes related, but not identical, to those within the PI3K domain. Tra1-L3733A was characterized in more detail, as it caused the most specific growth defects. We suggest that the functional changes of Tra1-L3733A are due to a role for the FATC domain in maintaining a stable form of the protein since the steady state level of Tra1-L3733A was 25% of that seen for wild-type Tra1, and suppressor mutations that partially restored function of Tra1-L3733A increased its concentration to a similar extent. These findings demonstrate the importance of the FATC domain in the structure/function of Tra1 and emphasize the pronounced consequences of any regulatory mechanism that targets the FATC domain of the Tra1/TRRAP proteins.

Materials and methods

Yeast strains and growth

Yeast strains are listed in Table 1. CY4060 is a derivative of BY4743 in which one copy of *TRA1* has been gene replaced with *tra1-L3733A* that contains a *HIS3* allele at the downstream *Bst*BI site. CY4060 was sporulated to generate MAT**a** and MAT**α** haploid strains (CY4103 and CY4057, respectively) that were then crossed to deletion derivatives

Table 1 Strains used in this study

Strain	Genotype	*TRA1* plasmid(s)	Reference
KY320	*MAT***a** *ura3-52 ade2-101 trp1-Δ1 lys2-801 his3-Δ200 leu2::PET56*		Chen and Struhl (1988)
CY1021	Isogenic to KY320 except *tra1::Tn10LUK*	*myc-TRA1*	Saleh et al. (1998)
CY2706	Isogenic to CY1021	*myc_9-TRA1*	This work
CY2707	Isogenic to CY1021	*myc_9-tra1-L3721D*	This work
CY3003	Isogenic to CY1021	*myc_9-tra1-L3733A*	This work
CY3019	Isogenic to CY1021	*myc-TRA1 myc_9-tra1-G3745*	This work
CY3020	Isogenic to CY1021	*myc-TRA1 myc_9-TRA1*	This work
CY3083	Isogenic to CY1021	*TAP-FLAG-TRA1*	This work
CY3084	Isogenic to CY1021	*TAP-FLAG-tra1-L3721D*	This work
CY3085	Isogenic to CY1021	*TAP-FLAG-tra1-L3733A*	This work
CY4055	Isogenic to CY1021	*myc_9-tra1-L3733A/N3677D*	This work
CY4056	Isogenic to CY1021	*myc_9-tra1-L3733A/T3716A*	This work
BY4743	*MAT***a**/α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*		Winzeler and Davis (1997)
BY4741	*MAT***a** *ura3Δ0 met15Δ0 his3Δ0 leu2Δ0*		Winzeler and Davis (1997)
BY4742	*MAT*α *ura3Δ0 lys2Δ0 his3Δ0 leu2Δ0*		Winzeler and Davis (1997)
TAP-ADA2	Isogenic to BY4741 except *TAP-ADA2-HIS3*		Ghaemmaghami et al. (2003)
CY2998	Isogenic to TAP-ADA2 except *trp1::URA3*		This work
TAP-SPT7	Isogenic to BY4741 except *TAP-SPT7-HIS3*		Ghaemmaghami et al. (2003)
CY3001	Isogenic to TAP-SPT7 except *trp1::URA3*		This work
TAP-ESA1	Isogenic to BY4741 except *TAP-ESA1-HIS3*		Ghaemmaghami et al. (2003)
CY3002	Isogenic to TAP-ESA1 except *trp1::URA3*		This work
CY1524	Isogenic to CY1021	*$Flag_3$-$TRA1_{SB}$*	Mutiu et al. (2007a)
CY1531	Isogenic to CY1021	*$Flag_3$-TRA1-SRR3413*	Mutiu et al. (2007a)
BY7240	Isogenic to BY4741 except *yaf9::Kan^r*		Winzeler and Davis (1997)
CY4060	Isogenic to BY4743 except *TRA1/tra1-L3733A-HIS3*		This work
CY4057	*MAT*α *ura3Δ0 his3Δ0 leu2Δ0tra1-L3733A-HIS3*		This work
CY4103	*MAT***a** *ura3Δ0 his3Δ0 leu2Δ0 tra1-L3733A-HIS3*		This work
CY4318	*MAT*α *ura3Δ0 his3Δ0 leu2Δ0 tra1-A3727S-HIS3*		This work
CY4324	*MAT***a** *ura3Δ0 his3Δ0 leu2Δ0 tra1-F3740A-HIS3*		This work
CY4350	*MAT***a** *ura3Δ0 his3Δ0 leu2Δ0 tra1-F3744A-HIS3*		This work
CY4353	*MAT***a** *ura3Δ0 his3Δ0 leu2Δ0 TRA1-HIS3*		This work
FY630	*MAT*α *his4-917 lys2-173 R2 leu2*		Gansheroff et al. (1995)
FY1093	Isogenic to FY630 except *spt7::LEU2*		Gansheroff et al. (1995)
QY204	*MAT***a** *his3Δ200 trp1Δ63 ura3-52 leu2Δ1 lys2-128Δ*		Nourani et al. (2004)
QY202	Isogenic to QY204 except *yng2::Kan^r*		Nourani et al. (2004)

of BY4741 and BY4742 and sporulated to analyze the double mutant strains. CY4353, CY4318, CY4324, and CY4350 are similarly engineered strains containing wild-type *TRA1*, *tra1-A3734S*, *tra1-F3740A,* and *tra1-F3744A*.

TRA1 alleles contained on *TRP1* centromeric plasmids were transformed into CY1021 (Saleh et al. 1998) and the wild-type copy on a *URA3*-centromeric plasmid displaced by plasmid shuffling. Growth comparisons were performed on plates at 30° unless stated otherwise. Assays were performed in duplicate on independently constructed strains. Scoring of FATC domain mutations was relative to CY2706, which contains *$TRA1_{WT}$*, the background allele used to construct the mutations. *$TRA1_{WT}$* is N-terminally myc_9-tagged and contains a *BamH*I site that converts N3580A.

TAP-tagged *ADA2* (YSC1178-7500046), *SPT7* (YSC1178-7499287) and *ESA1* (YSC1178-7502907) (Ghaemmaghami et al. 2003) BY4741, and BY7042 (*yaf9::Kan^r*) were purchased from Open Biosystems. These strains were made *trp1::URA3* and *leu2::HIS3* using pTU10 and pLH7, respectively (Cross 1997).

Table 2 Oligonucleotides used in this study

Name	Sequence (5′–3′)	Description
2346	ATACGAGCTCTTTGAGGCTTTCTCTACCTTC	Outside end of *TRA1*[a]
2323-2	TCGGGATCCGCATGGGCCCAATGGGGTTT	
4249-3	GCGGCCGCAAACGCAGCGCATGATGATG	
2583-1	CGGGATCCGTATTTACTTTAGAAATGTTAC	*BamHI*/*TRA1*
2583-2	GAGGATCCAGAGGTTTTGTCAACATGG	
5138-3	TCATCCTAGCCTGTATTGGCAGCGCTG	L3721D
5138-4	GCCAATACAGGCTAGGATGAACTGGGTTG	
5138-1	CAGTTCATCGATGACTGTATTGGCAGCGC	D3722A
5138-2	CAATACAGTCATCGATGAACTGGGTTGTGAC	
55-IDT	GCTCTAGAACCATGGCATGAAGTTGACGTCTGT TCTTGCTAAGTTTCTTGGACTGACAGAGCTGCC AATACAGTCTAGG	A3727S
5185-1	TCTGTTCTTGCTGCGTTTCTTGGACTGACAGCG	L3733A
5185-1	TCCAAGAAACGCAGCAAGAACAGACGTGAAC	
5138-5	TAGCAAGAACATACGTGAACTTCATGCCA	D3737Y
5138-6	AAGTTCACGTATGTTCTTGCTAAGTTTCTT	
5714-1	GAACCATGGCATGGCGTTCACGTCTGTTCTTGC	F3740A
5747-1	CCCGAGCTCTTGATTGTTAGCAATACCG	F3744A
5747-2	CATGCCATGGGCCTAGAGCTGATACATGGGGT	
5164-1	ATACGAGCTCCTACCCGAACCATG	G3745
5088-1	GGCCGATGAACAAAAATTGATTTCTGA	*myc*-tag
5088-2	GGCCGCCAAATCTTCTTCAGAAATCAA	
5295-1	AACTGCAGTTTAGTACTAACAGAGACTTTTG	*MET3* promoter
5295-2	CCCAAGCTTAATTATACTTTATTCTTGTTATTAT	
3203-1	CGGGATGGTTAAGAGGCCAACGTCGATG	*RPL35a* promoter
3203-3	CCAATTGAGAAGCCAATTGTTC	
5669-1	AATTGAAGGGGCGGAATTCAGGGGCGGAG	STRE
5669-2	CCGCCCTGAATTCCGCCCCTTC	
5583-1	GTATCGATTCATTTAGACATTGC	*PHO5* CHIP
5583-2	ATCCGAAAGTTGTATTCACCAAG	
5526-1	GCTGGTAAATTCAACGAGGCGGATAATTCCTTAA CCAGGGGCCA	*ADE17*
5526-2	GGAACCCCAGACTTGGTCCAACTTGGTGAAAGCA GCTTCGGTCC	*RPL4b*

[a] *TRA1* sequence that enables the PCR and cloning of an internal restriction fragment

DNA constructs

The *PHO5-LacZ* reporter constructs in the *LEU2* centromeric plasmid YCp87 were described previously (Mutiu et al. 2007b). *Rpl35a-LacZ* was similarly constructed using the oligonucleotides indicated in Table 2. A *HIS3-LacZ* fusion regulated by two STRE elements (*SRE/his3-LacZ*) in place of the Gcn4 binding site was constructed by annealing oligonucleotides 5669-1 and 5669-2 and inserting the fragment into the *Eco*RI and *Sac*I sites of *his3-Δ88-LacZ* (Brandl et al. 1993).

myc$_9$*-TRA1-YCplac111* was constructed through consecutive ligation of oligonucleotides 5088-1 and 5088-2 (see Table 2) into *myc-TRA1-YCplac111* (Saleh et al. 1998). A *BamH*I site at position 10734 of *TRA1* was introduced by two-step PCR using oligonucleotide pairs of 2583-1 with 2346 and 2583-2 with 2323-2 and cloned as an *Apa*I–*Sac*I restriction fragment into full-length *TRA1* to give *myc*$_9$*-TRA1*$_{WT}$. Mutations of L3721D, D3722A, L3733A, D3737Y, and G3745 were similarly engineered. Initial reactions contained a listed oligonucleotide and the appropriate outside flanking primer with a unique cloning site (oligonucleotides 2346 and 4249-3). Fragments were moved into *myc*$_9$*-TRA1*$_{WT}$ *-YCplac111* using *Bam*HI–*Sac*I restriction sites. T3714I and I3720D were serendipitously isolated in sequencing of random alleles.

