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Polyamines regulate their synthesis by inducing expression and blocking degradation of ODC antizyme

R Palanimurugan[1], Hartmut Scheel[2], Kay Hofmann[2] and R Jürgen Dohmen[1,]*

[1]Institute for Genetics, University of Cologne, Cologne, Germany and [2]Bioinformatics Group, Memorec Biotec GmbH, Cologne, Germany

Polyamines are essential organic cations with multiple cellular functions. Their synthesis is controlled by a feedback regulation whose main target is ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis. In mammals, ODC has been shown to be inhibited and targeted for ubiquitin-independent degradation by ODC antizyme (AZ). The synthesis of mammalian AZ was reported to involve a polyamine-induced ribosomal frameshifting mechanism. High levels of polyamine therefore inhibit new synthesis of polyamines by inducing ODC degradation. We identified a previously unrecognized sequence in the genome of *Saccharomyces cerevisiae* encoding an orthologue of mammalian AZ. We show that synthesis of yeast AZ (Oaz1) involves polyamine-regulated frameshifting as well. Degradation of yeast ODC by the proteasome depends on Oaz1. Using this novel model system for polyamine regulation, we discovered another level of its control. Oaz1 itself is subject to ubiquitin-mediated proteolysis by the proteasome. Degradation of Oaz1, however, is inhibited by polyamines. We propose a model, in which polyamines inhibit their ODC-mediated biosynthesis by two mechanisms, the control of Oaz1 synthesis and inhibition of its degradation.

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Introduction

Proteolysis by the 26S proteasome is the main pathway for ATP-dependent nonlysosomal degradation of intracellular proteins in eukaryotes (Hershko and Ciechanover, 1998). Usually, the substrates destined for degradation by this multi-subunit protease are marked by the attachment of polyubiquitin chains, which are recognized by binding sites in the 19S caps (Verma *et al*, 2004). Several exceptions to this principle of ubiquitin-dependent degradation by the proteasome have been reported. The first described and best-studied substrate of ubiquitin-independent degradation by the proteasome is ornithine decarboxylase (ODC) (Coffino, 2001b). Other examples are c-jun in mammals and Rpn4 in *Saccharomyces cerevisiae* (Jariel-Encontre *et al*, 1995; Xie and Varshavsky, 2001). In the latter two cases, however, ubiquitylation has been shown to be relevant to proteolysis *in vivo* (Treier *et al*, 1994; Ju and Xie, 2004). ODC thus remains the only characterized *bona fide* substrate of ubiquitin-independent degradation by the proteasome.

ODC is the rate-limiting enzyme in the biosynthesis of the polyamines spermine and spermidine (Coffino, 2001b; Wallace *et al*, 2003). It catalyses the conversion of ornithine derived from arginine into the diamine putrescine, which is converted to spermidine by spermidine synthase. Spermine is derived from spermidine by the action of spermine synthase (Wallace *et al*, 2003). These polyamines are essential organic polycations that have been implicated in stabilization of chromatin and the cytoskeleton, as well as in processes ranging from DNA replication, transcription and translation: ion transport, to the regulation of cell growth and apoptosis (Coffino, 2001b; Childs *et al*, 2003; Wallace *et al*, 2003).

The effects of lowering the intracellular production of polyamines have been extensively studied in transgenic models (Janne *et al*, 2004). Inactivation of the ODC gene in mice, for example, led to embryonic lethality (Pendeville *et al*, 2001). *S. cerevisiae* mutants lacking the *SPE1* gene encoding ODC are viable but cease to grow and become morphologically abnormal upon transfer to polyamine-free media (Schwartz *et al*, 1995). Studies that employed overexpression of genes involved in polyamine synthesis in mice on the other hand demonstrated that too high levels of polyamines are detrimental as well and result in a variety of defects including sterility and the promotion of malignant transformation (Janne *et al*, 2004). Consistent with the latter notion, elevated levels of ODC have been associated with cancer, and polyamine analogues as well as drugs that influence intracellular polyamine levels have been considered as anticancer agents (Childs *et al*, 2003; Wallace *et al*, 2003).

The intracellular concentration of polyamines is controlled at several steps, including their uptake and their biosynthesis. The latter is mainly achieved by controlling the cellular ODC activity via an unusual mechanism involving ODC antizyme (AZ) (Hayashi *et al*, 1996; Coffino, 2001b). This protein was first identified in mammals, where it is now known to exist in several isoforms (AZ1, AZ2, AZ3, AZ4) (Heller *et al*, 1976; Ivanov *et al*, 1998a, 2000). AZ1 disrupts enzymatically active ODC homodimers by forming ODC/AZ heterodimers (Mitchell and Chen, 1990; Li and Coffino, 1992). AZ1 binding moreover mediates ODC degradation by the 26S proteasome (Li and Coffino, 1992; Murakami *et al*, 1992; Elias *et al*, 1995). In contrast, binding of AZ2 does not result in degradation of ODC (Zhu *et al*, 1999). The modes of action of AZ3, whose expression appears to be limited to the testis, and of AZ4 have not been analysed in detail (Ivanov *et al*, 2000;

*Corresponding author. Institute for Genetics, University of Cologne, Zülpicher Str. 47, 50674 Cologne, Germany. Tel.: +49 221 470 4862; Fax: +49 221 470 1631; E-mail: j.dohmen@uni-koeln.de

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Coffino, 2001a). AZ1-dependent degradation of ODC was shown both *in vitro* and *in vivo* not to require its ubiquitylation (Rosenberg-Hasson *et al*, 1989; Murakami *et al*, 1992). It was reported that, instead, a C-terminal degradation signal in ODC is exposed upon AZ1 binding that mediates binding to a ubiquitin recognition site in the 19S cap of the proteasome (Zhang *et al*, 2003).

AZ levels increase with rising intracellular polyamine concentrations. Polyamine induction of AZ thus constitutes a feedback control in polyamine homeostasis. For mammals, it was shown that polyamines mediate increased AZ expression by promoting +1 ribosomal frameshifting during decoding of the mRNA (Matsufuji *et al*, 1995). ODC AZ is widespread among eukaryotes ranging from fungi to mammals. Despite the availability of the complete genome sequence of *S. cerevisiae* since 1996, previous attempts to identify a sequence encoding an ODC AZ orthologue in this model eukaryote have been unsuccessful (Zhu *et al*, 2000). Experimental data, however, indicated that regulation of ODC in *S. cerevisiae* appears to be similar to that in mammals in that treatment with polyamines induces its rapid degradation. These data suggested the presence of a functional analogue of AZ in *S. cerevisiae* (Toth and Coffino, 1999; Gupta *et al*, 2001).

In this study, we used a profile-based sequence analysis method to identify an *S. cerevisiae* ODC AZ orthologue, termed Oaz1. Oaz1 resembles its mammalian counterparts in that it mediates degradation of ODC and in that its synthesis involves polyamine-controlled ribosomal frameshifting. We show that polyamines in addition control the level of AZ by inhibiting its ubiquitin-dependent degradation, thereby providing a mechanism that allows cells to rapidly reinitiate polyamine synthesis after its transient inhibition by Oaz1.

Results

Identification of an ODC AZ homologue (Oaz1) in S. cerevisiae

The family of obvious AZ orthologues detectable in the databases currently comprises more than 40 members. An inspection of their alignment revealed a conserved architecture with two homologous regions that are separated from each other by divergent linker sequences of variable length (shown in Figure 1C for selected AZ orthologues). A conserved N-terminal sequence (D1) is centred at the +1 frameshifting site present in all AZs (Figure 1C). The larger C-terminal region D2 has been linked to ODC binding and degradation (Ichiba *et al*, 1994; Chen *et al*, 2002).

