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A PLAC8-containing protein from an endomycorrhizal fungus confers cadmium resistance to yeast cells by interacting with Mlh3p

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ABSTRACT

Cadmium is a genotoxic pollutant known to target proteins that are involved in DNA repair and in antioxidant defence, altering their functions and ultimately causing mutagenic and carcinogenic effects. We have identified a PLAC8 domain-containing protein, named OmFCR, by a yeast functional screen aimed at identifying genes involved in cadmium resistance in the endomycorrhizal fungus *Oidiodendron maius*. OmFCR shows a remarkable specificity in mediating cadmium resistance. Both its function and its nuclear localization in yeast strictly depend on the interaction with Mlh3p, a subunit of the mismatch repair (MMR) system. Although proteins belonging to the PLAC8 family are widespread in eukaryotes, they are poorly characterized and their biological role still remains elusive. Our work represents the first report about the potential role of a PLAC8 protein in physically coupling DNA lesion recognition by the MMR system to appropriate effectors that affect cell cycle checkpoint pathways. On the basis of cell survival assays and yeast growth curves, we hypothesize that, upon cadmium exposure, OmFCR might promote a higher rate of cell division as compared to control cells.

INTRODUCTION

The integrity of genomic DNA is constantly challenged by genotoxic insults originating from either normal cellular metabolic processes or environmental factors. At the cellular level, protection from DNA damage is provided by faithful damage surveillance as well as coordinated and integrated checkpoint cascades enforcing cell cycle arrest, thus facilitating repair pathways, apoptosis or cellular senescence (1). Alterations or loss of genes involved in the damage response pathways can lead to genomic instability, inactivation of negative growth stimuli, resistance towards apoptosis and, in mammalian systems, cancer susceptibility syndromes. In *Saccharomyces cerevisiae*, the major defence mechanism against DNA damage is represented by the DNA repair system, consisting of three evolutionary conserved pathways that, although partially interwoven, respond to different kinds of DNA lesions: the nucleotide excision repair (NER), the base excision repair (BER) and the mismatch repair (MMR) pathways (2). BER mainly corrects non-bulky lesions produced by alkylation, oxidation or deamination that cause relatively minor distortions to the DNA helix. Conversely, NER acts on more substantially damaged areas of DNA including those caused by ultra-violet (UV) light and chemical mutagens. The MMR pathway repairs base-base mismatches and insertion/deletion loops that arise from DNA duplication, as well as mismatches in heteroduplexes that are formed during recombination. Homologous recombination repair (RR) and translesion synthesis (TLS) also provide routes by which yeast cells can continue replication despite the presence of replication fork-blocking adducts (3), but are often regarded as DNA damage-tolerance pathways.

Cadmium is known to disturb and inhibit all three major DNA repair pathways (4). In particular, it reduces the capacity of MMR to recognize small misalignments and base-base mismatches by binding to the MSH2–MSH6 complex and disrupting its structure and function (5–8). Unlike many other genotoxic metal ions, cadmium, in fact, does not inflict direct damage on DNA, proteins and lipids through the generation of reactive oxygen species (ROS), but it targets proteins that are directly or indirectly involved in DNA repair and in antioxidant defence, altering their functions and ultimately causing toxic, mutagenic and carcinogenic effects. Cells have developed two major cell cycle checkpoints that delay or halt cell cycle progression to allow repair of cadmium-damaged cellular components: the SCF^{Met30}/

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Met4p pathway and the Mec1p/Rad53p phosphorylation cascade, the latter being specifically activated in response to DNA damage (9).

Cadmium and cadmium compounds are genotoxic pollutants that accumulate in the environment as a consequence of several anthropogenic activities, such as nickel-cadmium battery and pigments manufacture, the application of rock phosphatic fertilizers, soil amendments with municipal sewage sludge and deposits of ash from fossil-fuel combustion (10). The massive input of cadmium in the environment and its potential adverse consequences for human health are triggering new research studies on the mechanism of toxicity, the risk assessment and the biological understanding of this mutagen. We undertook the present study to analyze the transcriptional response to cadmium exposure of the ericoid mycorrhizal fungus *Oidiodendron maius* strain Zn, which was isolated in Poland from plots contaminated with heavy metal dusts from the electro-filters of six industrial plants (11). The functional screening of an *O. maius* cDNA library in the *S. cerevisiae* cadmium-sensitive mutant y*ap1* led to the isolation of a PLAC8 domain-containing protein, hereafter referred to as OmFCR. This protein confers strong cadmium resistance to yeast cells through the interaction with Mlh3p, a subunit of the MMR system. Here we suggest that OmFCR may take part to the fairly unexplored role of the MMR system in connecting the DNA lesion recognition with downstream signaling cascades that ultimately lead to cell cycle checkpoints. Moreover, the discovery that OmFCR interacts with the MMR pathway provides new elements that may help to decipher the function of the PLAC8 domain, which, despite being widespread and evolutionary conserved in all eukaryotic kingdoms, has no assigned biological role.

MATERIALS AND METHODS

Fungal and yeast strains and growth conditions

O. maius strain Zn was isolated in the Niepolomice Forest (Krakow, Poland) from the roots of *Vaccinium myrtillus* growing in experimental plots treated with metal-containing dusts (12). The fungus was grown in Czapek mineral medium supplemented with 2% (w/v) glucose as described by Abbà and colleagues (13).

WYT yeast *yap1* deletion strain (genotype, MATa his3 can1-100 ade2 leu2 trp1 ura3 yap1::TRP1) was compared to the near-isogenic DY wild-type strain [genotype, MATa his3 can1-100 ade2 leu2 trp1 ura3::(3xSV40AP1-lacz)] for screening tests of cadmium resistance (14). The DY strain was kindly provided by Prof. D. Inzé of the University of Ghent, Belgium. Yeast *ycf1*, *hog1*, *skn7* and *cup1* deletion strains were in the parent MATα BY4741 background (genotype, MATα *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and were kindly provided by Prof. S. Ottonello (University of Parma, Italy) (15). The EY39 wild-type (genotype, *his4-r1 leu2-r1 MATa ade1-1 TRP5 CYH2 MET13 lys2::insE-A_{14} CANS ura3-1*) and the VC29 mutant (genotype, *his4-r1 leu2-r1 MATa ade1-1 TRP5 CYH2 MET13 lys2::insE-A_{14} CANS ura3-1 mlh3Δ::hphMX4*) strains were kindly provided by Prof. R. Borts (University of Leicester, UK) (16). Yeast *rad9* and *dun1* mutants and *mec1 sml1* double mutant were in the W303 background (*Mata; ade2-1; can1-100; 112his3-11,15; leu2-3; trp1-12; ura3*) and were kindly provided by Prof. J. Svejstrup (Cancer Research UK London Research Institute). Yeast strains were grown at 30°C on synthetic dextrose (SD) medium without uracil.

cDNA library construction and screening

An *O. maius* strain Zn cDNA library was prepared by pooling the RNA extracted from fungal mycelia exposed to a final concentration of 15 μM $CdSO_4$ for 24 h, 4 and 18 days. The cDNA library was cloned into the yeast over-expressing vector pFL61 and then transformed into the *yap1* deficient yeast strain following the lithium acetate/salmon sperm carrier DNA/PEG method (17). Transformants were selected on SD plates lacking uracil. The transformed yeast cells were spread both on SD-agar plates containing a linear concentration gradient (0–100 μM) of $CdSO_4$ and on SD-agar plates with concentrations of 50, 60, 70, 80 and 100 μM $CdSO_4$. After 4 days of growth, plasmids from the surviving yeasts were rescued in *Escherichia coli*, extracted, and sequenced.

Synthesis of EGFP-tagged OmFCR constructs for transformation of *yap1*

The open reading frame (ORF) of *OmFCR* was amplified by PCR using the plasmid isolated from the library screening as template. Both primers contained HindIII tails and the reverse primer was modified to remove the stop codon. The PCR product was HindIII digested and inserted in frame with the EGFP into the HindIII site of the pEGFP-N1 vector (Invitrogen, Carlsbad, CA, USA). The OmFCR-EGFP fragment was then PCR amplified with NotI-tailed primers and cloned into the NotI-cut pFL61.

The EGFP-OmFCR construct was obtained by fusion PCR following the protocol described by Kuwayama and collaborators (18). Three PCR reactions were set up: two primary reactions to amplify OmFCR and EGFP and a secondary reaction intended to fuse the two fragments into a single 1303 bp-long amplicon. The two primary PCR reactions were carried out in a final volume of 50 μl containing 200 μM of each dNTP, 5 μM of each primer, 5 μl 5× Phusion HF buffer and 0.5 U of Phusion High-Fidelity DNA Polymerase (Finnzymes, Finland). The PCR program was as follows: 30 s at 98°C for 1 cycle; 10 s at 98°C, 45 s at 60°C, 30 s at 72°C for 35 cycles; 10 min at 72°C for 1 cycle.

OmFCR and EGFP were amplified with primers 1-2 and 3-4, respectively (see Supplementary Table S1). Primer 2 was designed to remove the EGFP stop codon. During the fusion PCR, the 3′ region of the EGFP was joined to the 5′ region of OmFCR and the final PCR product was amplified with the NotI-tailed primers 1 and 4. The fusion PCR reaction was carried out using 30 ng of the purified OmFCR and EGFP PCR products. Construction of the N-terminal EGFP tagged OmFCR was confirmed by DNA sequencing. The two EGFP constructs were NotI digested, ligated into the pFL61 vector

and transformed into *yap1* mutant. Yeast nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI).