To integrate *tra1-A3727S*, *L3733A*, *F3740A,* and *F3744A* into the genome, mutations were introduced into the 3′ *Sph*I–*Fsp*I fragment of the gene and flanking region using the oligonucleotides listed in Table 2 and the terminal

*Nco*I site for cloning. The DNA contained *HIS3* at the *Bst*BI site to allow selection in yeast. A plasmid copy of YHR100C was transformed into the strains to ensure that this gene was not affected by the integration.

A 495-bp fragment of the *MET3* promoter flanked by *Pst*I and *Not*I sites was cloned into the molecules expressing *TRA1* after PCR with oligonucleotides 5295-1 and 5295-2. TAP-tagged *TRA1* molecules were cloned into a *LEU2* derivative of the *YCpDed-TAP* construct described previously (Mutiu et al. 2007a).

β-Galactosidase assays

Yeast strains containing *RPL35a-LacZ* were grown in YPD to an A_{600} of ~1.5, pelleted, washed in LacZ buffer, and concentrated fivefold. β-Galactosidase was determined using *o*-nitrophenol-β-D-galactosidase as substrate, standardizing to cell density (Ausubel et al. 1988). For analysis of *PHO5-LacZ* under inducing conditions, overnight cultures were washed three-times in water then grown 15 h in YPD depleted of phosphate (Han and Grunstein 1988). *STRE/his3-LacZ* and *his3-Δ88-LacZ* were assayed after growing tenfold dilutions of saturated cultures (from minimal media) for 15 h in YPD containing 4% ethanol.

RNA purification and gene profiling

Yeast cells, CY2706, and CY3003, were grown at 30° in YP media containing 2% glucose to an $A_{600} = 2.0$. RNA was purified from 10^8 cells after glass bead disruption as described previously (Mutiu and Brandl 2005). RNA integrity numbers of greater than 8.9 were determined for each RNA sample using an Agilent 2100 Bioanalyzer at the London Regional Genomics Centre. mRNA-Seq libraries were constructed and sequencing were performed on the Illumina/Solexa Genome Analyzer II platform at the DNA Facility at Iowa State University. The CY2706 and CY3003 samples were each run on a single Illumina GAII lane, producing 13014880 reads for CY2706 and 11156078 reads for CY3003 of 35 nucleotides. The *S. cerevisiae* genome sequence and the general features format file (saccharomyces_cerevisiae.gff) were obtained from Saccharomyces Genome Database on May 1, 2009. The sequencing reads were mapped onto the genomic sequence using the novoindex and novoalign programs with the default parameters, except that reads mapping to two or more places in the genome were placed at one position at random. With this option novoalign marks a read as uniquely or repetitively mapping in the genome; only uniquely mapping reads were used for the subsequent analysis (83 and 84% for CY2706 and CY3003, respectively). Mapped reads were placed into bins composed of protein-coding genes, tRNA and rRNA genes as defined by the gff file. Only reads that did not overlap the start or end position of the gene were counted and reads mapping to the top and bottom strands were tabulated separately. The relative occurrence of each ORF annotated in the Saccharomyces Genome Database as a ratio of its length was calculated after normalization to 10 million reads for each sample, similar to the normalization outlined in Mortazavi et al. (2008). Genes with ≥0.05 reads per base pair of gene length were considered for further analysis. Agglomerative hierarchical clustering based on the average linkage of uncentered correlations was performed using CLUSTER 3.0 software (Eisen et al. 1998) on the profiles from strains within the compendium data set (Hughes et al. 2000) the data sets of strains containing deletions of NuA4 (Krogan et al. 2004) and SAGA components (Ingvarsdottir et al. 2005) and with *tra1-SRR3413* (Mutiu et al. 2007a). Genes not appearing in at least two of the profiles were excluded. The data were visualized using MAPLETREE (http://rana.lbl.gov/EisenSoftware.htm).

RNA dot blots with probes for *ADE17* and *RPL4a/b* (Table 2) were performed on Hybond-N membrane (Amersham) using 10 and 2.5 μg of total RNA, essentially as described by the manufacturer. Hybridizations were performed in buffer containing 5× standard saline citrate (SSC), 5× Denhardt's solution, 0.5% sodium dodecyl sulphate (SDS), and 90 μg/ml denatured herring sperm DNA at 52°. Washes in 2× SSC plus 0.1% SDS and 1× SSC plus 0.1% SDS were performed at 42°.

Chromatin immunoprecipitation assays

Assays for acetylated histones were performed essentially as described previously (Hoke et al. 2008a). Cells were grown in YPD media to an $A_{600} \sim 2.0$. Antibodies were purchased from Abcam Inc. (anti-H3, ab1791; anti-AcH4/K8, ab1760).

Western blotting

Yeast extract prepared by grinding in liquid nitrogen or by lysis with glass beads (Saleh et al. 1997) was separated by SDS-PAGE and transferred to PVDF membrane (Roche Applied Science). Anti-myc (Evan et al. 1985), anti-Mcm2 (Santa Cruz Biotechnology, Cat. # sc-6680; kindly supplied by Megan Davey), and anti-calmodulin-binding protein (CBP) antibodies (Millipore Corp., Cat. # 07-482) were used at ratios of 1:5000, 1:4000, and 1:1000, respectively. Secondary antibody (anti-Mouse IgG HRP, Promega; anti-Goat IgG HRP, Sigma; anti-Rabbit IgG HRP, Promega) used at a ratio of 1:10000 was detected using SuperSignal West Pico Chemiluminiscent Substrate (Thermo Scientific). Densitometric scanning of films was performed using AlphaImager 3400 software (Alpha Innotech, Inc.).

TAP purification

Whole cell extracts were prepared by grinding in liquid nitrogen (Saleh et al. 1997). Tandem affinity purification (Rigaut et al. 1999) with 1 l of extract grown in minimal media lacking tryptophan to an $A_{600} \sim 2$ was carried out as described previously (Mutiu et al. 2007a).

Genome-wide localization studies

Genome-wide localization studies were performed essentially as described (Yu et al. 2004) for yeast strain CY2706 grown at 30° in YPD. Immunoprecipitations were performed in triplicate with 10 µl of anti-myc antibody (9E11) and using pan-mouse IgG Dynal beads (Invitrogen). Antibody was pre-incubated with the beads in 1× phosphate-buffered saline containing 5 mg/ml BSA for a minimum of 2 h. *P* values were calculated using an error model provided by Rosetta Resolver. The genome-wide occupancy was expressed as the ratio of fluorophore intensities from chromatin fragments enriched by immunoprecipitation versus that of the input chromatin fragments. Spots with a *P* value threshold of 0.02 and a ratio of intensity >1.0 were included in the final dataset (Online Resource 1).

Isolation of intragenic suppressors of *tra1-L3733A*

C-terminal sequences of *tra1-L3733A* downstream of the *Apa*I site at base pair 9175 were mutagenized by PCR, cloned back into the full-length molecule, and shuffled into yeast strain CY4018 by selection on 5-FOA. Individual colonies were selected for growth on YPD plates containing 4% ethanol; the plasmids were isolated, sequenced, and verified for plasmid dependency of the selection by repeating the selection process after transformation into CY1021.

Gal4 affinity chromatography

Interaction of myc_9-Tra1 constructs from yeast strain CY2998 with recombinant activation domain of Gal4 was performed as described by Mutiu et al. (2007a).

Results

Characterization of mutations within the FATC domain of Tra1

The C-terminal region of the Tra1/TRRAP family contains three conserved domains: FAT (FRAP-ATM-TRRAP), phosphatidylinositol 3-kinase-like (PI3K) and FATC (FAT C-terminal; Fig. 1a). To identify key residues required for function, we introduced mutations into the FATC domain. To identify residues to target, we analyzed an alignment of the FATC domains of Tra1/TRRAP from five species (Fig. 1b, upper alignment) and an alignment of *S. cerevisiae* Tra1 with the FATC domains from members of the PIKK family (lower alignment). L3733 and A3727 (numbering for *S. cerevisiae* Tra1) are highly conserved throughout the PIKK family, as are hydrophobic residues at positions equivalent to I3720, I3724, F3740, W3743, and F3744. An acidic residue is conserved at D3737 within Tra1/TRRAP, but is aromatic in the broader family. Other positions are conserved in the fungal forms of Tra1. We constructed alleles of *S. cerevisiae TRA1* with changes to these different classes of residues (see Fig. 1b). The L3733A change was of particular interest because the comparable mutation in SMG-1 results in loss of kinase activity (Morita et al. 2007). Some of the changes, for example, L3721D and D3737Y, were made to resemble the residues found in the PIKK family. Another allele, which we have termed *tra1-G3745*, was constructed with a glycine codon following the terminal phenylalanine codon to analyze the importance of the positioning of the terminal carboxyl group.