In order to identify a sequence encoding a presumptive ODC AZ in *S. cerevisiae*, we searched its genome with generalized profiles that were based upon a multiple alignment of AZ sequences derived from various fungal species. The generalized profile method constitutes a sensitive means to identify distant homologues based on family-wide conserved sequence features (Bucher *et al*, 1996; see Supplementary data). After a few iterations of profile construction, we found the previously uncharacterized *S. cerevisiae* open reading frame (ORF) 'YPL052w' to encode a polypeptide related to C-terminal parts of AZs in other species. Obvious orthologues of 'YPL052w' are also present in the genome of other closely related Hemiascomycetes (Figure 1 and Supplementary Figure S1). Upon close inspection, the genomic locus of 'YPL052w' and its upstream sequences exhibit several features that are strikingly similar to those found in known AZ genes (Figure 1A). A putative ATG start codon is located 274 base pairs upstream of the annotated start codon of ORF 'YPL052w'. The upstream ATG represents the translational start site of a short ORF that ends with a TGA stop codon after 207 bases. This ORF, which we refer to as 'ORF1', is too short to be annotated in the *Saccharomyces* genome database. The stop codon is embedded in a sequence stretch of ~20 nucleotides, which is well conserved around the established ribosomal frameshifting site in mammalian AZ mRNAs (Figure 1B). If an analogous +1 ribosomal frameshifting that results in the omission of the U in the UGA stop codon in the mRNA is assumed in *S. cerevisiae,* translation would shift to a second reading frame (ORF2) to produce a putative 292 residue polypeptide. The C-terminal 202 amino acids of this protein are encoded by the annotated ORF 'YPL052w'. In support of the inferred frameshifting site, the resulting protein sequence that is encoded by the sequence encompassing this site exhibits a striking similarity to the D1 motif in known AZs (Figure 1C). The presumed AZs of *S. cerevisiae* and its relatives moreover display homology to the D2 domain of established AZ orthologues from other eukaryotes, which is likely to have an αβα fold (Figure 1C). Taken together, these data led us to hypothesize that *S. cerevisiae* ODC AZ is encoded by a locus, termed *OAZ1* for ODC AZ, which extends from the beginning of ORF1 to the end of 'YPL052w' on chromosome XVI. According to this hypothesis, and similar to its counterparts in other eukaryotes, synthesis of Oaz1 in *S. cerevisiae* would also involve a ribosomal frameshifting event.

Oaz1 mediates degradation of ODC by the proteasome

To test our hypothesis that *S. cerevisiae OAZ1* encodes a putative ODC AZ experimentally, we first asked whether this gene, as some of its counterparts in other eukaryotes including mammals (see Introduction), is indeed required for the regulated turnover of ODC. To address this question, we generated genomic tags, leading to an expression of ODC marked with three copies of the ha epitope at the C-terminus. These tags were introduced into a wild-type strain and a congenic strain deleted for sequences encompassing the annotated ORF 'YPL052w', which encodes about two-thirds of the presumptive Oaz1. The steady-state levels of ODC in these two strains grown with and without treatment with 100 μM of the polyamine spermidine for 3 h were determined by anti-ha Western blot analysis (Figure 2A). Quantification of the signals revealed that the wild type (wt) contained only ~50% of the ODC as compared to the *oaz1*-Δ strain (Figure 2B). The presence of 100 μM spermidine in the culture medium resulted in a further reduction of ODC levels in wt to ~12%, whereas no changes were observed in *oaz1*-Δ. Next we studied the kinetics of ODC disappearance after addition of spermidine to a culture of wild-type cells. ODC levels dropped rapidly to an extent that they were below detection already 60 min after spermidine addition (Figure 2C). In the *oaz1*-Δ mutant, in contrast, ODC levels were not affected by spermidine. We conclude that the presence of Oaz1 is required for controlling the levels of ODC in response to changes in polyamine concentrations, consistent with its presumed function of an ODC AZ and a role in ODC turnover. To verify that the observed effect reflects differences of ODC

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ATGTATGAAGTAATACAGAAAAGGAAAACAAAAATAATAAACGTTTTACAGAGTCCTGAACTCATGAGGCTCATAGAGGACCCATCAAATCTGGGTATT  99 ORF1
M  Y  E  V  I  Q  K  R  K  T  K  I  I  N  V  L  Q  S  P  E  L  M  R  L  I  E  D  P  S  N  L  G  I    33

TCTTTACATTTTCCAGTAAGTTCACTGCTAAAAAGTAATAAGTGCACACCAATGCCTAAACTTTCTACGTATAGTTTGGCTAGTGGGGGATTTAAGGAT 198
S  L  H  F  P  V  S  S  L  L  K  S  N  K  C  T  P  M  P  K  L  S  T  Y  S  L  A  S  G  G  F  K  D    66

TGGTGCGCGTGACATCCCTCTAGACGTTCCACCAGAGATTGATATCATCGATTTTTACTGGGATGTTATTTTATGCATGGAATCTCAATTCATATTAGA 297 ORF2
W  C  A  *
          D  I  P  L  D  V  P  P  E  I  D  I  I  D  F  Y  W  D  V  I  L  C  M  E  S  Q  F  I  L  D    99

TTACAATGTTCCGTCAAAAAATAAGGGGAACAATCAGAAGTCTGTTGCTAAGCTGTTGAAAAATAAGCTTGTAAACGATATGAAAACTACGTTAAAAAG 396
 Y  N  V  P  S  K  N  K  G  N  N  Q  K  S  V  A  K  L  L  K  N  K  L  V  N  D  M  K  T  T  L  K  R 132

ACTAATTTATAATGAAAATACCAAGCAATATAAAAATAATAATAGCCACGATGGTTACAATTGGAGAAAACTAGGCTCGCAGTATTTCATACTGTATCT 495
 L  I  Y  N  E  N  T  K  Q  Y  K  N  N  N  S  H  D  G  Y  N  W  R  K  L  G  S  Q  Y  F  I  L  Y  L 165

TCCCCTATTTACGCAGGAACTGATTTGGTGTAAACTTAATGAAAACTATTTCCATGTTGTATTACCATCTTTACTGAATAGTAGGAACGTTCATGATAA 594
 P  L  F  T  Q  E  L  I  W  C  K  L  N  E  N  Y  F  H  V  V  L  P  S  L  L  N  S  R  N  V  H  D  N 198

CCACAGTACCTATATAAATAAAGATTGGTTACTTGCCCTTTTAGAGCTAACTTCCAACCTGAACCAAAACTTCAAATTCGAATACATGAAATTGAGATT 693
 H  S  T  Y  I  N  K  D  W  L  L  A  L  L  E  L  T  S  N  L  N  Q  N  F  K  F  E  Y  M  K  L  R  L 231

GTATATTTTAAGAGATGATTTAATTAATAATGGTTTGGATCTTTTGAAAAATCTTAACTGGGTCGGTGGGAAACTGATTAAAAATGAAGATAGAGAAGT 792
 Y  I  L  R  D  D  L  I  N  N  G  L  D  L  L  K  N  L  N  W  V  G  G  K  L  I  K  N  E  D  R  E  V 264

CTTGTTGAACTCGACCGATTTAGCTACGGATTCTATTTCTCATTTATTAGGTGATGAAAACTTTGTTATTTTAGAGTTTGAATGCTAA           880
 N  L  L  S  T  D  L  A  T  D  S  I  S  H  L  L  G  D  E  N  F  V  I  L  E  F  E  C  *             292
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B

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                                                       ▼
Saccharomyces cerevisiae   AGTTTGGCTAGTGGGGATTTAAGGATTGGTGCGCGTGACATCCCTCTAGACGTTCCACC
Saccharomyces paradoxus    AGTTTGACTGGCGGCGGCTTTAAGGATTGGTGCGCGTGACATCCCTCTAGACCTTCCTCC
Saccharomyces bayanus      AGTTTGACTAGTAGTGGGTTTAAGGATTGGCGCGCGTGACGTCCCTTTAGATCTTTCTGC
Saccharomyces mikatae      AGTTTGGCTAGCTGCGGCTTTAAGGATTGGCGCGCGTGACATCCCTTTGAATATTCCTCT
Saccharomyces castellii    TCTTTAACGAGCAGTGGATTTAAGGATTGGTATGCGTGACATCGGAGACGGGATCATCTT
Saccharomyces kluyverii    TACACTTTGAAAGATGGCACCTACCAGTGGTGTCCGTGACATGAGAGCCACTTTTATGCC
Kluyveromyces waltii       TACACTGTGCGGATGGGTGTGAGGAGTGGTGCCCGTGACACCGGACCTTGGATTGACTA
Ashbya gossypii            TGCTCCTCGAGGACCGGGTACGCGGAATGGTTGGCGTGACAGGGGGGCGGGGGCGCAGTC