The localization of EGFP and DAPI fluorescence was observed using a Leica TCS SP2 confocal microscope, using a long-distance 40× water-immersion objective (HCX Apo 0.80). The Ar laser band of 488 nm was used to excite the EGFP and to collect transmitted light images of the samples. An emission window of 500–525 nm was used to image EGFP fluorescence. An excitation wavelength of 405 nm was used to excite the DAPI, whose fluorescence was recorded between 430 and 500 nm.

Dilution spot assays

Dilution spot assays were used to monitor cell growth at various drug concentrations. For all conditions, overnight yeast cultures were diluted to $OD_{600} = 0.1$ and cultured at 30°C until $OD_{600} = 0.2$. Subsequently, 5 µl of serial dilutions (5×10^6, 5×10^5 and 5×10^4 cells/ml) of each strain were spotted in triplicate onto drug-amended SD medium and incubated at 30°C for 6 days.

The tested toxic substances were purchased from Sigma (St. Louis, Missouri, USA) as $3CdSO_4*8H_2O$, $ZnSO_4*7H_2O$, $CuSO_4$, $NiSO_4*6H_2O$, $Na_2HAsO_4*7H_2O$, menadione sodium bisulfite, NaCl, buthionine sulfoximine (BSO), hydroxyurea and from InvivoGen (San Diego, California, USA) as phleomycin. Stock solutions were made in distilled water and sterilized by filtration through 0.22-pm-pore-size membrane filters (Millipore Corp., Bedford, MA, USA).

To investigate the response of the empty vector- and OmFCR-transformed cells to UV–C light exposure, cells were first spotted onto SD medium, then positioned at 10 cm from the light source and finally irradiated by a G30-T8 UV–C lamp (Sankyo Denki, Japan).

Measurement of Cd content in yeast

Logarithmically growing *yap1*-cell cultures were diluted to $OD_{600} = 0.4$ with fresh SD medium containing 50 µM Cd(II) and grown for 48 h at 30°C. Cells were then divided in two aliquots, pelleted by centrifugation and washed for 1 h, in either 10 ml distilled H_2O or 10 ml 0.5 M HCl. The HCl wash was used to remove loosely associated cadmium ions from yeast cell walls and distinguish them from the Cd content within the cells.

Cadmium content was measured after 6 M nitric acid digestion at 90°C for 1 h by the emission spectroscopic technique ICP-OES (Inductively Coupled Plasma-Optical Emission Spectrometry). Analyses were performed by the 'Laboratorio di analisi chimiche elementari' (Mineralogy and Petrology Department, University of Turin, Italy).

Yeast two-hybrid assay

The yeast two-hybrid screen was performed using the DupLEX-A yeast system (Origene Technologies, Rockville, MD, USA). The OmFCR coding sequence was cloned in frame with the DNA binding domain of LexA into the pEG202 vector. Ten-million clones were screened from a *S. cerevisiae* genomic library constructed in pJG4-5. Twelve clones, which were both leucine and X-Gal positive on galactose plates, and leu negative and X-Gal negative on glucose plates, were sequenced.

OmMlh3 isolation

A *blastx* search against the *O. maius* cDNA library using the yeast Mlh3p sequence as a query led to the identification of the *O. maius* Mlh3p homolog, hereafter named OmMlh3. Two EcoRI-tailed primers (EcoOmMlh3f 5′-TTCCGAATTCTGCGATAACTTACCGTTTGA-3′; EcoOmMlh3r 5′-TAATGAATTCCTCAAGACTCGCT ACTGTCA-3′) were designed for the PCR amplification of the OmMlh3 C-terminal region that corresponds to the yeast Mlh3p fragment isolated from the yeast-two hybrid screening. The PCR program was as follows: 30 s at 98°C for 1 cycle; 10 s at 98°C, 45 s at 60°C, 30 s at 72°C for 35 cycles; 10 min at 72°C for 1 cycle. The PCR product was EcoRI digested, ligated in frame with the activator domain of B42 into the pJG4-5 vector and transformed into the yeast strain EGY48, containing both the reporter plasmid pSH18–34 and the bait plasmid pEG202 with *OmFCR*.

Cell survival and Can^R mutator assay in *ycf1* mutant

Base–base substitutions, deletions, insertions and large chromosomal rearrangements can be revealed by the canavanine (Can^R) assay, which was extensively used to study both cadmium-induced mutations and mutations caused by alterations in the MMR system proficiency (7,19,20).

The *ycf1* strain was chosen instead of the *yap1* strain to perform experiments on mutation frequencies, because *yap1* bears a mutation on the *CAN1* gene that makes it resistant to canavanine.

The OmFCR and the pFL61-*ycf1* cells were cultured overnight in 5 ml of SD medium at 30°C to an optical density $(OD)_{600} \sim 1$. Cells were then diluted to $OD_{600} = 0.15$ in 40 ml of fresh medium and split in two cultures: one was treated with 50 µM $CdSO_4$ and the other was left untreated. The OD_{600} of untreated and treated cell cultures was measured after 2, 3, 7, 24 and 48 h of incubation at 30°C. Untreated cultures reached saturation within 48 h, so only treated cell cultures were further sampled.

After 7 h, when the OD_{600} of the treated OmFCR-*ycf1* cells was statistically different ($P < 0.01$) from the OD_{600} of the treated pFL61-*ycf1* cells, a fraction of treated and untreated cell cultures was harvested by centrifugation, washed and resuspended in sterile water to an $OD_{600} = 0.4$ (which corresponds to nearly 1.0×10^7 cells/ml). One millilitre of each culture was plated onto selective medium lacking arginine but containing canavanine at 60 mg/l. Mutation frequencies were determined after 3 days at 30°C as the number of colony forming units per number of plated cells and expressed as averages ± standard deviations.

Cell survival was measured by plating about 200 cells (calculated through appropriate dilutions based on the initial OD) onto SD medium after 7, 24, 48 and 120 h of cadmium exposure and expressed as ratio of the number of colonies grown from cadmium-exposed cultures to the number of colonies grown from unexposed cultures. Cell

survival of unexposed OmFCR-*ycf1* and pFL61-*ycf1* cells was similar and considered as 100% survival. Colonies were counted after 2 days at 30°C.

Cell survival and Can^R mutator assays were carried out with three biological replicates and three technical replicates for each biological replicate.

Statistical analyses

Statistical analyses were carried out through one-way analysis of variance (one-way ANOVA) and Tukey's post hoc test, using a probability level of $P < 0.05$.

Sequence data from this article have been submitted to GenBank under the following accession numbers: HQ839765 for *OmFCR* and HQ839766 for *OmMLH3*.

RESULTS

Identification of Cd(II)-resistance genes

An *O. maius* cDNA library was introduced into the yAP1-null (*yap1*) yeast mutant strain DY, which harbours an inactivated gene normally required for Cd resistance. Approximately 4×10^5 primary transformants were plated onto (i) SD agar plates containing a linear $CdSO_4$ concentration gradient (0–100 μM) and (ii) SD agar plates with fixed cadmium concentrations (50–100 μM). Empty vector-transformed *yap1* cells (pFL61-*yap1* cells) were unable to grow at 50 μM Cd(II), so this limit was used as a concentration threshold for testing cadmium resistance.

Transformed cells growing better than pFL61-*yap1* cells were selected and their plasmids rescued and sequenced. All the sequenced plasmids contained the same fungal cDNA insert encoding a predicted 179-amino acid long protein. We named this sequence OmFCR (*O. maius* Fungal Cadmium Resistance) after the AtPCR (*Arabidopsis thaliana* plant cadmium resistance) protein family (21), with whom OmFCR shared the capacity of conferring cadmium resistance to hypersensitive yeast mutants and a significant sequence similarity (*E*-value $<1e{-}06$). OmFCR-*yap1* cells grew until the third serial dilution at 80 μM Cd(II) (Figure 1A), while complete growth arrest was recorded at 120 μM Cd(II) (data not shown).

Computational analyses

A *blastx* search against NCBI (National Center for Biotechnology Information, NIH, Bethesda) databases revealed the presence of a Cys-rich conserved domain of unknown function called PLAC8 between AA 50–154 of the OmFCR sequence. Significant similarities were found with other unknown PLAC8-containing proteins from filamentous fungi, plants and animals. Interestingly, the PLAC8 domain is not present in any *S. cerevisae* proteins. Most of the *blast* hits were classified as 'hypothetical proteins' and the PLAC8 domain covered almost the entire length of the deduced protein. Only four groups of PLAC8 domain-containing proteins have been experimentally characterized, but none of them have been assigned a specific function so far: the aforementioned *A. thaliana* AtPCR proteins involved in cadmium and zinc resistance (21–23) and their homologs in other plants; the *A. thaliana* AtMCA1 protein (24), which is part of a stretch-activated Ca^{2+}-channel system; the Cell Number Regulator (CNR) genes (25), which may cooperate directly or indirectly in cell number regulation; the mammalian onzins (synonyms: PLAC8 protein, cornifelin, C15 protein), whose deregulation leads to marked apoptotic resistance, loss of G2/M checkpoint control, enhanced proliferation and tumorigenic conversion (26–30).

The *Pongo abelii* onzin (NP_001124833.1), the *Zea mays* CNR10 (NM_001157656.1) and the *A. thaliana* AtPCR3 (NM_112731) were selected as the most similar proteins to OmFCR within each group and were aligned with OmFCR by T-Coffee (31) (Figure 2). AtMCA1 (AB196960.1), being a much longer protein, was aligned only on its PLAC8 domain. The similarity among the five proteins was scattered and limited to specific amino acids, especially cysteine residues.