The initial *tra1* alleles analyzed (T3714I, I3730D, L3721D, D3722A, L3733A, D3737Y and G3745) were introduced on *TRP1*-centromeric plasmids into *S. cerevisiae* strain CY1021, which contains a disruption of the genomic copy of *TRA1*, complemented by wild-type *TRA1* expressed from a *URA3*-containing centromeric plasmid. Interestingly, *tra1-G3745* and to a lesser extent *tra1-L3733A*, resulted in slow growth in combination with the wild-type allele (not shown). The alleles were examined for their ability to support viability by shuffling out wild-type *TRA1* on media containing 5-fluoroorotic acid. The six alleles with single residue changes supported growth, whereas *tra1-G3745* did not. Of the viable strains, only the strain containing *tra1-L3733A* had obvious growth defects.

The *tra1-L3733A* allele resulted in several phenotypes shared with strains having mutations in the *ada* genes (Fig. 1c; Table 3). These phenotypes included slow growth on media containing ethanol, Calcofluor white, or tunicamycin. Interestingly, however, it did not display the classic *ada* phenotype of resistance to overexpression of VP16 (Berger et al. 1992). The *tra1-L3733A* allele decreased growth on media containing *tert*-butylhydroperoxide, or lacking inositol, both characteristics of defects in Spt function (Gansheroff et al. 1995). A dichotomy was seen for NuA4-related phenotypes, as the *tra1-L3733A* strain was sensitive to benomyl but not methylmethane sulphonate.

The phenotypes of the *tra1-L3733A* strain were similar but not identical to those of the *tra1-SRR3413* strain (Mutiu et al. 2007a). Similarities included slow-growth on media containing ethanol, Calcofluor white, benomyl, rapamycin, geneticin, and chloramphenicol; whereas, differences in

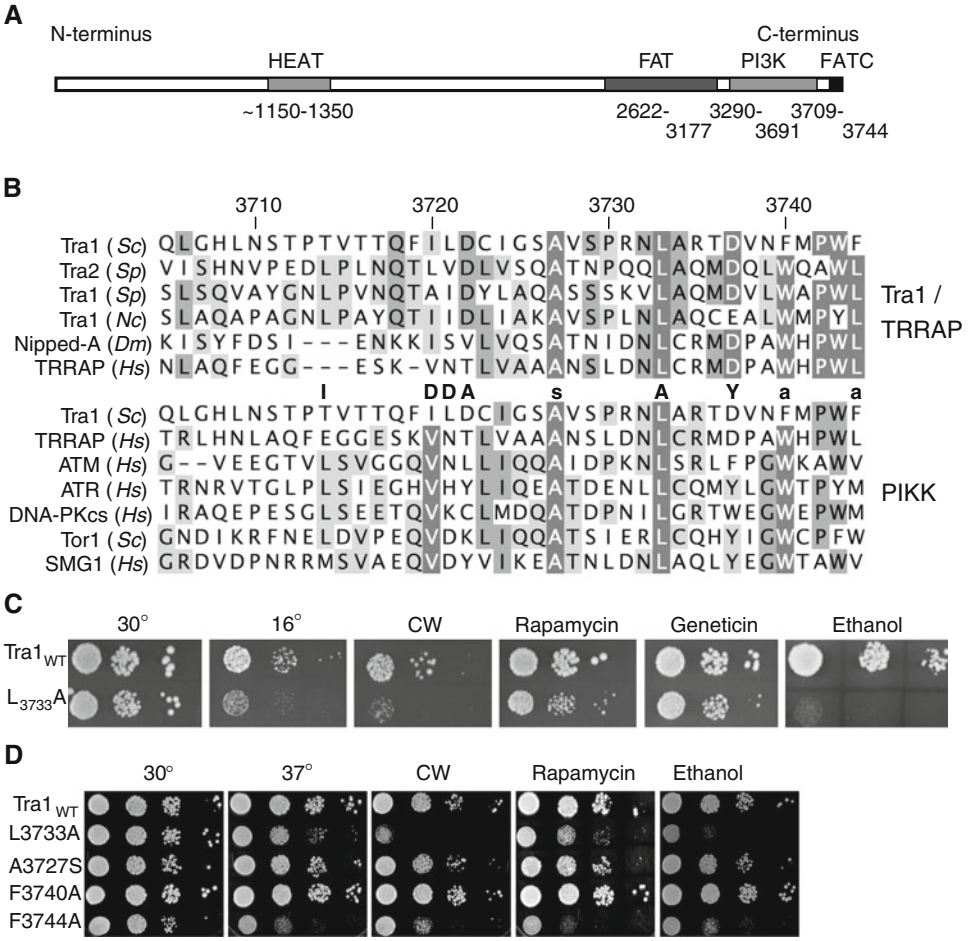


Fig. 1 FATC domain mutants. **a** Schematic diagram of the architecture of the Tra1/TRRAP family indicating the approximate positions of the HEAT, FAT, PI3 K, and FATC domains (Bosotti et al. 2000; Murr et al. 2007). Numbering is for the 3744 residues of *S. cerevisiae* Tra1. **b** Sequence alignment of FATC domains from PIKK family proteins. *Top panel* Sequences are *Saccharomyces cerevisiae* Tra1 (NP_011967), *Schizosaccharomyces pombe* Tra2 (Q10064), *Schizosaccharomyces pombe* Tra1 (NP_595777), *Neurospora crassa* Tra1 (CAC18279), *Drosophila melanogaster* Nipped-A (NP_001097192), *Homo sapiens* TRRAP (NP_003487). *Lower panel* Sequences from *Saccharomyces cerevisiae* Tra1 (NP_011967), Tor1 (CAA52849) and *Homo sapiens* TRRAP (NP_003487), ATM (Q13315), ATR (Q13535), DNA-PKcs (P78527) and SMG-1 (NP_055907). Residues indicated between the alignments in bold capitals are those changes that were analyzed through plasmid shuffling. Lower case letters indicate residues analyzed by gene integration, see c. Numbering is for *S. cerevisiae* Tra1. Alignments were performed using the default parameters of the ClustalW utility [http://www.ebi.ac.uk/clustalw/] and the terminal 49 residues of each protein. **c** Growth of the *tra1-L3733A* strain. Yeast strains containing centromeric plasmids expressing *tra1-L3733A* and $TRA1_{WT}$ were grown to saturation and ten-fold serial dilutions plated onto YP media containing 2% glucose and grown at 30° or 16°, or at 30° on YPD containing 5 μg/ml Calcofluor white (CW), 1 nM rapamycin, 20 μg/ml geneticin, or 4% ethanol. **d** Analysis of integrated *tra1* alleles. $TRA1_{WT}$, *tra1-L3733A*, *tra1-A2727S*, *tra1-F3740A* and *tra1-F3744A* were integrated into BY4743. Haploids containing the integrated allele were obtained after sporulation. Saturated cultures were serially diluted and plated onto YPD at 30° or 37°, or at 30° on YPD containing 5 μg/ml Calcofluor white (CW), 1 nM rapamycin, or 6% ethanol. We note that the BY4742/4741 background is less sensitive to ethanol than KY320, so ethanol sensitivity was assayed at a concentration of 6%

sensitivity to *tert*-butylhydroperoxide and tunicamycin were observed. In addition, defects in telomere maintenance or elongation were not observed using the plasmid linearization assay of Lundblad and Szostak (1989; not shown).

To examine the importance of the highly conserved alanine at 3727 and the hydrophobic residues, F3740 and F3744, we constructed yeast strains in which *tra1-A3727S*, *tra1-F3740A*, *tra1-F3744A,* as well as *tra1-L3733A* were integrated into the genome of the wild-type strain BY4741/4742 and analyzed growth under a variety of conditions (Fig. 1d). Similar to the plasmid copy, the integrated allele of *tra1-L3733A*, expressed from its native promoter, resulted in slow growth at 37° and in media containing Calcofluor white, rapamycin, and ethanol. The *tra1-A3727S* strain was slightly sensitive to each of these conditions; in comparison, the *tra1-F3740A* strain was relatively unaffected. Mutation of the terminal phenylalanine to alanine (F3744A) resulted in a general reduction in viability in all conditions assayed, including rich media at 30°. This

Table 3 Phenotypes of *tra1-L3733A* and *tra1-SRR3413* strains

Condition[a]	*tra1-L3733A*[b]	*tra1-SRR*[c]	*ada2Δ0*	*spt7Δ0*	*yng2Δ0*	*yaf9Δ0*
Ethanol	**S**[d, e]	S	**S**	**S**	N	N
Calcofluor white	**S**	S	**S**	**S**	s	N
Tunicamycin	**S**	N	**S**	s	N	N
Overexpression of VP16	N	N	R	N	N	S
tert-Butylhydroperoxide	**S**	N	N	**S**	N	N
Depleted of inositol	s	S	N	S	nd	nd
Benomyl	**S**	S	N	N	**S**	**S**[f]
Methylmethane sulphonate	N	s	N	N	S	N
Rapamycin	**S**	S	N	nd	**S**	**S**
Geneticin	S	S	N	N	N	s
Chloramphenicol	S	S	N	s	N	nd

[a] Growth was assessed at 30°C on YPD media containing 4% ethanol, 5 μg/ml Calcofluor white, 1.5 μg/ml tunicamycin, 1.0 nM rapamycin, 0.015% *tert*-butylhydroperoxide, 20 μg/ml benomyl, 0.025% methylmethane sulphonate, 20 μg/ml geneticin, or 0.6 μg/ml chloramphenicol

[b] Yeast strains (and their relevant controls) are the following: *tra1-L3733A,* CY3003 (CY2706)*; tra1-SRR,* CY1531 (CY1524); *ada2Δ0*, CY947 (KY320); *sptΔ0*, FY1093 (FY630); *yng2Δ0*, QY202 (QY204); *yaf9Δ0*, BY7240 (BY4741)

[c] *tra1-SRR3413,* see Mutiu et al. (2007b)

[d] *S* slow growth as compared with wild-type, *s* partial slow growth, *N* same sensitivity as wild-type, *R* resistant, *nd* not determined

[e] Phenotypes of *tra1-L3733A* shared with *ada2Δ0*, *spt7Δ0*, *yng2Δ0,* or *yaf9Δ0* are in bold