S. pombe                   GGCTCTACGCCGGCAGGGGGCGCGGAATGGTGCTCCTGAGGCGCTTGAGCGCTCAAGACC AF217277
Neurospora crassa          TGCACTACCGGCGTCACAGGGGCCGAGTGGTATTCCTGAGGTCCCTTCGACGGGACTTCC BX294028
C. elegans                 TGCTCGACCCAGCCCGGCGACGTCGGATGGTGTTTTTGACGCCCCCCATGGCGTGCTCAC AF217278
D. melanogaster            CGCATCTCTCTCGGCGTAGGGCCTCTGTGGTGGTCCTGATGTCCCTGTCCACCACAGAAC U29529
Homo sapiens AZ1           TGTAGTAACCCGGGTCCGGGGCCTCGGTGGTGCTCCTGATGCCCCTCACCCACCCCTGAA U09202
Homo sapiens AZ2           TGCAGGCACATTGTTCCAGGGCCTCTGTGGTGCTCCTGATGCCCCTCACCCACTGTCGAA AF057297
Homo sapiens AZ3           TTGACACTCCAGCCCCGTTCCTGCCTCCAGTGCTCCTGAGTCCCTATTACCTTTACTGTT AF175296
Homo sapiens AZ4           TGTCGTCACCCGGGTCCGGGGCCTCGGTGGTGCTCCTGATGTCCCTCACCCACCCCTGAA AF293339
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C

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               --------D1---------          -----------------------------------------------D2---------------------------------------
PHD            LL.LLLLLLL...LLLLL           .....       EEEE.LLLLLL........HHHHHHHHHHHH       ....EEEEEEELLL.......HHHHHHHHH.LL..L
                             ▼
S.cer.  56 TYSLASGGFKDWCADIPL.( 78).NWRKL.( 5).ILYLPLFTQELIW.(30).KDWLLALLELTS.(6).KFEYMKLRLYILRDDL.(4).LDLLKNLNWVGGKLI
S.par.  56 TYSLTGGGFKDWCADIPL.( 78).NWRNL.( 5).ILYLPLFTQELIW.(30).KDWLLALLELTS.(6).KFEYMKLRLYILRDDL.(4).LDLLKNLNWVGGKLI
S.bay.  56 AYSLTSSGFKDWRADVPL.( 78).NWRNL.( 5).ILYLPLFTQELIW.(30).KDWLLALLELTS.(6).KFEYMKLRLYILRDDL.(4).LDLLKNLNWVGGKLI
S.mik.  56 TYSLASCGFKDWRADIPL.( 78).NWRNI.( 5).ILYLPLFSQELIW.(30).KDWLLALLELTS.(6).KFEYMKLRLYILRDDL.(4).LDLLKNLNWVGGKLI
S.cas.  59 SYSLTSSGFKDWYADIET.( 60).SWRGL.( 5).MVYLPLIFDDLIW.(28).KEWLLSLLELSN.....SLDLQYLRLYLRRNDS.(5).TTLLRNLNWIGGRIL
S.klu.  53 HYYTLKDGTYQWCPDMSH.( 73).LWRQV.( 5).VLYNPSLFNMPLW.(19).KQLLLSLLEYAD.....MVSVQFLRIYLMREFQ.(2).KELLHNLNWLGGYMV
K.wal.  54 YYYTVRDGCEEWCPDTDL.( 64).VWRQI.( 5).LLFCPEWAQRPIW.(20).KQLLMSLLEYAA.....TLDLTWLRLSLVRDVA.(2).RELLRNLSWLGGRVV
A.gos.  50 YRCSSRTGYAEWLADRAG.( 88).KWVQS.( 5).VLMDERAAGRVDL......KRWLVMMMELVD.(4).LRGCQALQVYVSREDL.(3).KDLLKNLNWIGGELV

S.pom.  54 VYGSTPAGGAEWCSEALE.( 66).YWHGI.(10).LFLIPESWEDVHL......KEGLVAIIDLAV.(1).RLHCSKLVLFVDKNNS.(2).PYLVKSLHWVGFEPL
N.cra.  53 HYCTTGVTGAEWYSEVPS.(155).LWDYV.( 7).VVAEEMETGEKTL.(14).KKALVALIELAD.(1).PLACSHMVICLDRSIP.(4).VPLMKGLQWAGFSMT
C.ele.  29 SPCSTQPGDVGWCFDAPH.( 19).NWRVT.( 7).AIMIPHDQPVLGI.( 1).KKNFVDLLEFAE.(3).EMERVLAVFEKARINP.(1).EGFPRTLRYVGFRPY
D.mel.  42 SYRISLGVGPLWWSDVPV.( 91).NWNTI.( 8).YVALPKDLPPAGS......KQTFISLLEFAE.(3).EVDGIVMVMPKDQPDR.....ARLIEAFLFMGFEPL
AZ1     55 HCCSNPGPGPRWCSDAPH.( 56).NWRTV.( 6).YIEIPGGALPEGS......KDSFAVLLEFAE.(3).RADHVFICFHKNREDR.....AALLRTFSFLGFEIV
AZ2     19 QCCRHIVPGPLWCSDAPH.( 55).SWDAV.( 6).FVEIPDGLLADGS......KEGLLALLEFAE.(3).KVNYVFICFRKGREDR.....APLLKTFSFLGFEIV
AZ3    203 EDLTLQPRSCLQCSESLV.( 52).HWHGL.( 6).FLDIPYQALDQGN......RESLTATLEYVE.(3).NVDSVFVNFQNDRNDR.....GALLRAFSYMGFEVV
AZ4     53 HGCRHPGPGPRWCSDVPH.( 56).SWRAV.( 6).YVELPAGALPEGC......KDSLAVLLEFAE.(3).RAEHVFICFPKNREDR.....AALLRTFGFLGFELV
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Figure 1 Sequence analysis of *S. cerevisiae* Oaz1. (**A**) Coding sequence of the *OAZ1* genomic locus together with the encoded polypeptides. ORF1 including a TGA stop codon (shown in bold) encompasses the sequence until position 210. Upon a predicted ribosomal frameshifting resulting from skipping the first nucleotide in this stop codon (marked by an asterisk), translation continues in the +1 frame (ORF2). The ATG codon of the annotated ORF 'YPL052w' is shown in bold. (**B**) Alignment of genomic or cDNA sequences encompassing the frameshifting sites in established AZ genes (lower part, accession numbers are given on the right side), as well as of previously unrecognized orthologues in Hemiascomycetes (upper part, sequences found as homologues of YPL052w in the *Saccharomyces* genome database, www.yeastgenome.org). A triangle marks the base that is presumed to be skipped during +1 ribosomal frameshifting. Conserved positions are printed on black background. (**C**) Alignment of AZs orthologues from the same species as in (B). A triangle indicates the position of the frameshifting site. Conserved residues are printed on black background, and positions assigned to amino acids with similar physicochemical properties are shaded in grey if supported by at least 50% of a total of 40 AZ family members that were compared in this analysis (only eight are shown). The sequences of a region termed D1 were aligned such that the frameshifting sites occupy equivalent positions. In the top row, the secondary structure as calculated via PHD (Rost, 1996) is presented. Only positions with an expected average accuracy >82% were considered. The abbreviations denote the following secondary structure types: E, extended (beta-sheet), A, alpha-helix and L, loop.

half-life, we performed pulse-chase analyses to determine ODC turnover rates in the same strains. As shown in Figure 2D and quantified in Figure 2E, addition of spermidine to the culture of the wild-type strain resulted in the induction of a rapid turnover of ODC ($t_{1/2}\sim 9$ min). Deletion of *OAZ1* resulted in a drastic stabilization of ODC, with spermidine