The internet service for protein sequence analysis PredictProtein (32) predicted the existence of a single transmembrane helix spanning from amino acids 99 to 116 of the OmFCR sequence (Table 1), while more than 50% of the protein was predicted to be devoid of regular secondary structures. In addition, the solvent accessibility composition suggested that OmFCR might be a globular protein. According to the WoLF PSORT localization program (33), it might be a nuclear protein, even though neither a standard nuclear localization signal (NLS) nor a DNA binding motif could be identified. Three disulfide bonds in the $C(X)_4CCPC$ motif and a potential threonine phosphorylation site on T163 were predicted by DISULFIND (34) and the NetPhos 2.0 Server (35), respectively (Table 1).

Oligonucleotide-directed site-specific mutagenesis and cadmium resistance in *yap1* mutant

To identify critical amino acid residues in OmFCR, some of the conserved amino acids identified by sequence alignment were primarily used as targets for oligonucleotide-directed site-specific mutagenesis (Figure 2). All the mutations are illustrated in Table 1. Briefly, the substitutions of R123, C142 and C145 with alanines yielded the R123A mutant, showing a single amino acid substitution, and the AXXA mutant, characterized by the simultaneous replacement of the two cysteines (Table 1). Cysteines within the CXXC motifs are found in a wide variety of metal-binding proteins and are considered redox residues as well as potential metal ion coordinators (36).

As the CPX motif is supposed to be specifically involved in heavy metal ions transduction in P1B-type ATPases (37), the possible role of the $C(X)_4CCPC$ region in mediating Cd resistance was investigated by combinations of multiple Cys, Pro → Ala replacements. The importance of the $C(X)_4CCPC$ region, the CXXC motif and the putative transmembrane helix domain was further tested by two C- and two N-terminal protein truncations.

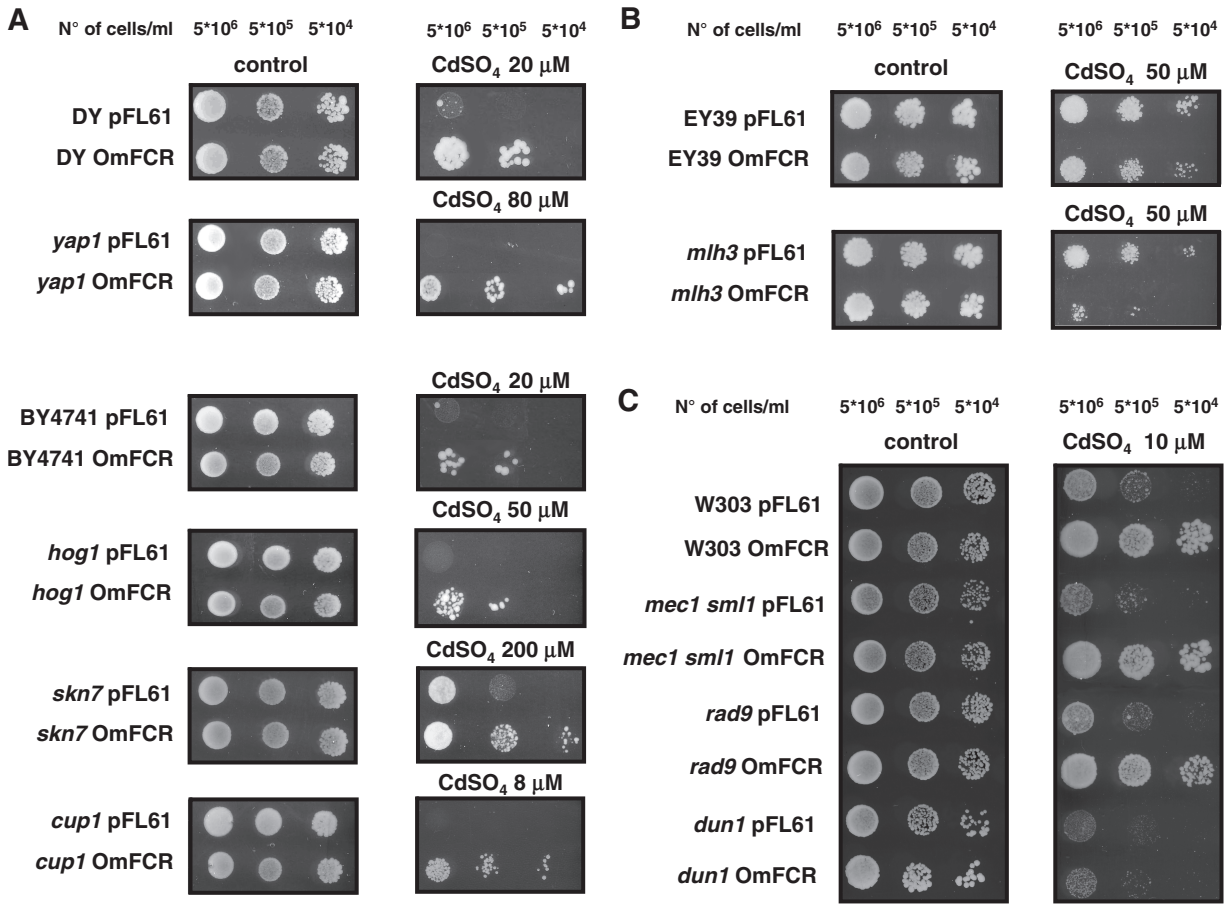


Figure 1. Sensitivity of *S. cerevisiae* strains to cadmium on solid medium. Each yeast strain was transformed with the empty vector (pFL61) and the OmFCR construct (OmFCR) and plated in three serial dilutions onto SD medium with (right column) or without (control, left column) $CdSO_4$. The concentrations of cadmium used in spot assays are indicated above each row. Plates were incubated at 30°C for 6 days. (**A**) DY wild-type strain and its isogenic *yap1* mutant, BY4741 wild-type strain and three isogenic mutants (*hog1*, *skn7*, *cup1*) on control and cadmium-amended medium. (**B**) EY39 wild-type strain and the isogenic *mlh3* mutant on control and 50 μM cadmium-amended medium. (**C**) W303 wild-type strain, the isogenic *mec1 sml1* double mutant, *rad9* mutant and *dun1* mutant on control and 10 μM cadmium-amended medium.

The non-conserved putative protein kinase C phosphorylation site T163 and the following residue D164 were also subjected to single amino acid substitution with alanines, giving rise to T163A and D164A mutants, respectively.

The 13 mutated OmFCR constructs listed in Table 1 were cloned in the yeast expression vector pFL61, transformed in *yap1* cells and plated onto a wide range of cadmium concentrations (50–320 μM).

The multiple replacements of Cys with Ala in the CPX motif and the upstream/downstream cysteines correlated with a progressive reduction in cadmium resistance, which could either depend on the number of the disrupted putative disulphide bonds or on the number of disrupted cadmium-binding residues. By contrast, replacement of both C142 and C145 with Ala in the CXXC motif caused the yeast cadmium resistance to be almost doubled [200 μM Cd(II)] with respect to the wild-type OmFCR. The single replacements involving T163 and D164 in the putative phosphorylation site caused a further increase of cadmium resistance up to 320 μM. No growth was observed for the R123A and the four truncated mutants at 50 μM Cd(II).

Testing other stress-inducing substances on OmFCR-*yap1* cells

To understand if the molecular mechanisms underlying the increased cadmium resistance of OmFCR-transformed cells rely on the presence of glutathione, cadmium-amended media were supplemented with 2 mM BSO, an inhibitor of glutathione (GSH) synthesis (38). GSH levels are already low in *yap1* cells (39), because yAP1 is a transcriptional activator of the γ-glutamylcysteine synthetase (GSH1) that catalyzes the first step in GSH biosynthesis. As demonstrated for AtPCR1-*ycf1* cells (21), cadmium resistance of OmFCR-*yap1* cells was not affected by the presence of BSO (see Supplementary Figure S1C), indicating that OmFCR likely confers GSH-independent cadmium resistance. On the contrary, *yap1* cells transformed with the empty vector became more susceptible to 8 μM Cd(II) when BSO was added to the medium (see Supplementary Figure S1C). This result suggested that a small amount of GSH was still present in *yap1* cells and that BSO had effectively decreased the GSH content of the cells.