[f] Le Masson et al. (2003)

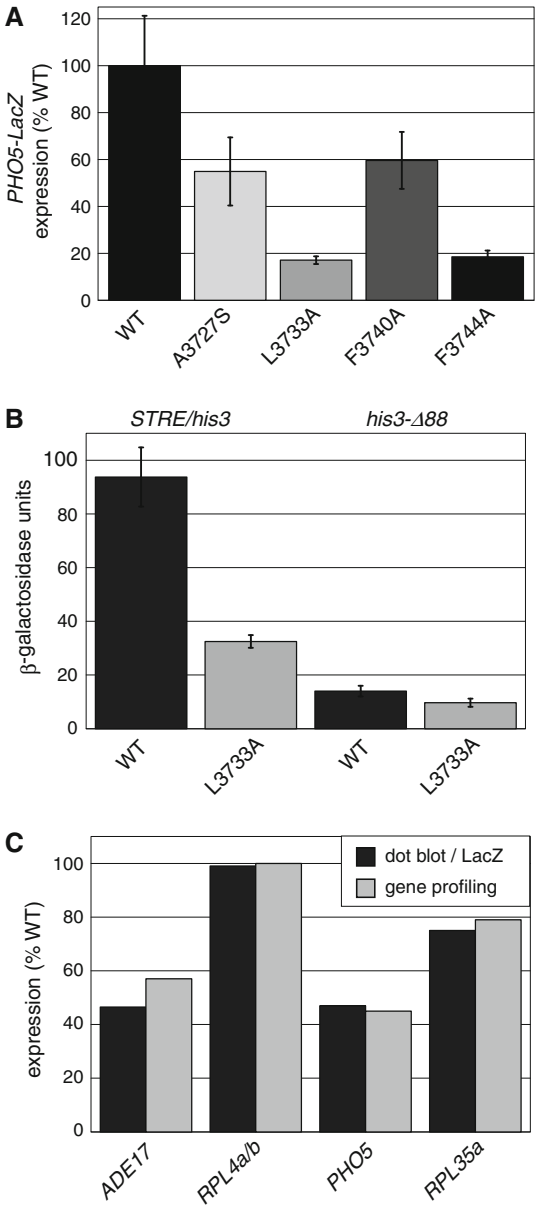
reduced viability demonstrates the importance of the terminal residue for Tra1 function and is consistent with the inability of *tra1-G3745* to support viability. Interestingly, *tra1-F3744A* did not result in as pronounced specific phenotypes as seen with *tra1-L3733A*. In fact, the *tra1-F3744A* strain was slightly less sensitive to Calcofluor white and ethanol than the *tra1-L3733A* strain.

Transcriptional effects of mutations within the FATC domain

The effects of the FATC domain mutations on transcription were initially assayed by determining the expression of the SAGA and NuA4-dependent *PHO5* promoter (Barbaric et al. 2003; Nourani et al. 2004). *LacZ* assays were performed under inducing conditions with the integrated *tra1* alleles (wild-type, L3733A, A3727S, F3740A and F3744A; Fig. 2a). The effect of these alleles on *PHO5* expression followed a similar pattern to their effects on growth. Tra1-L3733A and F3744A reduced *PHO5* expression to ~20% of wild-type. Tra1-A3727S and F3740A had a more modest effect, reducing expression to ~60% of wild-type. We note that this compares with *PHO5-LacZ* expression of <5% of the wild-type level seen upon deletion of either the SAGA component, Spt7 or NuA4 component, Yng2 (not shown).

The phenotypes of the *tra1-L3733A* strain suggested a partial inability to respond to environmental change and stress. Though multiple factors are involved in the cellular response to stress, a general stress response involves transcriptional induction upon binding of transcription factors Msn2 and Msn4 to stress response elements (STRE elements) (Gasch et al. 2000; Harbison et al. 2004; Martinez-Pastor et al. 1996). To determine if *tra1-L3733A* affected activation through STRE elements, we constructed a hybrid promoter containing two STRE elements at the position of the Gcn4-binding site in the *HIS3* promoter, and assayed transcription when cells were grown in YPD containing 4% ethanol. As shown in Fig. 2b, expression of *STRE/his3* was reduced threefold in the *tra1-L3733A* strain. The effect of the L3733A mutation was dependent on the stress response elements as the comparable promoter lacking the STRE elements (*his3-Δ88*) was only slightly affected.

To analyze for transcriptional effects of the *tra1-L3733A* allele on a broader scale, we compared the gene expression profiles of wild-type (CY2706) and *tra1-L3733A* (CY3003) strains grown in YPD using next generation sequencing. The full data set has been submitted to the Gene Expression Omnibus at the National Center for Biotechnology: accession number GSE18591. After normalization, expression of 11 genes was elevated ≥2-fold; 79 genes were decreased ≥2-fold (Table 4). While no over-riding patterns were apparent, of the 11 genes with elevated expression, *HSP26*, *PIR3*, *DDR2,* and *GRE1* have roles in the cellular response to stress. Confirmation of the general profile seen by sequencing was obtained by the analysis of *LacZ*-reporter fusions and dot blotting for selected genes as shown in Fig. 2c.



◀ **Fig. 2** Gene expression in the *tra1-L3733A* strain. **a** The promoter region of *PHO5* was cloned as a *LacZ* reporter fusion into the *LEU2* centromeric plasmid YCp87 and transformed into yeast strains CY4353 (*TRA1*), CY4318 (*tra1-A3727S*), CY4103 (*tra1-L3733A*), CY4324 (*tra1-F3740A*) and CY4350 (*tra1-F3744A*), containing a plasmid copy of YHR100C. β-Galactosidase activity was determined after growth in low phosphate media for 15 h at 30°. Expression is shown as a percentage of that found for CY4353. Measurements were made in triplicate with the standard deviation indicated. **b** Expression from stress response elements. A cassette of two STRE elements was cloned into the *Eco*RI and *Sac*I sites of *his3-Δ88-LacZ* (Brandl et al. 1993) to give *STRE-his3-LacZ*. These elements replace the normal Gcn4 binding site in *HIS3*. *STRE-his3-LacZ* and *his3-Δ88* were transformed into CY2706 and CY3003, and β-galactosidase assays performed after growth of cells in YPD containing 4% ethanol. **c** Expression levels determined by gene profiling (GP, mRNA-Seq) as compared with RNA dot blots (*ADE17* and *RPL4a/b*) and *LacZ* reporter fusions (*PHO5* and *RPL35a*). RNA was prepared from yeast strains CY3003 (*tra1-L3733A*) and CY2706 ($TRA1_{WT}$). mRNA-Seq libraries were constructed and sequencing performed on the Illumina/Solexa Genome Analyzer II platform. Comparisons between the strains were made as outlined in "Materials and methods". Similarly prepared RNA was spotted onto Hybond-N membrane and probed with single-stranded DNAs complementary to *ADE17* and *RPL4a/b* RNA. Hybridization was detected by autoradiography and quantitated using AlphaImager 3400 software and shown as a percentage of wild-type expression. Expression of *PHO5-LacZ* and *RPL35a-LacZ* fusion reporters were determined after growth of CY3003 and CY2706 in YPD. Assays were performed in triplicate

To address whether the expression changes determined for *tra1-L3733A* resembled patterns seen with other mutations, we performed a hierarchical cluster analysis with the compendium dataset of Hughes et al. (2000), the datasets of strains containing deletions of NuA4 (Krogan et al. 2004) and SAGA components (Ingvarsdottir et al. 2005), and with the dataset of the PI3K-domain mutation *tra1-SRR3413* (Mutiu et al. 2007a) (Fig. 3a). Of the approximately 300 comparisons in the analysis, the gene expression profile of the strain containing *tra1-L3733A* clustered closest to *tra1-SRR3413*. Other components of SAGA and NuA4 did not cluster within this leaf suggesting that the effects of *tra1-L3733A* result from the combined alteration of SAGA and NuA4 complexes and/or that Tra1 has one or more roles outside these complexes.

The genome-wide occupancy profile of myc_9-Tra1 was determined to help assess whether the effect of Tra1 on gene expression is direct. As shown in Fig. 3b, there is a positive correlation between genomic occupancy of Tra1 and transcriptional frequency (Holstege et al. 1998), suggesting that Tra1 is recruited to actively transcribed genes. In addition, there was a positive correlation for the top quartile of Tra1 binding and Fhl1 ($p = 2.2 \times 10^{-7}$) and Rap1 ($p = 1.1 \times 10^{-5}$), likely related to the involvement of these factors and the NuA4 complex in regulating expression of ribosomal protein genes (Lieb et al. 2001; Rudra et al. 2005; Reid et al. 2000).