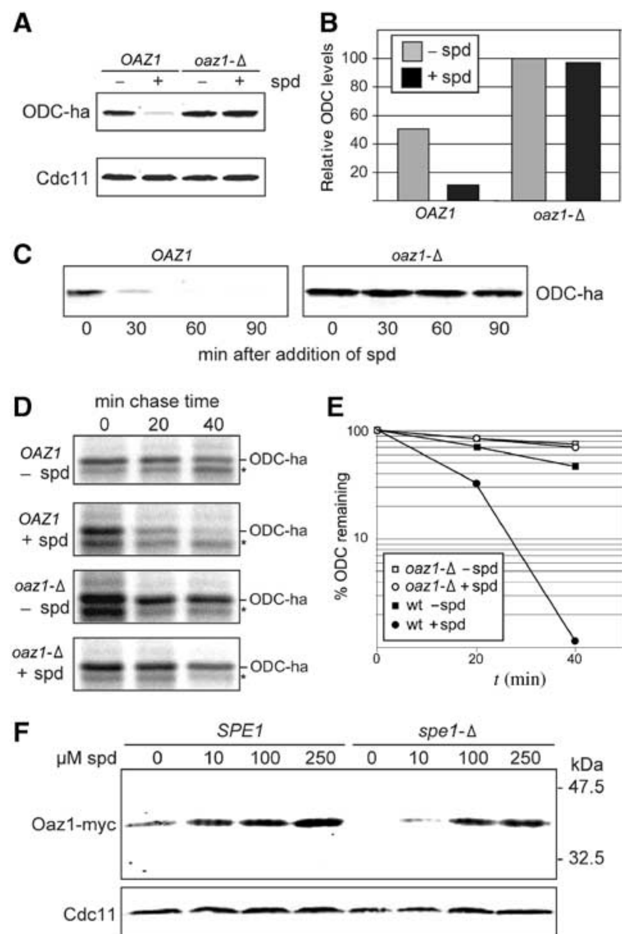

Figure 2 Oaz1 mediates degradation of ODC. (**A**) Steady-state levels of ODC-ha in strains PMY1 (wt) and PMY2 (*oaz1*-Δ) grown for 3 h in the absence (−spd) or presence of 100 μM spermidine (+spd) were analysed by anti-ha Western blotting. The blot was simultaneously probed with anti-Cdc11 antibodies to control for differences in protein loading. (**B**) Quantitation of fluorescence signals shown in (A). Values were normalized using the data obtained for Cdc11 and are given in % of the signal detected for *oaz1*-Δ grown in the absence of spermidine, which was set to 100%. (**C**) 'Spermidine chase' of ODC-ha. ODC-ha in cell extracts was detected as in (A), but at the indicated time points after adding spermidine to a concentration of 100 μM to the media. (**D**) Pulse-chase analysis of ODC turnover. The same strains as in (A) were grown for 3 h in the absence or presence of 100 μM spermidine before labelling. An asterisk marks the position of a nonspecific band. (**E**) Quantitation of radioactive ODC-ha signals shown in (D), which were normalized using data for nonspecific background bands. (**F**) Spermidine induction of Oaz1-myc. Extracts from chromosomally tagged wt or *spe1*-Δ (=*odc*-Δ) strains (Table I) were analysed by anti-myc Western blotting. Both strains were incubated in the presence of the indicated concentrations of spermidine in the media.

having no effect on its stability. The observation that spermidine-induced degradation of ODC required the presence of the *OAZ1* gene suggested that levels of Oaz1 are controlled by polyamines. To test for spermidine induction of Oaz1, we fused a sequence encoding three copies of the myc epitope in frame to the 3′ end of ORF2 on chromosome XVI. When extracts of the so-marked strain were assayed with anti-myc antibodies, a protein with an apparent molecular weight of ∼40 kDa was detected (Figure 2F). The size is consistent with the calculated molecular weight of the predicted Oaz1-myc_3 polypeptide encoded from the inferred ATG start codon of ORF1 to the end of ORF2 (34 kDa) plus the C-terminal myc tag (∼6 kDa). The level of Oaz1-myc_3 increased when spermidine was added to the culture media. The effect was even more striking when the *spe1*-Δ mutant lacking ODC was used in the experiment. In the absence of supplemented spermidine, this strain, which is unable to synthesize spermidine, did not contain detectable amounts of Oaz1 (Figure 2F). Addition of high amounts of spermidine, however, also resulted in a strong induction of Oaz1 in this mutant. Together, these results demonstrated that a regulation of Oaz1 levels is underlying the observed effects of spermidine on ODC stability.

Consistent with previous reports (Elias *et al*, 1995; Gandre and Kahana, 2002; Hoyt *et al*, 2003), we observed that an intact proteasome is required for spermidine-induced degradation of ODC. In strains carrying either the *cim3-1/rpt6* mutation affecting an essential ATPase subunit (Rpt6) of the 19S activator complex of the 26S proteasome, or in *pre1-1* and *pre2-1* mutants, in which the β4 or β5 subunits of the 20S proteasome core are affected, spermidine-induced degradation of ODC was severely impaired (Figure 3A). We conclude that Oaz1 is required for spermidine-induced degradation of ODC by the proteasome establishing its role as an ODC AZ in *S. cerevisiae*. Degradation of ODC in mammals has been the paradigm of ubiquitin-independent degradation by the proteasome. It has been reported recently that degradation of

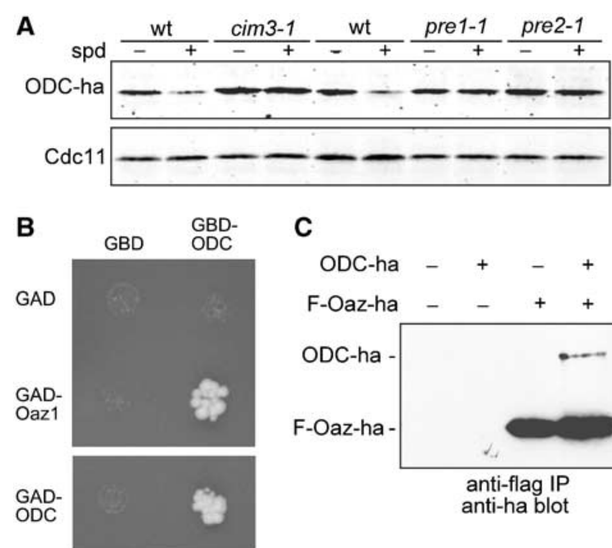

Figure 3 ODC binding to Oaz1 underlies its degradation by the proteasome. (**A**) Steady-state levels of ODC-ha expressed from P_{ODC} in the centromeric plasmid pPM67 in wt or proteasome mutants grown for 3 h in the absence (−spd) or presence of 100 μM spermidine (+spd) were analysed by anti-ha Western blotting. Lower part, anti-Cdc11 loading control. (**B**) Two-hybrid interaction of Oaz1 and ODC. Strain PJ64-4A was transformed with plasmids expressing Oaz1 or ODC as fusions to the Gal4-activating domain (GAD) or the Gal4 DNA-binding domain (GBD). Plasmids expressing just GAD or GBD were used as controls. Interaction was assayed on SD media lacking histidine to monitor the expression of a *HIS3* reporter gene that is under the control of P_{GAL1}. Colony growth indicates interaction. (**C**) ODC coimmunoprecipitates with Oaz1. Extracts from strain YHI29/1 (*pre1-1*) expressing either ODC-ha, flag(F)-Oaz1-ha or both were subjected to immunoprecipitation with anti-flag resin. Immunoprecipitated proteins were analysed by SDS–PAGE and anti-ha Western blotting.

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ODC by the proteasome in *S. cerevisiae* does not require ubiquitin either (Gandre and Kahana, 2002; Hoyt *et al*, 2003). Together, these data indicated that regulated AZ-mediated and ubiquitin-independent degradation of ODC is conserved from *S. cerevisiae* to humans.