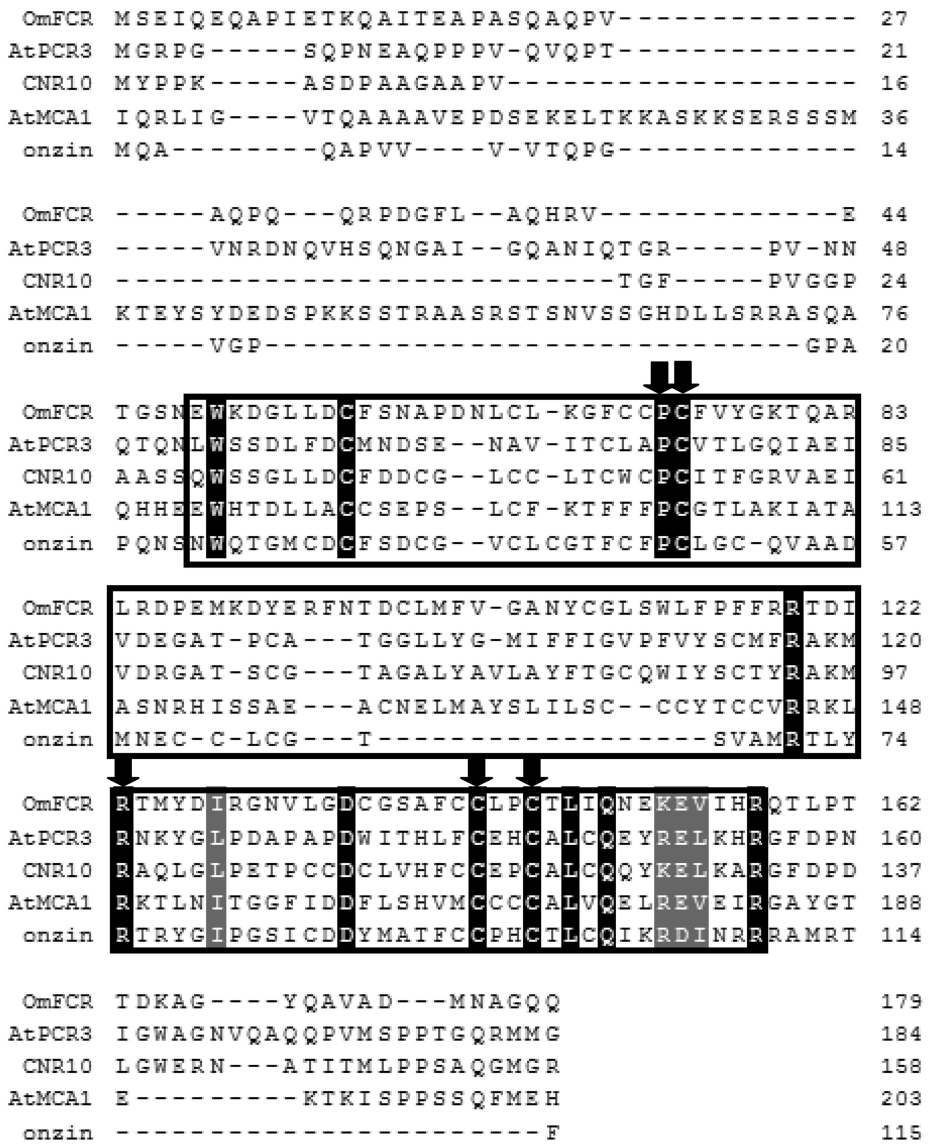


Figure 2. Sequence alignment of four PLAC8 proteins with OmFCR. The *Pongo abelii* onzin (NP_001124833.1), the *Zea mays* CNR10 (NM_001157656.1), the *Arabidopsis thaliana* AtPCR3 (NM_112731) and the *A. thaliana* AtMCA1 (AB196960.1) were aligned with OmFCR by T-Coffee and visualized by Multiple Align Show. AtMCA1, being a much longer protein in comparison to the other four, was aligned including only its PLAC8 domain. Black-shaded amino acids are identical, gray-shaded amino acids are functionally equivalent. Arrows point to the conserved amino acids which were primarily used as targets for oligonucleotide-directed site-specific mutagenesis. The box shows the PLAC8 domain.

The expression of OmFCR and its cadmium hypertolerant mutation D164A did not change the resistance of *yap1* cells to 20 mM Zn(II), 1.3 mM Cu(II), 0.8 mM Ni(II), 120 mM menadione, 1.6 M NaCl, 10 s at 51°C (heat shock), 40 mM hydroxyurea, 15 s UV–C light exposure (see Supplementary Figure S1A). Unexpectedly, pFL61-*yap1* cells were more resistant to 1 mM As(V) and 50 μg/ml phleomycin than OmFCR-*yap1* cells (see Supplementary Figure S1A).

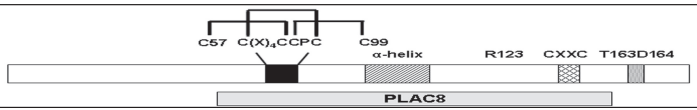
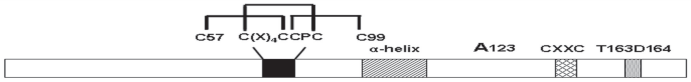
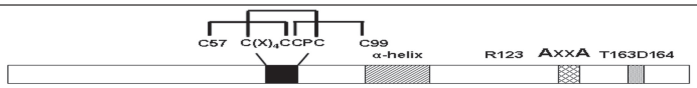
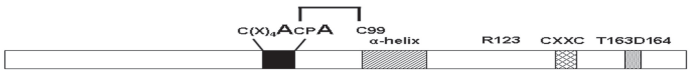
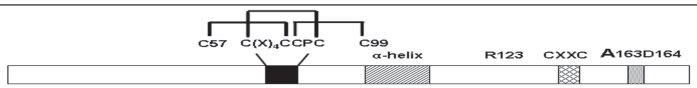
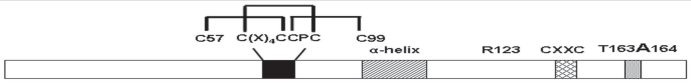
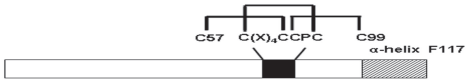
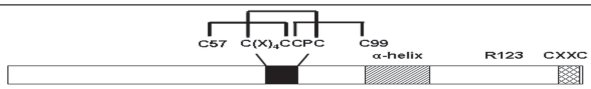
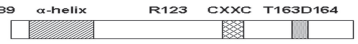
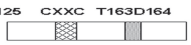
OmFCR transformation of other yeast strains and cadmium resistance

To test whether OmFCR-mediated cadmium resistance requires specific stress-activated pathways, we used yeast mutants carrying single deletions of *HOG1*, *SKN7* and *CUP1* redox stress responsive transcription factors and of the vacuolar glutathione S-conjugate transporter *YCF1*. All OmFCR-transformed cells grew better than the corresponding empty vector-transformed cells on Cd-containing medium (Figure 1A). A similar result was also observed in the OmFCR-transformed wild-type strains, DY and BY4741, even though the growth difference between OmFCR- and empty vector-cells in wild-type yeasts was much slighter than the one detected in the mutant yeasts (Figure 1A).

ICP-OES analysis

The *A. thaliana* AtPcr1 protein was suggested to mediate cadmium resistance by decreasing Cd uptake (21), so our first hypothesis was that OmFCR might have a similar

Table 1. Oligonucleotide-directed site-specific mutagenesis of the OmFCR sequence

Mutations	Structures	[Cd(II)] (μM)
Wild-type		120
R123A		<50
AXXA		200
$C(X)_4CAPA$		90
$C(X)_4ACPA$		80
$A(X)_4CAAA$		80
$C(X)_4AAAA$		70
$A(X)_4AAAA$		50
T163A		320
D164A		320
F117[a]		<50
E151[a]		<50
M89[b]		<50
M125[b]		<50

First column: name of OmFCR mutation. Second column: schematic representation of the wild-type and the mutated OmFCR sequences. The $C(X)_4$ CCPC region, putative disulfide bonds, the putative α-helix, the CXXC motif and the putative phosphorylation sites (TD) are indicated. The position of the PLAC8 domain is shown only for the wild-type OmFCR. Single or multiple amino acid substitutions are highlighted in bold. Third column: cadmium concentration that causes growth inhibition of the second and third yeast dilution in dilution spot assays. < 50 indicates complete growth inhibition at any dilution at 50 μM Cd(II).

[a]C-terminal truncated mutants, the final amino acid before the stop codon is shown.

[b]N-terminal truncated mutants, the starting methionine is shown.

biological role. Thus, the ICP-OES method was applied to determine Cd contents in Cd-treated OmFCR-, D164A- and pFL61-expressing yeast cells. However, no significant difference ($P > 0.05$) was recorded in the total Cd level of the three yeast strains grown for 48 h in 50 μM Cd(II)-containing medium, neither in water- nor in HCl-washed cells (Table 2).

Subcellular localization

The cellular localization of OmFCR was investigated in *yap1* cells by transformation with two enhanced green fluorescent protein (EGFP)-tagged OmFCR constructs. The N-terminal tagging probably interfered with the OmFCR function, as EGFP-OmFCR transformed cells did not grown on 50 μM Cd(II) (see Supplementary Figure S1B), which corresponds to half the growth limiting concentration of the untagged OmFCR. Conversely, yeasts transformed with the C-terminal EGFP tag (OmFCR-EGFP) were almost as cadmium resistant as the untagged OmFCR-transformed ones (see Supplementary Figure S1B). Western blots of OmFCR-EGFP transformed cells extracts probed with an anti-GFP monoclonal antibody showed that the fusion protein was of the predicted size (data not shown).

EGFP-*yap1* cells were used as negative control both for cadmium resistance assays of EGFP-tagged OmFCR constructs and for OmFCR subcellular localization. No growth of EGFP-*yap1* cells was recorded on 50 μM Cd(II)-containing medium (see Supplementary Figure S1B) and a diffused EGFP fluorescence was observed in the cytoplasm of these cells (Figure 3F). By contrast, the green fluorescence of the OmFCR-EGFP fusion protein was detected mostly as a single bright spot within the cytoplasm, which corresponded to the nucleus, as demonstrated by DAPI counterstaining (Figure 3A–D). No difference in the localization was recorded between 50 μM Cd(II) exposed- and non-exposed-cells (Figure 3B and E, respectively).

Yeast two-hybrid analysis

OmFCR was used as a bait in a yeast two-hybrid screening of a *S. cerevisiae* DupLEX-A Yeast Two-Hybrid genomic library to identify genes encoding proteins capable of interacting with OmFCR.

Table 2. Cadmium content in pFL61-, OmFCR- and D164A-*yap1* cells measured by ICP-OES after 48 h of 50 μM cadmium exposure

	Cd–H_2O[a]	Cd–HCl[b]
Empty vector	4.48*E*−06 ± 3.7*E*−07	7.37*E*−08 ± 3.7*E*−09
OmFCR	4.36*E*−06 ± 3.4*E*−07	8.20 *E*−08 ± 8.8*E*−09
D164A	4.93 *E*−06 ± 5.2*E*−07	6.01 *E*−08 ± 9.5*E*−09

The results are expressed in nmol/mg of dry weight as the average ± the standard deviation. No significant difference ($P > 0.05$) was recorded in the total Cd level of the three yeast strains, neither in water- nor in HCl-washed cells.
[a]Cadmium content of water-washed yeast cells.
[b]Cadmium content of HCl-washed yeast cells.