Intragenic suppressors of *tra1-L3733A*

As a tool to evaluate mechanisms by which the L3733A mutation may affect Tra1 function, we selected random intragenic suppressor mutations that enable growth on media containing ethanol. A library of approximately 200 independent alleles was constructed by PCR mutagenesis of the C-terminal 2060 base pairs of the *tra1-L3733A* allele. Mutations N3677D and T3716A were able to partially suppress the slow growth in YPD containing 4% ethanol caused by the L3733A mutation (Fig. 4a) and restore transcription of *PHO5-LacZ* to approximately 70 and 80% of wild type, respectively (Fig. 4b). Both of the suppressor

Table 4 Expression of genes most altered in CY3003 (*tra1-L3733A*)

Locus	Gene	Fold change[a]	Description[b]
YDR070C	*FMP16*	3.1	Unknown
YPL187W	*MF(α)1*	3.0	Mating pheromone α-factor
YBR117C	*TKL2*	2.7	Transketolase: synthesis of aromatic amino acids
YBR072W	*HSP26*	2.6	Heat shock protein: chaperone activity
YKL163W	*PIR3*	2.4	Required for cell wall stability
YGR109C	*CLB6*	2.3	B-type cyclin: DNA replication
YHR033W		2.3	Unknown
YOL052C-A	*DDR2*	2.2	Multistress response protein
YPL223C	*GRE1*	2.2	Stress induced hydrophilin
YCR007C		2.0	Integral membrane protein
YPR203W		2.0	Unknown
YHR136C	*SPL2*	−10.4	Similar to cyclin-dependent kinase inhibitors
YDR281C	*PHM6*	−9.7	Unknown
YEL035C	*UTR5*	−8.0	Unknown
YAR064W		−4.3	Unknown
YAR071W	*PHO11*	−4.0	Acid phosphatase
YDR379C-A		−4.0	Unknown
YHR215W	*PHO12*	−3.7	Acid phosphatase
YML123C	*PHO84*	−3.1	Inorganic phosphate and manganese transporter
YKL053C-A	*MDM35*	−3.1	Mitochondrial intermembrane space
YBR147W	*RTC2*	−2.9	Similarity to G-protein coupled receptor
YDL098C	*SNU23*	−2.8	Component of U4/U6.U5 snRNP
YKL120W	*OAC1*	−2.7	Mitochondrial inner membrane transporter
YBR050C	*REG2*	−2.7	Phosphatase regulatory subunit
YNL155W		−2.7	Unknown
YHR081W	*LRP1*	−2.6	Exosome-associated nucleic acid binding protein
YGL009C	*LEU1*	−2.6	Leucine biosynthesis
YLR297W		−2.4	Unknown
YLR346C		−2.4	Unknown
YNR050C	*LYS9*	−2.4	Lysine biosynthesis
YHR055C	*CUP1-2*	−2.3	Metallothionein
YKL099C	*UTP11*	−2.3	Subunit of U3-containing small subunit processome
YGR129W	*SYF2*	−2.3	Pre-mRNA splicing
YDL182W	*LYS20*	−2.3	Lysine biosynthesis
YLL009C	*COX17*	−2.3	Copper metallochaperone
YLR262C	*YPT6*	−2.3	Ras-like GTP binding protein involved in secretion
YJR057W	*CDC8*	−2.3	Thymidylate and uridylate kinase
YDR471W	*RPL27B*	−2.3	Ribosomal subunit
YNL214W	*PEX17*	−2.3	Peroxisomal membrane peroxin
YBR093C	*PHO5*	−2.3	Acid phosphatase
YOL014W		−2.3	Unknown
YDR288W	*NSE3*	−2.3	Mms21-Smc5-Smc6 complex subunit
YLR159C-A		−2.2	unknown
YJR159W	*SOR1*	−2.2	Sorbitol dehydrogenase
YMR294W	*JNM1*	−2.2	Component of the dynactin complex
YJL190C	*RPS22A*	−2.2	Ribosomal subunit
YLR442C	*SIR3*	−2.2	Chromatin silencing
YHR053C	*CUP1-1*	−2.2	Metallothionein
YIR034C	*LYS1*	−2.1	Lysine biosynthesis
YJL200C	*ACO2*	−2.1	Putative mitochondrial aconitase
YOR044W	*IRC23*	−2.1	Unknown

[a] Values are the fold change in expression of CY3003 (*tra1-L3733A*) relative to CY2706 (*TRA1*) as determined from sequence profiling

[b] From the Saccharomyces Genome Database

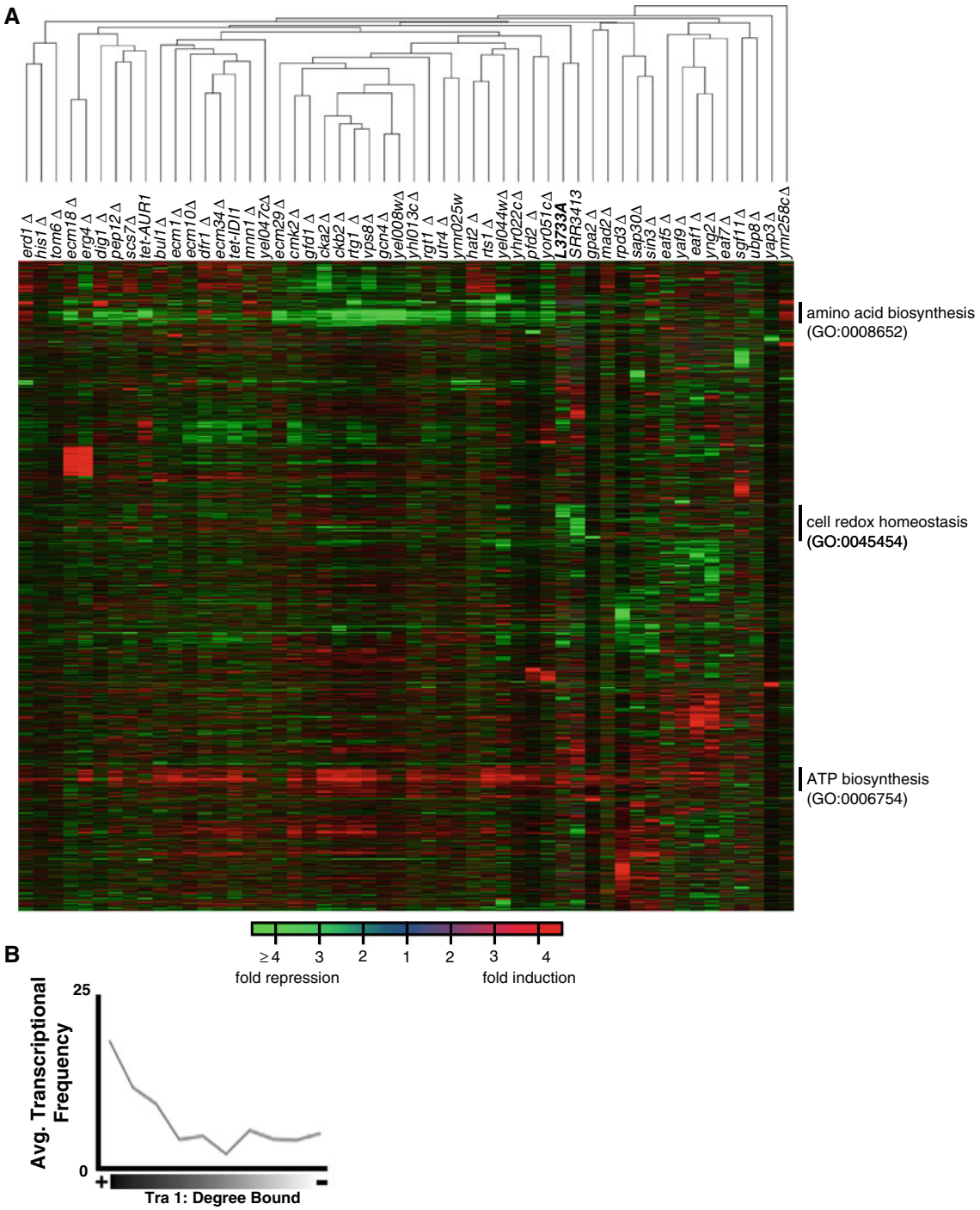


Fig. 3 Gene expression in the *tra1-L3733A* strain. **a** Hierarchical cluster analysis. Comparisons were initially made with the compendium data set (Hughes et al. 2000), profiles from strains with deletions of NuA4 (Krogan et al. 2004) and SAGA components (Ingvarsdottir et al. 2005), and with *tra1-SRR3413* (Mutiu et al. 2007a). The diagram shows those profiles clustering in closest proximity to *tra1-L3733A*. Gene families are indicated on the right. **b** Correlation between degree of Tra1-binding and average transcriptional frequency. Genome-wide localization studies were performed in triplicate for yeast strain CY2706 (*myc$_9$-TRA1*) grown at 30° in YPD, essentially as described (Yu et al. 2004). The average transcription frequency of each gene (Holstege et al. 1998) is plotted versus the relative binding of *myc$_9$-TRA1*

mutations occur at positions that are not highly conserved within the Tra1/TRRAP family. N3677 is at the C-terminal end of the PI3 K domain, while T3716 is within the FATC domain.

Expression of Tra1-L3733A and Tra1-G3745

We used Western blotting to determine the steady-state levels of N-terminally myc$_9$-tagged Tra1 in crude extracts of

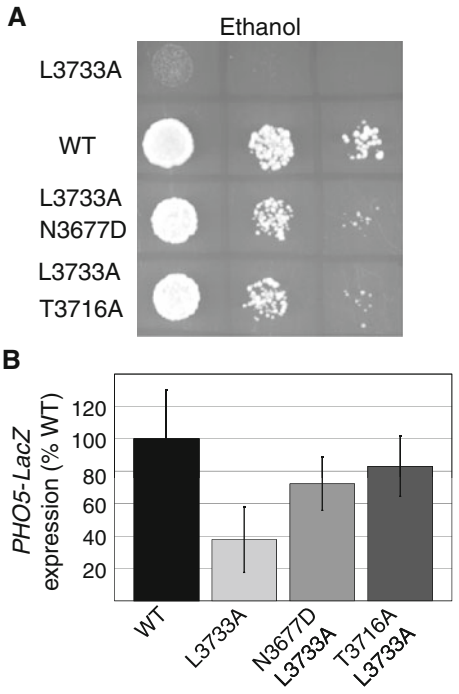


Fig. 4 Intragenic supressors of the *L3733A* mutation. **a** Yeast strains CY3003 (*tra1-L3733A*), CY2706 ($TRA1_{WT}$), CY4055 (*tra1-L3733A/N3677D*) and CY4056 (*tra1-L3733A/T3716A*) were grown in minimal media and tenfold serial dilutions spotted onto a YPD plate containing 4% ethanol. **b** *PHO5-LacZ* expression. The above strains containing YCp87-*PHO5-LacZ* were grown to saturation in minimal media lacking leucine, diluted sixfold in YPD depleted of phosphate and grown for 15 h at 30°. *β*-Galactosidase activity was calculated for each strain in triplicate and plotted as a percentage of that found for CY2706

yeast strains CY2706 ($Tra1_{WT}$ expressed from the *DED1* promoter) and CY3003 (Tra1-L3733A expressed from the *DED1* promoter). As shown in Fig. 5a, Tra1-L3733A was reduced compared with wild-type Tra1. The profile of proteolytic products also differed for the wild-type and mutant proteins (compare lanes 2 and 4). A similar reduction of Tra1-L3733A was seen when cells were disrupted under denaturing conditions (not shown). As shown in Fig. 5b and quantified in Fig. 5c, the second site mutations N3677D and T3716A partially restored Tra1 levels and to an extent that paralleled their restoration of function. This correlation suggests that the phenotype of *tra-L3733A* is related to the reduced steady-state level of the protein.