Oaz1 physically interacts with ODC

Having established that Oaz1 is required for regulated proteolysis of ODC in *S. cerevisiae*, we next asked whether its mode of action is similar to that of its counterpart AZ1 in mammals (see Introduction). We therefore tested whether *S. cerevisiae* ODC and Oaz1 interact *in vivo*. Using the two-hybrid assay, we detected a strong interaction of ODC with Oaz1, as well as of ODC with itself (Figure 3B). The latter result demonstrated that dimerization of yeast ODC could be detected with this procedure. The former result was consistent with a model, in which, in analogy to mammalian systems, heterodimer formation underlies the targeted degradation of ODC in *S. cerevisiae*. To confirm this result biochemically, we co-expressed epitope-tagged versions of ODC and Oaz1 in yeast cells. Both proteins carried ha epitopes at their C-termini. Oaz1 was in addition tagged with a flag epitope at the N-terminus. Immunoprecipitations were carried out with anti-flag antibodies. ODC-ha was detected in precipitates from extracts of flag-Oaz1-ha co-expressing cells, but was absent from cell extracts lacking it (Figure 3C). Together, these data showed that Oaz1 forms a complex with ODC in *S. cerevisiae*, consistent with its function as an ODC AZ.

Polyamines induce ribosomal frameshifting during translation of OAZ1 mRNA

As outlined above, the complex genomic structure of the *OAZ1* gene suggested that synthesis of Oaz1 protein involves a ribosomal frameshifting event. For ODC AZ in mammals, it has been demonstrated that translational frameshifting is induced by polyamines (see Introduction). The observed spermidine inducibility of Oaz1 suggested that a similar mechanism might operate in *S. cerevisiae* as well. To investigate the mechanism underlying the inducible synthesis of Oaz1, we generated fusions of a sequence encoding two copies of the myc epitope with the 5′ end of ORF1 and of a sequence encoding two copies of the ha epitope with the 3′ end of ORF2. Two otherwise identical constructs were generated that carried both tags but were distinguished either by the presence (*OAZ1*) or the absence of the frameshifting site (*OAZ1-if*; 'if' denotes an in-frame fusion of ORF1 and ORF2). To generate the latter construct, the T nucleotide within the TGA stop codon of ORF1 was deleted. Both constructs were expressed from the unrelated P_{CUP1} promoter to exclude any putative effects of polyamine on transcriptional regulation. In addition, we chose the proteasome-deficient *pre1-1* mutant (Heinemeyer *et al*, 1991) as a host strain because it turned out that Oaz1 is degraded by the proteasome and that this process is influenced by polyamines (described in a later section). The effects of spermidine on ribosomal frameshifting were therefore studied in *pre1-1* cells, which were transformed with centromeric plasmids carrying the constructs described above. The presence of tagged polypeptides in cell extracts was analysed by Western blotting (Figure 4A and B). The cells expressing the construct containing the frameshift site showed increasing myc-Oaz1-ha signals with rising spermidine concentration in the growth media. The strain expressing the in-frame fusion, in contrast, yielded myc-Oaz1-ha signals that did not respond to changes of the spermidine concentration in the media (Figure 4B). Note that the levels of tagged Oaz1 in the latter strain were ~10-fold higher than those obtained with the strain requiring induced frameshifting to synthesize Oaz1. In both strains, these signals were detectable with anti-myc as well as with anti-ha antibodies, supporting the notion that spermidine-induced ribosomal frameshifting had taken place during expression from the *OAZ1* construct. This

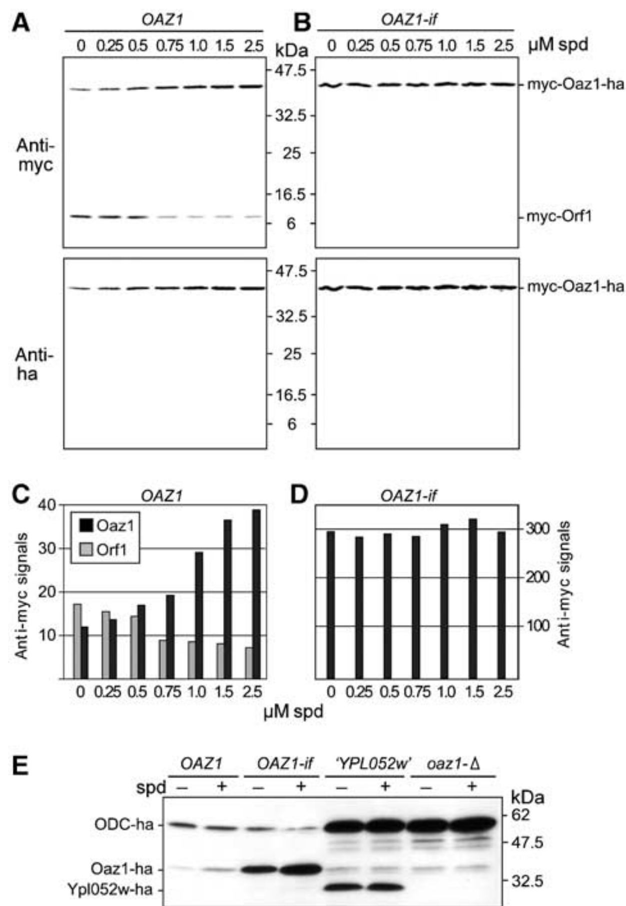

Figure 4 Synthesis of Oaz1 involves spermidine-inducible frameshifting. (**A, B**) Western blot analysis of *pre1-1* mutant strain YHI29/1 transformed with centromeric plasmids expressing, from P_{CUP1}, either (A) *OAZ1* including the frameshift site or (B) *OAZ1-if*, an in-frame fusion of ORF1 and ORF2 that was generated by deleting the T nucleotide marked with an asterisk in Figure 1A. Both constructs were fused at their 5′ ends to a sequence encoding two copies of the myc tag and at their 3′ ends to a sequence encoding two copies of the ha tag. Cells were grown in the presence of the indicated concentrations of spermidine and 100 μM $CuSO_4$ to induce expression from P_{CUP1}. Extracts were analysed simultaneously for myc of ha tagged proteins by immunoblotting. (**C, D**) Quantitation of fluorescence signals shown in (A, B), respectively. (**E**) An *OAZ1* gene lacking the frameshift mediates ODC degradation. Strain PMY2 (*oaz1-Δ ODC-ha*) was transformed with an empty vector or with plasmids expressing, from P_{CUP1}, either *OAZ1*, *OAZ1-if* or ORF 'YPL052w'. All three constructs were fused at their 5′ ends to a sequence encoding a flag-6His tag and at their 3′ ends to a sequence encoding two copies of the ha tag. Transformants were grown in the presence of 100 μM $CuSO_4$ and either in the absence or presence of 100 μM spermidine as indicated. Yeast cell extracts were analysed by anti-ha immunoblotting.

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conclusion was supported further by the detection of the faster migrating myc-Orf1 polypeptide, in this case only with the anti-myc antibody, in the extracts of the strain expressing *OAZ1* bearing the frameshifting site. The intracellular concentration of this polypeptide declined with increasing spermidine concentration in the growth media (Figure 4A and C). Together, these data proved our prediction that Oaz1 is expressed from ORF1 and ORF2 as a result of a spermidine-induced ribosomal frameshifting. This notion is supported by the ability of an *OAZ1-if* construct expressed from P_{CUP1} to complement the defect of the *oaz1*-Δ mutant in the degradation of ODC (Figure 4E). An otherwise identical construct bearing the authentic frameshifting site resulted in efficient complementation of *oaz1*-Δ even without supplemented spermidine. This result indicated that P_{CUP1} provided an expression level of *OAZ1* sufficient for efficient targeting of ODC without additional polyamines in the media for the induction of ribosomal frameshifting. A construct expressing only the annotated ORF 'YPL052w', in contrast, showed no complementation of *oaz1*-Δ, supporting the notion that this ORF does not encode a functional AZ. The experiment shown in Figure 4E also indicated that relatively low amounts of Oaz1 are sufficient to mediate degradation of the bulk of ODC. The residual ODC appears to be fairly resistant to Oaz1-induced degradation. The constructs used in Figure 4E carried N-terminal flag-His_6 tags. Similar results were obtained with N-terminal myc_2, although this tag appeared to reduce the efficiency of Oaz1 in ODC targeting (data not shown).