This screening led to the isolation of twelve positive prey clones: seven bore the 3′ terminus of the *MLH3* gene and five the 3′ terminus of the *MAS1* gene. Both fragments of genomic sequences were intron-free and corresponded to the last 252 C-terminal amino acids of Mlh3p and to the last 216 C-terminal amino acids of Mas1p. *MLH3* is a member of the MutL-homolog (*MLH*) family of DNA MMR genes (40), while *MAS1* encodes a subunit of the mitochondrial processing protease, which participates in protein cleavage during import into mitochondria (41).

OmFCR, Mlh3p and Mas1p constructs did not show any evidence of self-activation when assayed by co-transformation with empty vector or vector expressing an irrelevant protein such as OmSOD1 (42) (see Supplementary Figure S2). Each positive clone was repeatedly propagated on galactose containing-medium lacking leucine and tested for its ability of expressing β-galactosidase, as these properties were indicative of protein–protein association in the DupLEX-A system. The latter measurement could also be used to quantify the relative strength of any such interactions (43). As OmFCR/Mas1p association generated very pale blue colonies, we focussed our attention on the OmFCR/Mlh3p interaction (see Supplementary Figure S2).

Confirming the role of Mlh3p and its *O. maius* homolog OmMlh3 as OmFCR interactors in yeast

A *mlh3* mutant was used to determine whether the OmFCR-mediated cadmium resistance is strictly dependent on the interaction with Mlh3p. The yeast mutant was transformed with the empty vector and with OmFCR and plated onto 50 μM cadmium amended-medium. For the first time, the transformation with OmFCR resulted in a decreased cadmium resistance with respect to the empty vector-transformed cells, while no significant difference was observed in the corresponding isogenic wild-type cells (Figure 1B). This result indicates that, in the absence of Mlh3p, not only was OmFCR unable to confer cadmium resistance to yeast cells, but it also caused an overall reduction of yeast fitness with respect to the empty vector-*mlh3* cells.

Despite the absence of a nuclear localisation sequence, OmFCR-EGFP was localized in the nucleus in *yap1* cells (Figure 3A–D). By transformation of *mlh3* cells with the OmFCR-EGFP construct, we tested whether (i) OmFCR is driven to this subcellular compartment by its interaction with Mlh3p and (ii) the OmFCR inability to confer cadmium resistance to *mlh3* cells is potentially related to a non-nuclear localization. The fluorescence of the OmFCR-EGFP fusion protein in *mlh3* cells was observed as a diffuse cytoplasmic staining (Figure 3H), whereas the signal in the corresponding wild-type cells was predominantly nuclear (Figure 3G), as previously detected in *yap1* cells.

The *O. maius* homolog of the yeast *MLH3* gene was isolated by PCR with specific primers. The 3′ terminus of *OmMlh3*, which corresponds to the yeast *MLH3* gene fragment isolated by yeast-two hybrid, was cloned into the pJG4-5 plasmid and tested as a prey for a possible

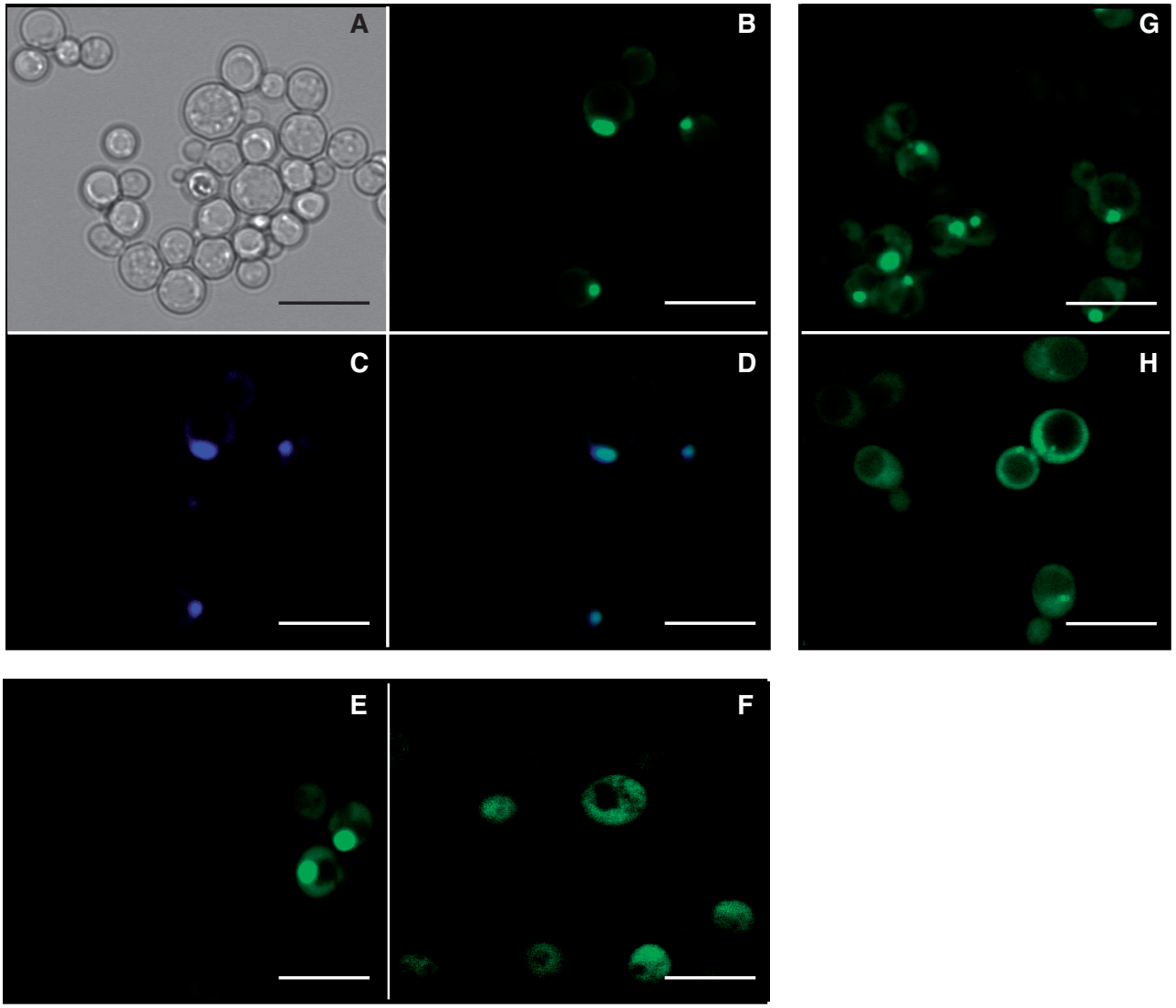


Figure 3. Localization of OmFCR in cells expressing the OmFCR-EGFP construct. Panels A–F: *yap1* mutant cells. (**A**) Cells are shown in transmitted light. (**B**) OmFCR-EGFP localization is seen as a single green spot within the cell. (**C**) DNA is stained in blue with DAPI. (**D**) Both probes label the nucleus, as seen in the superimposition of the signals. (**E**) OmFCR-EGFP nuclear localization after 24 h of cadmium exposure. (**F**) EGFP cytoplasmic localization. (**G**) In EY39 wild-type cells, the OmFCR-EGFP fluorescence is similar to the one observed in panel B. (**H**) In *mlh3* cells, OmFCR-EGFP localization is observed as a diffused cytoplasmic staining. Scale bars: 10 μm.

protein–protein interaction with OmFCR. Despite some sequence divergence with Mlh3p, OmMlh3 was also capable of strongly interacting with OmFCR (see Supplementary Figure S2).

Upon acute cadmium exposure no significant difference was observed in cell survival and in the mutation frequency between OmFCR- and pFL61-*ycf1* cells

Given the interaction of OmFCR with Mlh3p, we next investigated whether the capacity of OmFCR-transformed cells to grow upon cadmium acute exposure was due to a possible involvement of OmFCR in reducing the mutagenic effects of cadmium and/or in increasing cell survival.

The optical densities of OmFCR- and pFL61-*ycf1* cell cultures were monitored at different time-points (Figure 4A). After 7 h of 50 μM cadmium exposure, the difference between the OD_{600} of OmFCR-*ycf1* cells and of pFL61-*ycf1* cells began to be highly significant ($P < 0.01$) (Figure 4A, inset). From this time-point on, cell survival of Cd-treated cultures was measured and expressed as percentage relative to untreated cell cultures, which was demonstrated to be 100% (225.3 ± 30.6 and 217 ± 5.3 colonies for pFL61- and OmFCR-*ycf1* cells, respectively).