The cellular concentration of myc_9-Tra1-G3745 was analyzed in a strain containing untagged wild-type Tra1 since *tra1-G3745* does not support viability. The extreme slow growth of this strain made recovery of the protein difficult. We estimate that Tra1-G3745 was present at a level approximately 5% of wild-type (not shown) suggesting that the precise location of the C-terminal carboxyl group is critical for the stability of Tra1.

The NuA4 complex is localized to the *PHO5* promoter prior to gene activation (Nourani et al. 2004). As another measure of the relative level of Tra1-L3733A in vivo, we determined the extent of histone H4 acetylation at the *PHO5* promoter after growth of $TRA1_{WT}$ and *tra1-L3733A* strains in YPD. Chromatin immunoprecipitations were performed with anti-acetylated histone H4/K8 antibody and to allow normalization, with anti-histone H3 antibody. As shown in Fig. 5d, under conditions in which total histone H3 was relatively unchanged (lanes 2–4), the L3733A mutation reduced histone H4 acetylation at *PHO5* by approximately threefold (lanes 5–7).

If the effects of the L3733A mutation result primarily from decreased stability of Tra1, we would expect that reducing the wild-type protein would cause a similar phenotype. Cells containing wild-type Tra1 under control of the methionine-repressed *MET3* promoter (Mao et al. 2002) (*MET3*-$Tra1_{WT}$) were grown in minimal media with increasing concentrations of methionine and in the presence or absence of 3% ethanol. As shown in Fig. 6, in media lacking methionine *MET3*-$Tra1_{WT}$ supported growth in both media at a level comparable to *DED1*-expressed $Tra1_{WT}$. In as little as 5 μM methionine, reduced expression of *MET3*-$Tra1_{WT}$ resulted in decreased growth of the strain in YPD and increased sensitivity to ethanol, which resembled that seen for the L3733A mutation (expressed from the *DED1* promoter). We note that at elevated concentrations of methionine, fast-growing suppressors were evident with the strain containing *MET3*-$Tra1_{WT}$, likely arising from derepression of the *MET3* promoter or increased plasmid copy number. Given the number of generations required to obtain detectable amounts of Tra1, these suppressors made it difficult to compare the exact level of Tra1 in the presence of methionine.

Molecular interactions of Tra1-L3733A and Tra1-G3745

The ability of Tra1-L3733A to associate with SAGA and NuA4 components was compared to wild-type Tra1 and the phenotypically neutral Tra1-L3721D. myc_9-tagged Tra1-L3721D, Tra1-L3733A, and $Tra1_{WT}$ were expressed in a strain containing TAP-*ADA2* (Ghaemmaghami et al. 2003), and tandem affinity purification performed on crude yeast extracts. As shown in Fig. 7a, Tra1-L3721D and Tra1-L3733A co-purified with Ada2 at levels comparably to wild-type Tra1. Similarly, neither mutation affected interaction with TAP-Spt7 or with TAP-Esa1 (Fig. 7b). To determine if the L3733A mutation affects Tra1's ability to interact with transcriptional activators, we analyzed the binding of Tra1-L3733A to the activation domain of Gal4 ($Gal4_{AD}$). Myc_9-tagged $Tra1_{WT}$ and Tra1-L3733A were purified via association with TAP-tagged Ada2. The affinity-purified SAGA complex was then chromatographed on GST-$Gal4_{AD}$ columns and the association of Tra1 determined after elution with glutathione by Western blotting.

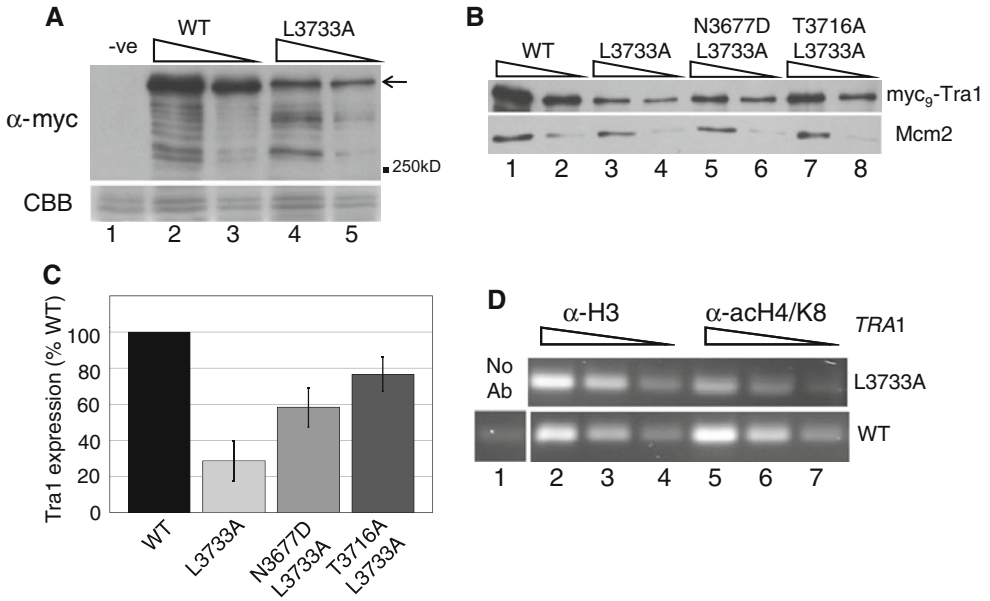


Fig. 5 Expression of the *tra1-L3733A* and *tra1-G3745* alleles. **a** Whole cell extracts were prepared from yeast strain KY320 (−ve, *lane 1*) or strains expressing myc_9-tagged versions of $Tra1_{WT}$ (*lanes 2, 3*) or Tra1-L3733A (*lanes 4*, *5*). Extract was separated by SDS PAGE (5%) and Western blotted with anti-myc antibody. The upper panel is the Western blot with anti-myc antibody. The full length myc_9-Tra1of ~450 kDa and the mobility of a 250-kDa marker protein are indicated with an *arrow* and *dot*, respectively. The *lower panel* is a portion of an equivalent gel stained with Coomassie Brilliant Blue (CBB). *Lanes 1, 3*, and *5* contain 50 μg of protein; *lanes 2* and *4*, 100 μg. **b** Levels of Tra1-L3733A suppressors. CY2706 (myc_9-$Tra1_{WT}$, *lanes 1*, *2*), CY3003 (myc_9-Tra1-L3733A, *lanes 3*, *4*), CY4055 (myc_9-Tra1-N3677D/L3733A *lanes 5*, *6*), and CY4056 (myc_9-Tra1-T3716A/L3733A *lanes 7*, 8) were grown to $A_{600} = 2.0$. Crude protein was isolated by bead lysis and 50 μg (even numbered lanes) and 100 μg (odd numbered lanes) was separated by SDS-PAGE and Western blotted with anti-myc or anti Mcm2 antibodies. Expression of *tra1-G3745*. **c** Quantitation of Tra1 levels. The Western blot as in B was performed in triplicate with independently grown cultures. Relative amounts of Tra1 were determined by densitometry using AlphaImager 3400 software. **d** Histone H4 acetylation. Chromatin was isolated from yeast strains CY2706 ($TRA1_{WT}$, *lower panel*) or CY3003 (*tra1-L3733A*, *upper panel*) grown to $A_{600} = 2.0$ in YPD. Chromatin was immunoprecipitated with antibody to histone H3 (*lanes 2–4*) and histone H4 acetylated at K8 (*lanes 5–7*), then analyzed by PCR with primers for *PHO5* promoter sequences. Consecutive samples represent twofold serial dilutions of the DNA to enable quantitation. *Lane 1* is a mock experiment with no histone antibody performed with a wild-type extract at 2× concentration

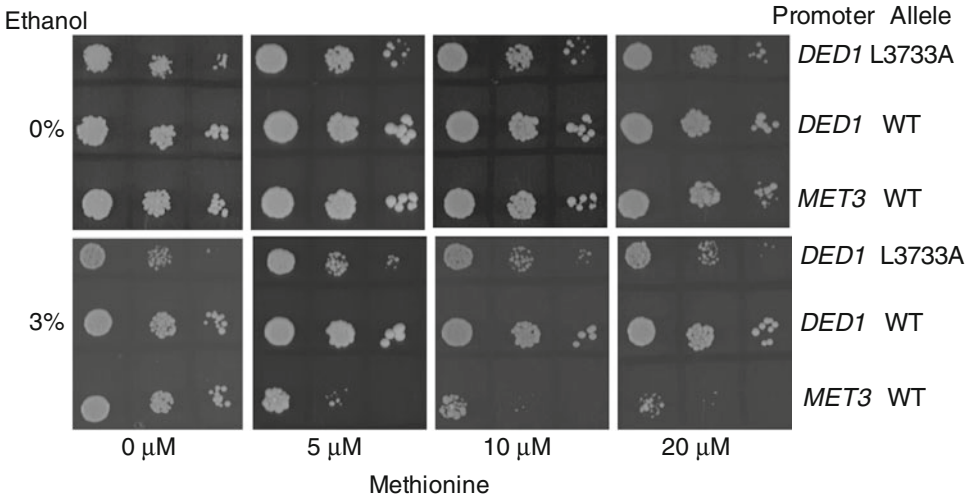


Fig. 6 Reduced expression of Tra1 results in ethanol sensitivity. Yeast strains CY3003 (*DED1* promoter: *tra1-L3733A*), CY2706 (*DED1* promoter*:* $TRA1_{WT}$), and CY3021 (*MET3* promoter*:* $TRA1_{WT}$) were grown in minimal media lacking methionine, and then serial dilutions were spotted onto minimal plates containing the indicated concentration of methionine and for the lower panel 3% ethanol

As shown in Fig. 7c, Tra1-L3733A interacted with the activation domain of Gal4 to approximately the same extent as wild-type Tra1.