Control of Oaz1 levels involves its ubiquitin-mediated degradation by the proteasome

While we were studying the effects of mutations affecting proteasome function on ODC levels, we observed that Oaz1 levels were also increased. As shown in Figure 5A for the *pre1-1* mutant, Oaz1 accumulated to much higher levels in such strains. We next asked whether degradation of Oaz1 by the proteasome was ubiquitin-dependent. To test this, we expressed Oaz1-ha from an in-frame fusion of ORF1 and ORF2 (Oaz1-if, see above) in a mutant with a temperature-sensitive ubiquitin-activating enzyme (*uba1*) and in mutants lacking various ubiquitin-conjugating enzymes (*ubc*) (Jentsch, 1992). Increased levels of Oaz1 were detected in the *uba1* mutant (grown at the semi-permissive temperature of 30°C) and in a strain lacking *UBC4* and *UBC5* (Seufert and Jentsch, 1990; McGrath *et al*, 1991) (Figure 5A). Mutants lacking other Ubc-encoding genes such as *ubc1*, *ubc2*, *ubc6 ubc7*, *ubc8*, *ubc10*, and *ubc13* had Oaz1 levels comparable to the wild type (data not shown). Pulse-chase experiments showed that the elevated steady-state levels in *pre1-1*, *uba1*, and *ubc4 ubc5* mutants were due to a drastic stabilization of the Oaz1 protein (Figure 5B and C). These data suggested that Oaz1 levels are directly controlled by Ubc4/Ubc5-mediated ubiquitin-dependent proteolysis by the proteasome. A prediction of this interpretation is that Oaz1 should be ubiquitylated prior to degradation. To verify this, we co-expressed Oaz1-ha with myc-tagged ubiquitin (myc-Ub) and performed anti-ha immunoprecipitations. Myc-Ub conjugates were precipitated from cells co-expressing these two tagged proteins, but were absent from control strains expressing either of the two proteins alone (Figure 5D). The more abundant of these conjugates was also detectable with anti-Ha antibody, but only after overexposing the Western blot (data not shown).

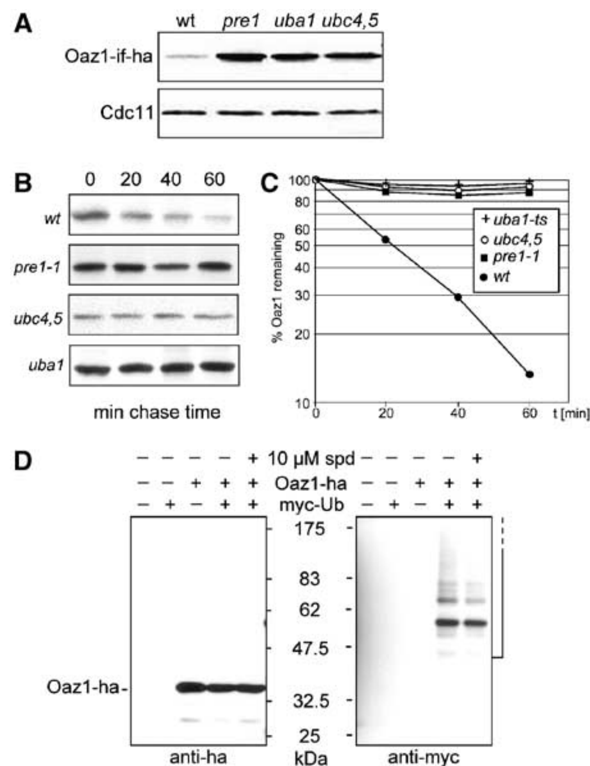

Figure 5 Ubiquitin-mediated degradation of Oaz1. (**A–C**) Degradation of Oaz1 requires ubiquitylation enzymes and the proteasome. (A) Oaz1-ha steady-state levels detected by anti-ha Western blotting in the strains indicated. *OAZ1-if-ha* was expressed from P_{CUP1} in plasmid pPM58. Lower part, anti-Cdc11-loading control. (B) Pulse-chase analysis of Oaz1 turnover in the same strains as in (A). (C) Quantitation of data shown in (B). (**D**) Oaz1 is ubiquitylated *in vivo*. JD47-13C transformants expressing either *OAZ1-if-ha*, myc-Ub, or both were grown in the presence of 100 μM $CuSO_4$. Where indicated, spermidine (10 μM) was added to the media 1 h before extraction. After immunoprecipitation with anti-ha antibodies, precipitates were analysed by anti-myc and anti-ha Western blotting. Ubiquitylated forms of Oaz1-ha are indicated by an open-ended bracket.

Since the extracts were boiled in the presence of 2% SDS prior to immunoprecipitation, we conclude that myc-Ub was covalently attached to Oaz1.

Polyamines block degradation of Oaz1

While we were studying ribosomal frameshifting during the synthesis of Oaz1, we observed that spermidine addition to the growth media increased Oaz1 levels even when it was expressed from the control construct (*OAZ1-if*) that lacked the frameshift (Figure 4E). This effect of spermidine on Oaz1 levels, however, was not detected in the proteasome-deficient *pre1-1* mutant (Figure 4A). Since expression of *OAZ1-if* was driven from P_{CUP1}, an effect of spermidine on the transcriptional regulation of this construct appeared unlikely. These observations suggested that ubiquitin-mediated degradation of Oaz1 by the proteasome is influenced by polyamine levels. To follow up on this initial finding, we applied spermidine in concentrations ranging from 0 to 10 μM to a culture expressing *OAZ1-if* from P_{CUP1}. As a result, we observed a dose-dependent increase in Oaz1 signal (Figure 6A and B). We therefore asked whether spermidine addition affected the half-life of Oaz1. Pulse-chase analyses demonstrated that

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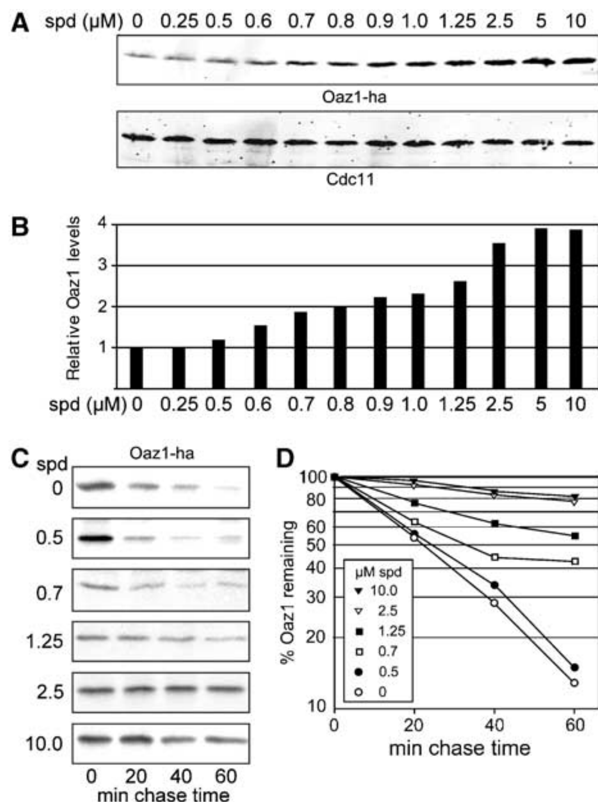

Figure 6 Spermidine inhibits degradation of Oaz1. (**A**) Analysis of the effects of spermidine on Oaz1 levels in the absence of ribosomal frameshifting. Wild-type strain JD47-13C transformed with pPM58 expressing *OAZ1-if-ha* was grown for 1 h in the presence of spermidine at the indicated concentrations. Extracts were analysed by anti-ha Western blotting. Cdc11 was detected simultaneously as a loading control. (**B**) Quantitation of data shown in (A). (**C**) Pulse-chase detection of concentration-dependent inhibition of Oaz1 degradation by polyamines in the same strain as in (A). Spermidine at the indicated concentrations was added 1 h before pulse labelling. (**D**) Quantitation of data shown in (C).

spermidine inhibited Oaz1 degradation in a dose-dependent manner (Figure 6C and D). In order to test whether the observed inhibitory effect of spermidine was specific for Oaz1 degradation or whether this polyamine acted as a general inhibitor of proteolysis, we used established test substrates of ubiquitin-mediated proteolysis. R-β-galactosidase (R-βgal) is degraded by the N-end rule pathway, whereas Ub-P-βgal is degraded by the ubiquitin-fusion degradation (UFD) pathway (Johnson *et al*, 1995; Varshavsky, 1996). M-βgal served as a stable control protein. In marked contrast to the effect on Oaz1, spermidine addition to the growth media of cells expressing the β-galactosidase (βgal) test proteins had no effects on their steady-state levels (Figure 7A and B) or their turnover rates (Figure 7C and D). Since, as described above, Oaz1 degradation is ubiquitin-dependent, we asked whether spermidine inhibited ubiquitylation of Oaz1 or whether the turnover of ubiquitylated Oaz1 is blocked. To address this question, we again co-expressed myc-Ub with Oaz1-ha, this time in the presence of spermidine in the medium. Addition of the polyamine resulted in a reduction of detectable ubiquitylated forms of Oaz1-ha (Figure 5D). These data indicated that spermidine interferes with degradation of Oaz1 at least in part by inhibiting its ubiquitylation.