After 7, 24 and 48 h of cadmium exposure, cell survival of both pFL61- and OmFCR-*ycf1* cells progressively decreased, but no statistically significant difference ($P > 0.05$) was observed between the two cultures (Figure 4B). After 120 h, the OD_{600} of treated OmFCR-*ycf1* cells was more than twice the OD_{600} of treated pFL61-*ycf1* cells (Figure 4A). At this time point, OmFCR-*ycf1* cells showed a slight, but significant, increase in the percentage of cell survival (4.0 ± 0.0%) as compared to the percentage recorded after 48 h

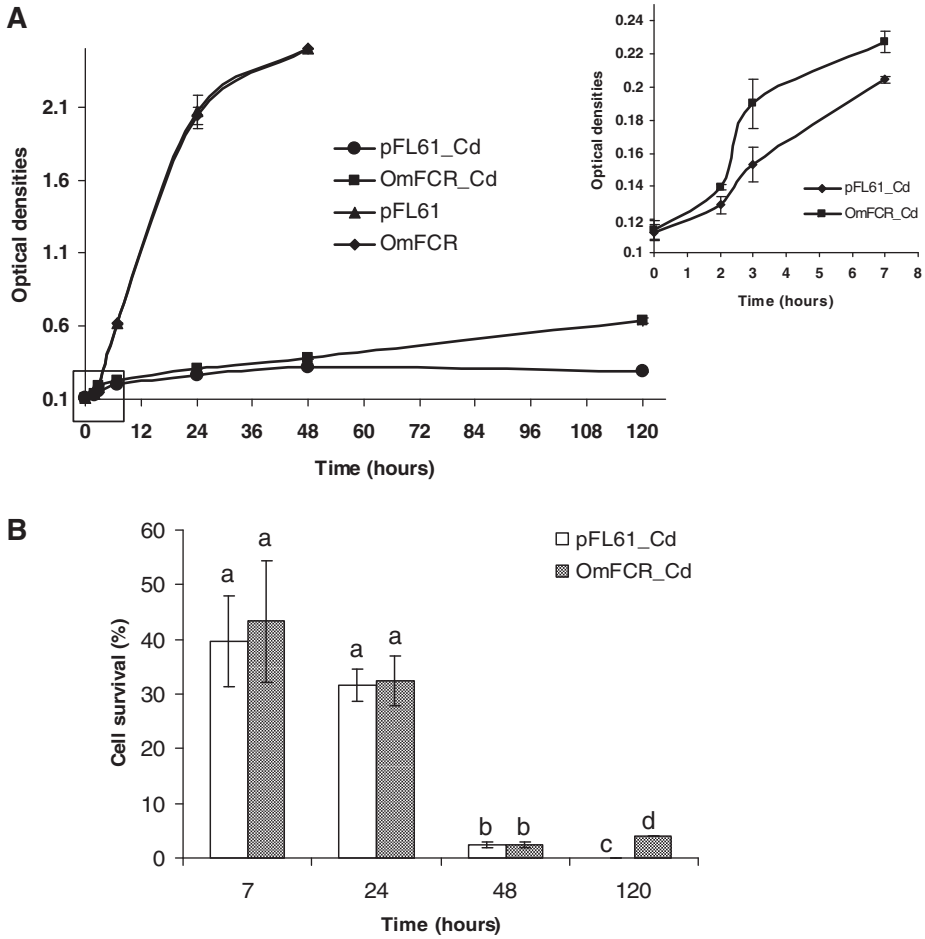


Figure 4. Yeast growth curves and cell survival percentages of unexposed and Cd-exposed cell cultures. (**A**) The optical densities of OmFCR- and pFL61-*ycf1* cell cultures was measured after 2, 3, 7, 24, 48 and 120 h of incubation at 30°C, either with or without 50 μM $CdSO_4$. Only treated cell cultures were also sampled at 120 h, because untreated cultures reached saturation within 48 h. After 7 h of incubation, the OD_{600} difference between Cd-treated OmFCR-*ycf1* cells (OmFCR_Cd) and Cd-treated pFL61-*ycf1* cells (pFL61_Cd) began to be highly significant ($P < 0.01$) (inset). The first points of Cd-treated OmFCR- and pFL61-growth curves were shown in details (inset). (**B**) From 7 h of incubation on, cell survival of Cd-treated cultures was measured and expressed as a percentage of the cell survival of the corresponding untreated cell cultures, which was demonstrated to be 100%. Different letters above each bar indicate statistically significant differences ($P < 0.05$).

(2.4 ± 0.6%), while the pFL61-*ycf1* cells were all, or almost all, dead (Figure 4B).

After 7 h of cadmium exposure, when cell survival was still acceptable to investigate cadmium-induced mutagenesis (39.6 ± 8.4% and 43.3 ± 11.1% for pFL61- and OmFCR-*ycf1* cells, respectively), nearly 1×10^7 cells from Cd-treated and untreated cell cultures were plated onto selective medium to examine the occurrence of Can^R mutations. As expected, a significant 2.2-fold increase in Can^R mutation frequency was recorded in cadmium-treated cells as compared to the corresponding non-treated cells. By contrast, no statistically significant difference ($P > 0.05$) in Can^R mutation frequency was observed between treated pFL61-*ycf1* cells and treated OmFCR-*ycf1* cells ($152.5 \times 10^{-7} \pm 19.5 \times 10^{-7}$ and $113.5 \times 10^{-7} \pm 38 \times 10^{-7}$, respectively).

For untreated cultures, no significant differences between pFL61- and OmFCR-*ycf1* cells were found in growth rate and in Can^R spontaneous mutation frequency ($49 \times 10^{-7} \pm 7.5 \times 10^{-7}$ and $45.5 \times 10^{-7} \pm 21.9 \times 10^{-7}$, respectively) at any time-point.

OmFCR appears to genetically interact with Dun1p

In *S. cerevisiae*, the phosphorylation cascade activated by Mec1p and involving Rad9p, Rad53p and Dun1p controls the G_2/M arrest response after DNA damage or replicational stress (44). To investigate whether the OmFCR-mediated response to cadmium may be related to this signalling pathway, mutants of this pathway were transformed with OmFCR. OmFCR-W303 wild-type cells, the isogenic *mec1 sml1* double mutant and the *rad9* mutant were demonstrated to be more resistant to cadmium than the corresponding pFL61-transformed cells (Figure 1C). By contrast, pFL61- and OmFCR-*dun1* cells exhibited the same cadmium sensitivity (Figure 1C).

DISCUSSION

The increased accessibility of high-throughput sequencing technologies is generating a tremendous amount of genomic data, but it is also widening the gap between

the experimentally characterized genes and the number of sequences without any functional assignment. The protein products without an assigned function are variously referred to as 'hypothetical proteins', 'predicted proteins' or even 'unknown proteins' and represent more than 40% of the entries within public databases (45). Some of them are widespread and evolutionarily conserved, so they are expected to play important biological roles (46). This might be the case of the PLAC8 family, formerly known as domain of unknown function (DUF) 614, which, according to the Pfam database (47), includes more than 500 proteins belonging to all the main eukaryotic kingdoms. Despite this wide phylogenetic distribution, the majority of the PLAC8 domain-containing proteins are classified as hypothetical proteins of unknown function by automated function prediction softwares.

Computational methods for predicting protein functions are certainly useful to create reasonable hypotheses on the biological functions of 'predicted proteins', but these hypotheses have to be ultimately verified through direct experimentation (48). Both approaches have been extensively used to characterize an *O. maius* cDNA that mediates a specific response of *yap1* yeast cells to cadmium exposure.

Amino acids that play a key role in OmFCR functionality

On the basis of *in silico* analyses, the OmFCR deduced amino acid sequence shares common traits with proteins involved in heavy metal detoxification, such as P1B-type ATPases (previously named CPx-ATPases) and metallothioneins, but the highest sequence similarity was recorded with fungal PLAC8-family members.

Similarly to P1B-type ATPases, OmFCR contains CPX and CXXC motifs. The CPX motif is a distinguishing feature of heavy metal-pumping ATPases and metal-binding proteins, including rubredoxins, ferredoxins, and metallothioneins, and it has been proposed to be specifically involved in metal ions translocation across the membrane (37). CXXC is the major redox motif utilized for formation, reduction and isomerization of disulfide bonds and is believed to be involved in the maintenance of protein conformation and protein–protein interactions (49).

The OmFCR CPX motif is localized at the beginning of the PLAC8 domain in the predicted transmembrane helix, while two CXXC motifs are recognizable at the opposite ends of the PLAC8 domain, the first encompassing the CPX motif within the $C(X)_4\underline{C}\underline{C}P\underline{C}$ region. Site-directed mutagenesis on Cys residues within the $C(X)_4CCPC$ region showed that an increase in the number of mutated cysteines corresponds to a decrease in Cd resistance. This OmFCR Cys-rich region corresponds to the onzin 11-amino-acid cysteine-rich region, which has been demonstrated to be fundamental for the interaction with phospholipid scramblase 1 (28). On this basis, we can hypothesize that the Cys-rich region might be also critical for the physical interaction between OmFCR and Mlh3p, which will be further discussed in the next paragraphs.

By contrast, the disruption of both cysteines in the CXXC motif located at the end of the PLAC8 domain represented a gain-of-function mutation, as the AXXA mutant could tolerate a Cd concentration 2-folds higher than the wild-type OmFCR.

At the C-terminus of the OmFCR sequence, beyond the predicted PLAC8 domain, there is a DKAGYQA motif in which three residues (D164, K165, G167) are identical to the DKTGTLT motif of P1B-type ATPases. The reversibly phosphorylated aspartic acid residue within this motif plays an essential role in the ATP hydrolysis and pumping cycle of P1B-type ATPases (50). Bioinformatic tools for the prediction of aspartic acid phosphorylation are not available so far, but the NetPhos 2.0 Server identified T163, the amino acid that immediately precedes the aspartic acid, as a possible phosphorylation site within the OmFCR sequence. Single amino acid substitutions involving T163 and D164 both determined a striking increase of the yeast Cd resistance, from 100 μM of the wild-type OmFCR to 320 μM of the two mutants. Although this result does not provide more clues about which amino acid could be phosphorylated, it suggests, together with the gain-of-function AXXA mutation, the presence of a negative regulatory domain in the C-terminal region of OmFCR. A similar conclusion was proposed for onzin by Rogulski and colleagues, who observed a more robust interaction of onzin with its partners, Mdm2 and Akt1, after the deletion of 72 C-terminal amino acids (27).