Since *tra1-G3745* does not support viability, interaction of myc_9-Tra1-G3745 with TAP-tagged Esa1, Spt7, and Ada2, was analyzed in strains also containing untagged wild-type Tra1. Crude extracts were tandem affinity purified and the level of myc_9-Tra1 determined by Western blotting. After normalizing for the level of input Tra1, the amount of Tra1-G3745 co-purifying with Spt7 and Esa1

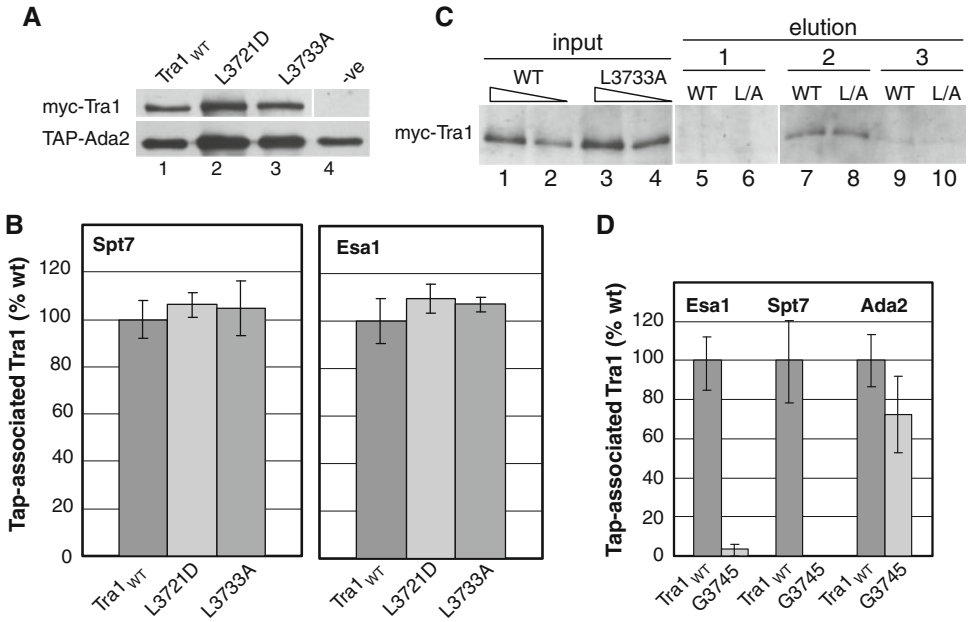


Fig. 7 Protein interactions of *Tra1-L3733A* and *Tra1-G3745*. **a** Interaction with Ada2. Crude extracts were prepared from an *ADA2-TAP* strain (Ghaemmaghami et al. 2003) expressing myc_9-$Tra1_{WT}$ (*lane 1*), myc_9-Tra1-L3721D (*lane 2*), myc_9-Tra1-L3733A (*lane 3*) or only the genomic *TRA1* (−ve, *lane 4*). Volumes corresponding to equal amounts of myc-tagged Tra1 were subject to tandem affinity purification and equal volumes Western blotted using anti-myc antibody (upper panel) or anti-CBP (*lower panel*). **b** Interaction with Spt7 and Esa1. Crude extracts of *SPT7-TAP* and *ESA1-TAP* strains expressing myc_9-$Tra1_{WT}$, myc_9-Tra1-L3721D, or myc_9-Tra1-L3733A were analyzed as above and scanned using AlphaImager 3400 software. The ratio of the Spt7 and Esa1-associated to input Tra1 is presented as percent of wild type. The experiments were performed in triplicate with the associated standard error indicated. **c** Interaction of Tra1-L3733A with GST-$Gal4_{AD}$. myc_9-$Tra1_{WT}$ or myc_9-Tra1-L3733A was expressed in yeast strain CY2998 containing TAP-tagged Ada2. After CBP affinity purification approximately equal amounts of $Tra1_{WT}$ (*lanes 1*, *2*) or Tra1-L3733A (*lanes 3*, *4*), as determined by Western blotting, were incubated with GST-$Gal4_{AD}$ bound to glutathione-Sepharose. After washing, three fractions were eluted with glutathione and Western blotted for the presence of $Tra1_{WT}$ (*lanes 5*, *7* and *9*) or Tra1-L3733A (*lanes 6*, *8* and *10*). The levels of bound $Tra1_{WT}$ and Tra1-L3733A correlated with the elution profile of GST-$Gal4_{AD}$ (not shown). **d** Tra1-G3745. Crude extracts were prepared from the *ESA1-TAP*, *SPT7-TAP,* or *ADA2-TAP* strains (Ghaemmaghami et al. 2003) expressing *myc_9-$TRA1_{WT}$* or *myc_9-TRA1-G3745*. Extracts were tandem affinity purified. Equal volumes were separated by SDS PAGE and Western blotted using anti-myc antibody to detect Tra1 or anti-CBP for Esa1, Spt7, or Ada2. AlphaImager 3400 software was used for densitometric analysis. The ratio between the purified and the input amounts is presented as percent of wild type. The experiments were performed in duplicate with the range as indicated

was diminished to <5%, of that found for wild-type Tra1 (Fig. 7d). Normal positioning of Tra1's C-terminal carboxyl group is thus required for formation of SAGA and NuA4 complexes. Interestingly, the additional C-terminal glycine only partially reduced (~70%) the interaction with Ada2, suggesting that Tra1 has more than one interaction site with components of SAGA.

Genetic interactions of *tra1-L3733A*

To investigate the relationship between the PI3K and FATC domains, we constructed the double-mutant allele, *tra1-SRR3413/L3733A* and examined its ability to support viability after plasmid shuffling in yeast strain CY1021. Transformation of a *TRP1* centromeric plasmid expressing Tra1-SRR3413/L3733A resulted in slow-growing colonies, suggesting a dominant negative effect of this allele (not shown). In addition, no colonies possessed the double-mutant allele (*tra1-SRR3413/L3733A*) after plasmid shuffling on 5-FOA. We conclude that the *tra1-SRR3413/L3733A* allele does not support viability and that the effects of PI3K and FATC domain mutations are additive.

tra1-L3733A was introduced into a group of the knockout collection of strains (Winzeler and Davis 1997) to examine genetic interactions with SAGA and NuA4 component genes (Table 5). Growth of the double mutants was compared with either single mutant on YPD and YPD containing 3% ethanol. (As shown above, in the BY4741/4742 strain background *tra1-L3733A* alone only causes a minor growth defect in 3% ethanol; sensitivity is seen at 6% ethanol.) In YPD media, synthetic slow growth/lethality was observed with deletions of some but not all components. *tra1-L3733A* was synthetically lethal with *spt20Δ* and severe slow growth was seen with *ada1Δ tra1-L3733A* and *eaf1Δ tra1-L3733A*. Ada1 and Spt20 have roles in the structural integrity of SAGA (Sterner et al. 1999; Wu and

Table 5 Growth of double mutants of *tra1-L3733A* with deletions of SAGA and NuA4 components

Deletion[a]	YPD[b]		YPD + 3% EtOH[d]	
	TRA1	*L3733A*	*TRA1*	*L3733A*
None (wt)	++++	+++	++++	+++
gcn5	+++	+/−	+	+/−
ada2	+++	+	+	+/−
ngg1	+++	+	+	+/−
spt3	+++	+++	+++	+
spt8	+++	+++	+++	+
ubp8	++++	+++	+++	+
sgf11	++++	+++	+++	++
sgf73	+++	+	+++	+/−
spt20	+	sl[c]	+/−	sl
ada1	++	+/−	+/−	−
sgf29	++++	++	+++	+
eaf1	++	+/−	+	−
eaf3	+++	++	++++	+
eaf7	+++	+++	+++	++

[a] Single mutants and the wild-type strain BY4741 (87) were obtained from Open Biosystems. Double mutants were made by mating of CY4057 or CY4103 with the indicated deletion strain from the consortium collection, followed by sporulation and selecting for HIS+ *Kan*^{r} spore colonies

[b] Strains were scored for relative growth on YPD or YPD plus 3% ethanol after 2 days at 30°. Scoring for the two sets of plates was done independently with ++++ being the most rapid growth observed in each condition

[c] Synthetic lethal, no HIS+ *Kan*^{r} spore colonies were obtained

[d] The *tra1-L3733A* alone in the BY4741/4742 strain background, only slightly affects growth in 3% ethanol; sensitivity is seen at 6%

Winston 2002), whereas Eaf1 is required for the integrity of the NuA4 complex (Auger et al. 2008; Babiarz et al. 2006). Similar synthetic lethality is found for double mutants of *ada1Δ* and *spt20Δ* with deletions of NuA4 components, and *eaf1Δ* with deletions of SAGA components (Lin et al. 2008, Mitchell et al. 2008). *tra1-L3733A* also resulted in synthetic slow growth in combination with disruptions of *gcn5*, *ada2*, *ngg1*, *sgf29,* and *sgf73*. In contrast, additive growth defects on YPD were not as pronounced with double mutants of *tra1-L3733A* with *spt3Δ*, *spt8Δ, sgf11Δ*, *ubp8Δ*, *eaf3Δ,* and *eaf7Δ*. Synthetic slow growth was observed for all the deletion combinations when cells were grown in media containing 3% ethanol. This implies that fully functional NuA4 and SAGA complexes are required under conditions of stress and that the effects of *tra1-L3733A* are additive with all functions of these complexes.