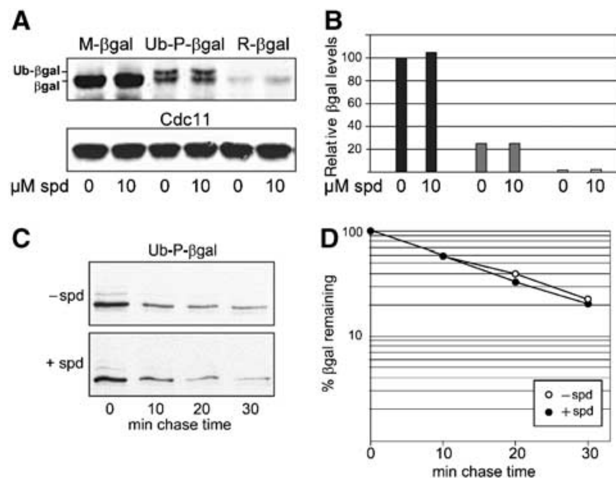

Figure 7 Spermidine has no general effect on turnover rates of proteolytic substrates. (**A**) Steady-state levels of proteolytic test substrate detected by anti-ha Western blotting (upper part). Anti-Cdc11 loading control (lower part). βgal variants were expressed as ubiquitin fusions (Ub-X-βgal). (**B**) Quantitation of data in (A). (**C**) Pulse-chase detection of Ub-P-βgal turnover rates. (**D**) Quantitation of data in (C).

Discussion

We report the discovery of *OAZ1*, an *S. cerevisiae* gene encoding an orthologue of mammalian ODC AZs. In addition, we detected closely related genes in the genomes of other Hemiascomycetes as well, thereby extending the AZ family by a set of fungal sequences, whose existence has been postulated (see Introduction). The sequence similarity, the conservation of the domain structure and the presence of a putative frameshifting site, all point to a common evolutionary origin of *S. cerevisiae OAZ1* and ODC AZ genes in other eukaryotes.

Conservation of regulation and function of ODC AZ

Similar to its extensively studied orthologues in mammals, *S. cerevisiae* Oaz1 mediates ubiquitin-independent degradation of ODC by the proteasome (Figures 2 and 3). Polyamine induces Oaz1 levels without affecting transcription of the *OAZ1* gene (data not shown). Synthesis of Oaz1 from two ORFs, instead, is controlled by polyamine-regulated programmed ribosomal frameshifting (Figure 4). Disruption of ORF2 resulted in abrogation of ODC degradation. Ectopic expression of the annotated ORF 'YPL052w' did not restore degradation. An in-frame fusion of ORF1 and ORF2, in contrast, resulted in a complementation of this defect in ODC degradation. These and related results established that ribosomal frameshifting is essential for synthesis of a functional Oaz1 in *S. cerevisiae*.

Whereas many viruses utilize programmed frameshifting (commonly −1) to decode their RNAs, there are only few examples for an employment of such mechanisms in the synthesis of cellular proteins. In *S. cerevisiae*, aside of the retrotransposon Ty, two genes have been proposed to utilize a programmed +1 ribosomal frameshifting in their decoding. *EST3* encodes a protein required for telomer replication (Morris and Lundblad, 1997). *ABP140* encodes an actin filament-binding protein (Asakura *et al*, 1998). In all these cases, a tRNA slippage at a CUU Leu codon in a sequence

(CUU AGG/A), in which it is followed by a slowly recognized codon, appears to underlie the frameshifting event (Sundararajan *et al*, 1999). A similar sequence element is absent from the frameshifting sites in AZ genes from yeast and other species (Figure 1B). Here an occlusion model has been proposed, in which sequences upstream of the frameshifting site and a downstream pseudoknot modify the structure in the A site of the ribosome occupied by the UGA termination codon, such that an Asp or a Glu codon in the $+1$ frame is recognized by the respective tRNAs (Namy *et al*, 2004). Despite the differences in the sequences of the frameshifting sites, $+1$ ribosomal frameshifting in decoding of both Ty1 and Oaz1 is modulated by polyamines (Balasundaram *et al*, 1994a, b) (Figure 4). Whether polyamines more generally stimulate $+1$ frameshifting in *S. cerevisiae*, however, is unclear as no data are available on the effects of polyamines on the rates of frameshifting in decoding of *EST3* and *ABP140*. It also remains to be investigated whether ribosomal frameshifting signals and their recognition are conserved among mRNAs of AZ orthologues. It was reported that decoding of a test construct containing the rat AZ cDNA in *S. cerevisiae* involves a -2 ribosomal frameshifting. Whether this event is stimulated by polyamines was not tested (Ivanov *et al*, 1998b). The discovery of the *OAZ1* gene in *S. cerevisiae* provides an easy to manipulate *in vivo* system that will help to understand underlying mechanisms and the sequence requirements for polyamine-induced ribosomal frameshifting.

Ubiquitin-mediated degradation of Oaz1 is inhibited by spermidine

We noticed that polyamine addition to the media resulted in increased Oaz1 protein levels even when an in-frame construct that did not require frameshifting was used. Our analysis revealed that Oaz1 is subject to ubiquitin-mediated proteolysis by the proteasome. Degradation of Oaz1 is inhibited by mutations affecting the ubiquitin-activating enzyme Uba1 (E1), the ubiquitin-conjugating enzymes Ubc4 and Ubc5, or the proteasome. Similarly, an E1 requirement was previously reported for degradation of AZ1 in mammalian cells, but ubiquitylated forms of it were not detected (Gandre *et al*, 2002). In yeast, we detected such forms for Oaz1, indicating that ubiquitylation is essential for its proteasomal degradation (Figure 5D). Upon addition of spermidine to the growth media, we observed a dose-dependent inhibition of Oaz1 degradation. How might polyamines interfere with Oaz1 degradation? There are examples in the literature both for the inhibition of proteasomal degradation of a ubiquitylated substrate and for the inhibition of the ubiquitylation of a protein by small organic compounds. Degradation of a ubiquitylated dihydrofolate reductase was shown to be inhibited by the folic acid analogue methotrexate (Johnston *et al*, 1995). Our results, in which we detected a reduction rather than a stabilization of ubiquitylated forms of Oaz1 upon spermidine addition, suggested that polyamines, in contrast, stabilize Oaz1 at least in part by interfering with its ubiquitylation (Figure 5D). Similar observations were made for mammalian spermidine/spermine *N*-acetyltransferase (SSAT), a key enzyme in polyamine catabolism. Ubiquitylation of SSAT was inhibited *in vitro* by polyamine analogues (Coleman and Pegg, 2001). It was proposed that binding of the analogue brings about a conformational change of SSAT that inhibits its ubiquitylation. A similar mechanism may underlie or contribute to the inhibition of Oaz1 degradation by spermidine.

Alternatively, the enzymes that mediate ubiquitylation of Oaz1 may be inhibited by spermidine. One example of an E3 ubiquitin ligase whose activity is regulated by a small organic compound is Ubr1 in *S. cerevisiae.* This ligase indirectly regulates the uptake of dipeptides by controlling the stability of the transcriptional repressor Cup9 that blocks expression of the *PTR1* dipeptide transporter gene. Dipeptides at low concentrations act as allosteric activators of the Ubr1 ligase activity towards Cup9 and thereby induce their uptake (Turner *et al*, 2000). In a diversion of such a mechanism, an as yet to be identified E3 enzyme responsible for Oaz1 ubiquitylation might be inhibited by polyamines.