Unlike P1B-type ATPases, which generally have 10 hydrophobic membrane-spanning helices, OmFCR is only 179 amino acid-long and has only one predicted transmembrane domain. In this regard, it is worth noting that much discrepancy arises among the results of different secondary structure prediction methods about the presence and the number of transmembrane domains.

All OmFCR truncated mutants showed that protein integrity is essential for function. On the contrary, onzin deletion mutants demonstrated that a 41-amino acid long fragment encompassing the 11-amino-acid Cys-rich region was necessary and sufficient for biological activity (28). In *Arabidopsis*, the essentiality of the Cys-rich segment is more controversial, as two C-terminal truncations of AtPcr1, both including the cysteine stretch, showed opposite phenotypes: the one with the longest truncation was not resistant to cadmium, while the second showed the same function as the full-length protein (21).

Finally, the single mutation on R123, a conserved amino acid in most PLAC8-containing proteins, caused a reduction in OmFCR cadmium resistance similar to the one observed in truncated mutants. Therefore, R123 could be itself a critical amino acid or be part of a fundamental region for OmFCR functionality.

OmFCR has not a direct role in cadmium detoxification

The yeast Yap1p has been demonstrated to play a crucial role not only in the response to oxidative stress, but also in the cadmium detoxification process through the transcriptional activation of *YCF1* (51). OmFCR has the capacity to reverse the cadmium-hypersensitive phenotypes caused by the single deletion of both *YAP1* and *YCF1* genes. Enhanced cadmium resistance was also observed in

other OmFCR-transformed yeasts that were not directly compromised for their response to this heavy metal, such as *skn7*, *hog1* and *cup1* mutants and DY and BY4741 wild-type strains. Although *skn7* mutant is hyper-tolerant to cadmium per se (52), OmFCR caused a further increase in the resistance to this heavy metal.

Similarly to AtPCR1 (21), it is likely that the OmFCR mechanism of action does not rely on glutathione, as cadmium resistance of the OmFCR-*yap1* cells did not change in the presence of the glutathione synthesis inhibitor BSO.

Cadmium contents measured by ICP-OES in pFL61-, OmFCR- and D164A-cells tend to exclude that OmFCR is either a membrane efflux pump or a heavy metal chelator, as the expression of an efflux pump would decrease the cellular Cd content in the cell, while an intracellular chelator would increase it (21). Although the possibility that Cys residues of OmFCR may bind Cd ions cannot be excluded, there are other arguments against the hypothesis that OmFCR might merely function as a chelator: (i) the E151 truncated mutant has the same number of Cys residues as OmFCR wt, but it showed a compromised phenotype on cadmium and (ii) the disruption of two Cys residues in the AXXA mutant caused an increased resistance to cadmium.

These data suggest that OmFCR does not have a direct role in cadmium detoxification, but it might confer a marked Cd-resistant phenotype through an interaction with yeast proteins and/or an involvement in some specific cadmium detoxification pathway.

OmFCR interacts with yeast Mlh3p and *O. maius* OmMlh3

The finding that OmFCR interacts with yeast Mlh3p and *O. maius* OmMlh3 is consistent with the nuclear localization of the OmFCR-EGFP construct. *MLH3* is, in fact, one of the four homologs of bacterial MutL that, in cooperation with the *MSH* genes, are involved in the correction of errors associated with DNA replication and recombination (53), especially during meiosis (54,55). In *S. cerevisiae* three MutL heterodimers, Mlh1p–Mlh3p, Mlh1p–Mlh2p and Mlh1p–Pms1p, have been identified so far. Interestingly, the fragment of Mlh3p that has been shown to interact with OmFCR corresponds to the Mlh1p-interactive carboxyl-terminal portion of this protein. The last 210 amino acids of yeast Mlh3p and the last 63 amino acids of *Homo sapiens* hMlh3 are, in fact, considered critical for the interaction with Mlh1p (56–58).

The Mlh1p–Mlh3p complex, in association with Msh2p–Msh3p, has been primarily ascribed a role in the repair of specific base-base mispairs as well as in the suppression of homology-mediated duplication and deletion mutations (40,59). Additionally, mutations in *MLH3* cause a small but significant increase in the rate of accumulation of single-base frameshift mutations in yeast (40).

The fact that OmFCR can confer a marked resistance only to cadmium-exposed cells is in agreement with the possible involvement of Mlh3p and the MMR system in the mechanism of action of OmFCR. Cadmium is in fact known to target all major DNA repair systems, as extensively reviewed by Giaginis and collaborators (4), but recent literature about cadmium mutagenic effects and cadmium-mediated carcinogenesis is mostly centered on its ability to inhibit especially the MMR proteins (5–8,60,61). In particular, ATP binding and hydrolysis by Msh2p–Msh6p have been clearly identified as targets of cadmium, while DNA binding affinity and mismatch recognition are less affected by this heavy metal (5,6). Wieland and collaborators discovered that the mechanism of ATP hydrolysis inhibition does not involve a particular site on the heterodimer, but it is rather due to the binding of a large number of cadmium ions to multiple sites, which leads to changes in protein conformation and loss of function (8). Interestingly, MMR-deficient cells showed an increase in the spontaneous mutation rates of long homonucleotide runs and other microsatellites similar to the phenotype observed in wild-type cells chronically exposed to cadmium, while lack of BER, NER or double-strand break repair had not such dramatic effects on those targets (7). This result strongly supports the hypothesis that MMR itself is a primary target of cadmium and the increase in frameshift mutations and microsatellite instability may account for cadmium genotoxicity. The relationship between cadmium exposure and the MMR system is so tight that the altered expression of the MMR genes in *A. thaliana* seedlings has been proposed by Liu and colleagues (62,63) as a possible bio-indicator of cadmium pollution.

Among the other stresses tested on OmFCR-*yap1* cells, copper, zinc, arsenic, menadione and heat shock are known to cause mainly macromolecular damage, depletion of cellular thiols and lipid peroxidation through oxidative damage (64–67), without involving specific DNA repair mechanisms. As regards the tested DNA-damaging agents, UV–C light exposure has been reported to induce DNA single-strand breaks that are mostly repaired by NER (68), while hydroxyurea and phleomycin are responsible for double-strand breaks (DBD) that are recognized and processed by homologous recombination or nonhomologous end joining repair (69,70).

A working model for OmFCR

Our results showed the existence of a tight relationship between Mlh3p and OmFCR in yeast, because the OmFCR-mediated cadmium resistance as well as the OmFCR localization within the cell require the presence of Mlh3p. It is therefore feasible that the nuclear localization of OmFCR may be fundamental for mediating cadmium resistance.

There is increasing evidence that the MMR system may have a direct or indirect role in the activation of the signal cascade that leads to cell cycle arrest in case of DNA lesion (71). In particular, the 'direct signalling model' proposes that MMR complexes have two distinct functions: one in DNA repair and the other in the DNA damage signal transduction that leads to cell cycle arrest and/or apoptosis, even independently of the MMR (72). The prokaryotic MutL and the eukaryotic Mlh proteins are considered the top candidates for the role of cellular ringmasters that bridge the DNA lesion recognition by

MutS/Msh proteins to downstream pathways (73). Yet, the identities of many of the MMR-associated accessory proteins and the details of MMR direct signaling pathways remain unclear (71,73).

In this scenario, we propose a working model (Figure 5) where OmFCR may represent an MMR-associated protein that physically couples DNA lesion recognition by the MMR system to appropriate effectors that affect cell cycle checkpoint pathways and ultimately determine cell fate. The results of the Can^R assays upon cadmium exposure are consistent with the fact that OmFCR is probably involved in non-DNA repair functions of the MMR system, rather than in DNA repair.

Yen and colleagues demonstrated that cells respond to cadmium exposure by inducing a cell cycle delay through the activation of the SCF^{Met30}/Met4p and the Mec1p/Rad53p cell cycle checkpoint pathways (9). The higher growth rate of OmFCR-transformed cells upon cadmium exposure might be explained by a possible alteration to the signaling of cell cycle delay. This cellular response appears to be directly or indirectly mediated by the genetic interaction of OmFCR with the kinase Dun1p, as demonstrated by the cadmium resistance experiments with yeasts mutated in the Mec1p-dependent phosphorylation cascade. Dun1p generally acts at the end of this checkpoint pathway, so the signaling pathway promoted by OmFCR seems to merge with the final part of the Mec1p-dependent phosphorylation cascade: in pFL61-transformed cells, Dun1p is likely to recruit effector proteins that cause cell cycle arrest, while the presence of OmFCR might enlist alternative effector proteins that ultimately allow the progression of cell division (Figure 5).

The potential phosphorylation site in the OmFCR sequence appears to be critical in cadmium response and may indicate for OmFCR an active role as a signal transducer in the phosphorylation cascade. The gain-of-function mutants T163A and D164A suggest that the phosphorylation of OmFCR might attenuate the signal that leads to cell cycle progression, representing either a negative feedback control system or a negative signal in case of excessive damage caused by cadmium.