Larschan and Winston (2001) found that deletions of *hda1* and *nhp10* suppress phenotypes resulting from disruption of *spt20*, an integral component of the SAGA complex. To determine if the effect of *tra1-L37733A* is related to a similar loss of function as *spt20*, we analyzed whether *hda1Δ0* and *nhp1Δ0* suppress *tra1*-L3733A. Growth of double-mutant strains was analyzed on YPD at 16°, 30°, and 37° and YPD containing 6% ethanol (Fig. 8). Under none of these conditions was the slow growth caused by *tra1*-L3733A suppressed by deletion of either *hda1* or *nhp10*. This result supports the view that the phenotypes arising from *tra1*-L3733A are not due solely to Tra1's action in the SAGA complex.

Discussion

Tra1 functions revealed by FATC domain mutations

Our studies demonstrate the consequences of reduced Tra1 function on gene expression. Mutation of L3733A resulted in decreased activation of *PHO5* and *STRE/his3* promoters, and a twofold or greater change in expression of ~90 genes in rich media. The effects of *tra1-L3733A* are likely mediated through partial loss of both SAGA and NuA4 function rather than loss of either individual complex. This is consistent with the phenotypic similarities between the *tra1-L3733A* strain and strains with deletions of components of NuA4 and SAGA, the additive effects of these mutations, and the inability of the *tra1*-L3733A allele to be suppressed by deletion of either *hda1* or *nhp10*.

When considered together, the phenotypes displayed by the *tra1*-FATC mutant strains reveal a role for Tra1 in responding to a variety of stress conditions. Growth defects included temperature sensitivity and slow growth in media containing ethanol, calcofluor white, *tert*-butylhydroperoxide, and tunicamycin. The latter three indicate deficiencies in pathways required for cell wall integrity, response to oxidative stress and the unfolded protein response, respectively. A requirement for Tra1 in responding to nutrient levels is apparent from the sensitivity to rapamycin. As evident by the reduced expression from *PHO5* and *STRE/his3* promoters in the *tra1-L3733A* strain, the stress-related phenotypes may be due to the inability of the FATC mutants to activate the expression of genes required to manage the stress. This interpretation agrees with the general finding that many SAGA-regulated genes fall into the category of stress-induced (Huisinga and Pugh 2004).

The *tra1-L3733A* strain did not possess all the phenotype characteristic of deletions of SAGA and NuA4 components. This suggests that some activities of the SAGA and NuA4 complexes are relatively unaffected by reduced levels of Tra1. For example unlike strains with deletions of the *ADA* genes (Berger et al. 1992), the *tra1-L3733A* strain is sensitive to VP16 overexpression. This could occur if sensitivity to VP16 requires minimal SAGA-mediated acetylation, or alternatively, sensitivity can result from Gcn5 activity independent of Tra1. The possibility of the latter is

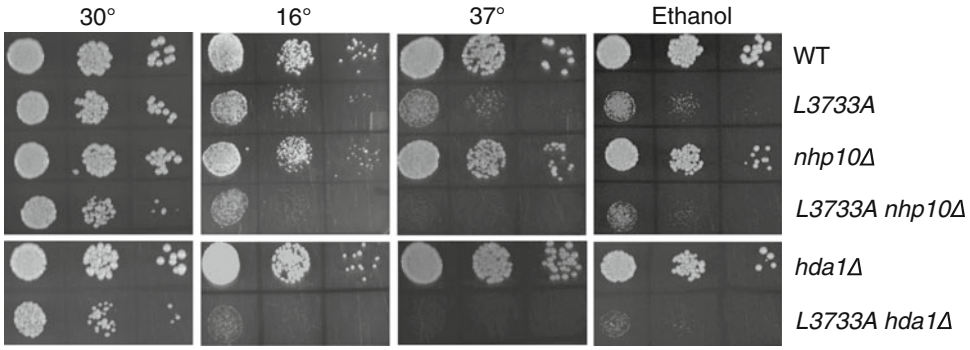


Fig. 8 Deletion of *hda1* or nhp10 does not suppress *tra1-L3733A*. Strains deleted for *hda1* or *nhp10* were generated by sporulation of diploid deletion strains obtained from Open Biosystems. Double mutants with *tra1*-L3733A were obtained by mating with CY4057 or CY4103 and sporulation. The wild-type strain BY4742, and the single- and double-mutant strains were grown in YPD and serial dilutions spotted onto YPD at 30°, 16° or 37°, or YPD containing 6% ethanol at 30°

consistent with biochemical evidence for an Ada complex (Eberharter et al. 1999; Saleh et al. 1997).

The FATC domain is required for Tra1 stability

We evaluated potential mechanisms for the decreased function of Tra1-L3733A. Since Tra1-L3733A interacted with the Gal4 activation domain and components of SAGA and NuA4 comparably to wild-type, the most straightforward explanation was its approximately fourfold reduced cellular concentration. Consistent with this model, similar growth defects are observed when the expression of wild-type Tra1 is decreased. Furthermore, intragenic suppressors of the L3733A mutation that increase activity restore Tra1 levels to a similar extent.

The reduced cellular concentration and altered proteolytic profile of Tra1-L3733A suggest a role for the FATC domain in maintaining the molecule's three-dimensional structure. Spagnolo et al. (2006) found that the FATC domain of DNA-PKcs is involved in a conformational change that place it in close proximity to HEAT repeat sequences found toward the N-terminus. We speculate that the FATC domain of Tra1 may directly or indirectly, have a comparable role in determining conformation and that destabilizing this structure would result in enhanced sensitivity to proteolytic cleavage. In such a model suppression of L3733A by T3716A and N3677D may occur through reducing proteoytic degradation, perhaps through stabilizing necessary molecular interactions.

To directly compare the half-life of Tra1-L3733A with wild-type Tra1 we analyzed protein levels after cycloheximide arrest of translation. Conclusions from these experiments were limited because of minimal turnover of Tra1 after cycloheximide arrest. Because of this we have also considered possible effects of the L3733A mutation on aspects of the expression of Tra1. It is unlikely that translational control is affected by L3733A because this would not easily account for the conservation of L3733 across species and in the PIKK family, the observed second-site suppression, or the altered proteolytic pattern. Nor does the inserted GCA codon show a negative bias. The level of Tra1-L3733A is also not likely the result of altered transcription since gene profiling data indicates only marginally reduced expression of *DED1* (the promoter for the plasmid copies of *TRA1*) and the phenotypes are observed when the L3733A mutation is expressed from its native promoter or the Met3 promoter (not shown). This being said, we cannot exclude contributions from more complex mechanisms: for example, the stabilization, processing or nuclear export of its mRNA transcript.

Dames et al. (2005) determined the structure of the Tor1 FATC domain in solution. They observed an extended α-helix that was interrupted by a hairpin followed by a candy cane-like loop for the terminal five residues, held in place by a disulfide bridge at positions that correspond to 3734 and 3741 of Tra1. In the Tor1 structure, the leucine equivalent to L3733 of Tra1 is positioned proximal to its terminal tryptophan where a hydrophobic interaction could potentially stabilize the conformation of the loop. We do not believe a similar structure exists for the FATC domain of Tra1 since the cysteines are not found in Tra1, nor is a glycine found within the loop that would facilitate the bend. In addition, while mutation of the terminal phenylalanine reduced growth, the phenotypic profile did not resemble *tra1-L3733A*. We also evaluated the possible importance of a hydrophobic patch created by extending the α-helix to the extreme C-terminus of Tra1 and the formation of a surface including L3733, F3740, and F3744. We conclude that if the extended helix is formed, the integrity of the hydrophobic patch is not likely important because mutation of F3740 to alanine did not result in obvious growth defects.

Integrity of the extreme C-terminus of Tra1 is essential for function

Being a 3744-residue protein, it seems unlikely that the C-terminus of Tra1 has a role in the innate folding of the protein. Rather, the reduced interaction of Tra1-G3745 with Esa1 and Spt7 support a model whereby the C-terminus is involved in protein–protein interactions necessary for function and stability of the protein. The extreme C-terminal sequences of Tra1 resemble the hydrophobic termini found for the interacting partners of PDZ domains (Tonikian et al. 2008) and the C-terminal phenylalanine of TraD, a protein required for bacterial F plasmid conjugation. Crystal structures of the TraD-TraM interaction show precise alignment of the TraD C-terminus with TraM (Lu et al. 2008). This interaction is disrupted by a glycine addition to TraD. The combination of the charged C-terminus on a hydrophobic residue creates a highly specific interaction site not otherwise found on the protein surface (Lu et al. 2008).

Sun et al. (2005) have shown that mutations within the FATC domain of ATM affect its interaction with Tip60. We analyzed whether a C-terminal fragment of Tra1 including PI3K and FATC domains is sufficient for interaction with Esa1, the yeast counterpart of Tip60, using both bacterially expressed proteins and two-hybrid analysis. In neither case was an interaction detected (not shown). This implies that other regions of Tra1 are also required for the interaction or that Esa1 is not a direct target of the FATC domain. Nevertheless, the finding that Tra1-G3745 associates poorly with Esa1 and Spt7 suggests that the C-terminus functions, at least in part, through protein–protein interactions.

tra1-G3745 and to a lesser extent *tra1-L3733A* act in a dominant negative fashion. This may seem inconsistent with their loss of function. We favor the idea that the dominant negative nature of these alleles is due to high levels of partial complexes and/or Tra1 proteolytic products since for *tra1-G3745* and *tra1-L3733A* there was an inverse relationship between the extent to which the allele was dominant negative and its cellular concentration.

Recently, Tra1 has been found associated with a group of proteins including Rvb1, Rvb2, Asa1, Tel2, Tti1, and Tti2, all of which are essential (Shevchenko et al. 2008). While the exact composition and function of this ASTRA (for ASsembly of Tel, Rvb and Atm-like kinase) complex (Shevchenko et al. 2008) is unknown, we cannot exclude the possibility that some of the deficiencies associated with the FATC mutants result from changes in their association with these proteins.

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