We favor a model in which spermidine binding to Oaz1 inhibits its ubiquitylation, based on our observation that SSATs were detected in searches that used AZ-based profiles. In a reverse approach, profiles constructed from acetyl transferases alignments moreover retrieved several AZs. In both cases, the sequence similarity, however, was below the threshold that would establish a clear evolutionary relationship. Secondary structure prediction for the putative homologous region in acetyl transferases and AZs, however, indicated that they share a common $\alpha\beta\alpha$ motif (data not shown). In the crystal structure of yeast *N*-acetyltransferase Gna1, this $\alpha\beta\alpha$ motif overlaps with the binding site for Acetyl-CoA and the substrate (Peneff *et al*, 2001). By analogy, this domain is likely to be involved in spermidine binding within SSAT enzymes. We hypothesize that the spermidine-dependent stabilization of AZ may be due to a direct binding of spermidine to an $\alpha\beta\alpha$ motif in the C-terminal region of AZ.

Based on our data, we propose that polyamines regulate their synthesis by controlling Oaz1 levels via two separate mechanisms. The first mechanism is the induction of ribosomal frameshifting in decoding of *OAZ1* mRNA by a mechanism that is poorly understood. The second mechanism is the inhibition of Oaz1 proteolysis. We propose that the latter mechanism ensures a rapid recovery from a state in which ODC is downregulated by AZ. In this model, a drop of polyamine levels below a critical threshold does not only prevent *de novo* synthesis of AZ but also results in a turnover of its existing pools, thereby allowing for a rapid recovery of ODC activity. Polyamines thus appear to mediate an efficient feedback control of their formation by affecting both the synthesis and turnover rates of ODC AZ.

Materials and methods

Yeast media

Yeast-rich (YPD) and synthetic (S) minimal media with 2% dextrose (SD) were prepared as described (Ramos *et al*, 1998). Spermidine (Sigma) was added to SD media at various concentrations as indicated.

Yeast strains and plasmids

Table I lists the strains used in this study. Strains expressing C-terminally ha_3- or myc_3-tagged variants of ODC or Oaz1 were constructed by a PCR-based method using short-flanking oligos and plasmids pYM2 and pYM5, respectively, as templates (Knop *et al*, 1999). The following plasmids were constructed in the background of the *CEN/URA3*-based plasmid YCplac33 (Gietz and Sugino, 1988). pPM52 expressed *flag-His$_6$-OAZ1-ha$_2$* from P_{CUP1}. Plasmid pPM53 was identical to pPM52, except that it expressed Oaz1 as an in-frame fusion of ORF1 and ORF2 (Oaz1-if). Plasmid pPM54

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Table I Yeast strains

Strain	Relevant genotype	Source/comment
BY4741	*MATa his3-Δ1 leu2Δ0 met15-Δ0 ura3-Δ0*	EUROSCARF ('wt')
BY4742	*MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0*	EUROSCARF ('wt')
Y02776	*oaz1-Δ::KanMX4 (=ypl052w-Δ)*	Derivative of BY4741, EUROSCARF
Y15034	*spe1-Δ::KanMX4 (='odc-Δ')*	Derivative of BY4742, EUROSCARF
PMY1	*ODC-ha$_3$::HISMX6*	Derivative of BY4741
PMY2	*ODC-ha$_3$::HISMX6 oaz1-Δ::KanMX4*	Derivative of BY4741
PMY17	*OAZ1-myc$_3$::HISMX6*	Derivative of BY4741
PMY15	*OAZ1-myc$_3$::HISMX6 spe1-Δ::KanMX4*	Derivative of Y15034
WGC4a	*MATα ura3 his3-11 leu2-3,112*	(Heinemeyer *et al*, 1991)
YHI29/1	*pre1-1*	Derivative of WGC4a (Heinemeyer *et al*, 1991)
YPH500	*MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52*	(Sikorski and Hieter, 1989)
CMY762	*cim3-1*	Derivative of YPH500 (Ghislain *et al*, 1993)
ubc4 ubc5	*ubc4-Δ::HIS3 ubc5-Δ::LEU2*	(Seufert and Jentsch, 1990)
JD47-13C	*MAT**a** his3-Δ200 leu2-3,112 lys2-801 trp1-Δ63 ura3-52*	(Ramos *et al*, 1998)
JD77-1-1	*uba1-Δ::HIS3 pRSts64-1(uba1-ts)*	Derivative of JD47-13C (McGrath *et al*, 1991)
PJ64-4A	*MAT**a** his3-Δ200 leu2-3,112 trp1-901 ura3-52 gal4-Δ gal80-Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	(James *et al*, 1996)

instead expressed *flag-His$_6$-YPL052w-ha$_2$*; pPM58 expressed *OAZ1-if-ha$_2$*. Plasmids pPM64 and pPM67 were based on the *CEN/LEU* plasmid pRS315 (Sikorski and Hieter, 1989) and expressed *ODC-ha$_3$* from P_{CUP1} or P_{ODC}, respectively. Plasmids pPM84 and pPM85 were based on the *CEN/URA3* plasmid pRS316 (Sikorski and Hieter, 1989). pPM84 expressed, from P_{CUP1}, *myc$_2$-OAZ1-ha$_2$* with a frameshift in the *OAZ1* ORF, whereas pPM85 expressed an otherwise identical in-frame fusion lacking the frameshift site.

Immunoblot analysis and determination of protein stability by pulse-chase analysis

For detection of steady-state levels, *S. cerevisiae* cells were grown at 30°C in SD media to an optical density measured at 600 nm (OD_{600}) of 1.0 ± 0.2. Proteins were extracted from yeast cells by boiling in loading buffer (0.0625 M Tris–HCl (pH 6.8), 2% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue) for 5 min. Extracts from cells corresponding to 0.5 OD_{600} ($\approx$15 μg) were loaded per lane. SDS–PAGE and immunoblots were performed as described (Ramos *et al*, 1998). Proteins were either detected using secondary anti-mouse or anti-rabbit IgG coupled to peroxidase (Boehringer Mannheim), Lumi-LightPLUS chemiluminescent substrate (Roche), and X-ray films (Figures 3C, 4E and 5D), or with anti-mouse, anti-rat, or anti-rabbit IgG coupled to near-infrared fluorophores (Rockland), and the Odyssey Infrared Imaging System. The latter system was also used for signal quantification. For detection of epitope tags, we used the following monoclonals as primary antibodies. The ha epitope was detected with 16B12 from mouse (Covance) in all experiments except those shown in Figure 4A and B, in which 3F10 from rat (Boehringer Mannheim) was chosen instead. Mouse monoclonal 9B11 (Cell Signaling Technology) was used for myc epitope detection. Cdc11 was detected with polyclonal rabbit antibodies (Santa Cruz Biotechnology), and βgal with a mouse monoclonal antibody (Promega). Pulse-chase analyses were carried out as described (Ramos *et al*, 1998).

Assay of ODC Oaz1 complex formation

Yeast two-hybrid assays were carried out as described (James *et al*, 1996). For coimmunoprecipitation, proteins were extracted from yeast cells by glass bead lysis in ice-cold lysis buffer (50 mM Na-HEPES (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing the 'Complete' Protease-Inhibitor cocktail (Roche). In all, 300 μg of protein was subjected to immunoprecipitation using anti-flag resin (Sigma). Precipitated proteins were washed six times with lysis buffer and eluted with 300 μg/ml flag peptide (Sigma).

Detection of ubiquitin conjugates

Yeast cells co-expressing *OAZ1-if-ha* (pPM58) and *myc-Ub* (plasmid YEp105) (Ellison and Hochstrasser, 1991), both from P_{CUP1}, were lysed by boiling for 5 min in lysis buffer (see above) with 2% SDS and 1% β-mercaptoethanol. Empty vector transformants were used as controls. Samples were diluted 1:20 with lysis buffer and subjected to immunoprecipitation with an anti-ha high-affinity matrix (Roche). Precipitated proteins were washed six times with a lysis buffer containing 0.1% SDS, eluted by boiling in loading buffer and analysed by SDS–PAGE and anti-myc and, after stripping, with anti-ha Western blotting.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

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