PLAC8 proteins and cell cycle control

Despite the poor conservation of amino acid sequences and the heterogeneity of experimental results on PLAC8 domain-containing proteins, some of them might be ultimately involved, either directly or indirectly, in cell cycle control. Guo and collaborators have recently suggested a possible association between the CNR proteins and the cell cycle, on the basis of an inverse relationship between the overexpression of CNR1 and the maize plant size and cell number (25). Investigations on FW2.2, which has been included in the CNR family (25), led to

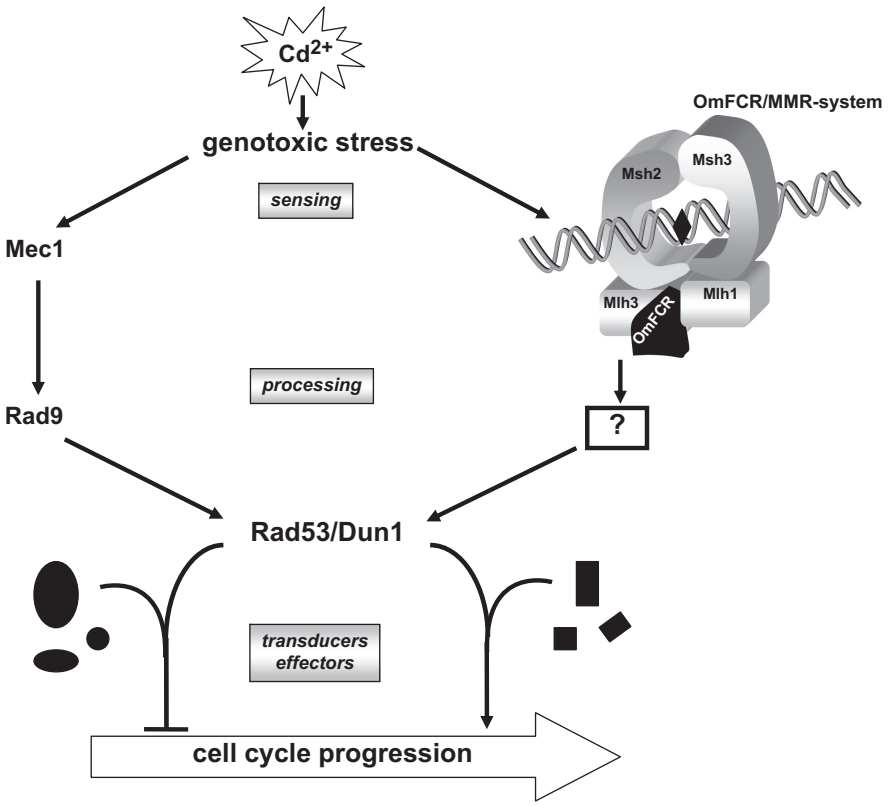


Figure 5. Working model for OmFCR. The genotoxic stress caused by cadmium might recruit the MMR system, which, in its turn, might promote the firing of OmFCR through protein–protein interactions with Mlh3p. The signaling pathway promoted by OmFCR appears to merge with the final part of the Mec1p-dependent phosphorylation cascade, at the Rad53p/Dun1p level. In pFL61-transformed cells (left) Dun1p is likely to recruit effector proteins that cause cell cycle arrest, while the presence of OmFCR (right) might enlist alternative effector proteins that ultimately allow the progression of cell division.

similar results, as its expression negatively affected cell number in tomato carpels (74). Furthermore, FW2.2 and GmFWL1 (*Glycine max* FW2.2-like 1) directly interact with casein kinases II (75,76), which are involved in the regulation of the signaling cascade that control cell division. Even mammalian onzins, whose deregulation leads to enhanced cell proliferation and tumorigenic conversion (28), appear to be involved in the same biological process. Therefore, it is likely that a large group of PLAC8 proteins is involved in the control of cell cycle checkpoints (77).

In conclusion, OmFCR is the first characterized fungal member of the PLAC8 family. The experiments demonstrate that OmFCR specifically mediates the yeast response to cadmium exposure through the interaction with Mlh3p and indicate the possible involvement of OmFCR in the signaling pathway that couples DNA lesion recognition by the MMR system and cell fate. Although further work is needed to elucidate the specific role of OmFCR in the cadmium response, our working model supports the hypothesis that a group of PLAC8 proteins is potentially involved in regulating cell division.

ACCESSION NUMBERS

GenBank HQ839765, HQ839766.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Hartwell,L. (1992) Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. *Cell*, **71**, 543–546.
2. Boiteux,S., Gellon,L. and Guibourt,N. (2002) Repair of 8-oxoguanine in *Saccharomyces cerevisiae*: interplay of DNA repair and replication mechanisms. *Free Rad. Biol. Med.*, **32**, 1244–1253.
3. Doetsch,P.W., Morey,N.J., Swanson,R.L. and Jinks-Robertson,S. (2001) *Yeast Base Excision Repair: Interconnections and Networks*. Academic Press Inc., San Diego.
4. Giaginis,C., Gatzidou,E. and Theocharis,S. (2006) DNA repair systems as targets of cadmium toxicity. *Toxicol. Appl. Pharmacol.*, **213**, 282–290.
5. Banerjee,S. and Flores-Rozas,H. (2005) Cadmium inhibits mismatch repair by blocking the ATPase activity of the MSH2-MSH6 complex. *Nucleic Acids Res.*, **33**, 1410–1419.
6. Clark,A.B. and Kunkel,T.A. (2004) Cadmium inhibits the functions of eukaryotic MutS complexes. *J. Biol. Chem.*, **279**, 53903–53906.
7. Jin,Y.H., Clark,A.B., Slebos,R.J.C., Al-Refai,H., Taylor,J.A., Kunkel,T.A., Resnick,M.A. and Gordenin,D.A. (2003) Cadmium is a mutagen that acts by inhibiting mismatch repair. *Nat. Genet.*, **34**, 326–329.
8. Wieland,M., Levin,M.K., Hingorani,K.S., Biro,F.N. and Hingorani,M.M. (2009) Mechanism of cadmium-mediated inhibition of Msh2-Msh6 function in DNA mismatch repair. *Biochemistry*, **48**, 9492–9502.
9. Yen,J.L., Su,N.Y. and Kaiser,P. (2005) The yeast ubiquitin ligase SCF^{Met30} regulates heavy metal response. *Mol. Biol. Cell*, **16**, 1872–1882.
10. McLaughlin,M.J., Parker,D.R. and Clarke,J.M. (1999) Metals and micronutrients - food safety issues. *Field Crops Res.*, **60**, 143–163.
11. Greszta,J., Braniewski,S., Chrzanowska,E., Nosek,A., Chlodny,J. and Zwolinski,J. (1987) The influence of dusts from chosen industrial lands on particular links of forest ecosystems of the Niepolomice forest. *Ekologia Polska*, **35**, 291–326.
12. Martino,E., Turnau,K., Girlanda,M., Bonfante,P. and Perotto,S. (2000) Ericoid mycorrhizal fungi from heavy metal polluted soils: their identification and growth in the presence of zinc ions. *Mycol. Res.*, **104**, 338–344.
13. Abbà,S., Khouja,H.R., Martino,E., Archer,D.B. and Perotto,S. (2009) SOD1-targeted gene disruption in the ericoid mycorrhizal fungus *Oidiodendron maius* reduces conidiation and the capacity for mycorrhization. *Mol. Plant-Microbe Interactions*, **22**, 1412–1421.
14. Kuge,S. and Jones,N. (1994) YAP1 dependent activation of TRX2 is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. *EMBO J.*, **13**, 655–664.
15. Ruotolo,R., Marchini,G. and Ottonello,S. (2008) Membrane transporters and protein traffic networks differentially affecting metal tolerance: a genomic phenotyping study in yeast. *Genome Biol.*, **9**, R67.
16. Cotton,V.E., Hoffmann,E.R. and Borts,R.H. (2010) Distinct regulation of Mlh1p heterodimers in meiosis and mitosis in *Saccharomyces cerevisiae*. *Genetics*, **185**, 459–467.
17. Gietz,R.D. and Woods,R.A. (2006) Yeast transformation by the LiAc/SS carrier DNA/PEG method. *Methods Mol. Biol.*, **313**, 107–120.
18. Kuwayama,H., Obara,S., Morio,T., Katoh,M., Urushihara,H. and Tanaka,Y. (2002) PCR-mediated generation of a gene disruption construct without the use of DNA ligase and plasmid vectors. *Nucleic Acids Res.*, **30**, e2.
19. Chen,C., Merrill,B.J., Lau,P.J., Holm,C. and Kolodner,R.D. (1999) *Saccharomyces cerevisiae* pol30 (proliferating cell nuclear antigen) mutations impair replication fidelity and mismatch repair. *Mol. Cell. Biol.*, **19**, 7801–7815.
20. Serero,A., Lopes,J., Nicolas,A. and Boiteux,S. (2008) Yeast genes involved in cadmium tolerance: identification of DNA replication as a target of cadmium toxicity. *DNA Repair*, **7**, 1262–1275.
21. Song,W.Y., Martinoia,E., Lee,J., Kim,D., Kim,D.Y., Vogt,E., Shim,D., Choi,K.S., Hwang,I. and Lee,Y. (2004) A novel family of cys-rich membrane proteins mediates cadmium resistance in *Arabidopsis*. *Plant Physiol.*, **135**, 1027–1039.

22. Song,W.Y., Choi,K.S., Kim,D.Y., Geisler,M., Park,J., Vincenzetti,V., Schellenberg,M., Kim,S.H., Lim,Y.P., Noh,E.W. *et al*. (2010) Arabidopsis PCR2 is a zinc exporter involved in both zinc extrusion and long-distance zinc transport. *Plant Cell Online*, **22**, 2237–2252.
23. Song,W.Y., Hörtensteiner,S., Tomioka,R., Lee,Y. and Martinoia,E. (2011) Common functions or only phylogenetically related? The large family of PLAC8 motif-containing/PCR genes. *Mol. Cells*, **31**, 1–7.
24. Nakagawa,Y., Katagiri,T., Shinozaki,K., Qi,Z., Tatsumi,H., Furuichi,T., Kishigami,A., Sokabe,M., Kojima,I., Sato,S. *et al*. (2007) *Arabidopsis* plasma membrane protein crucial for Ca2+influx and touch sensing in roots. *PNAS*, **104**, 3639–3644.
25. Guo,M., Rupe,M.A., Dieter,J.A., Zou,J., Spielbauer,D., Duncan,K.E., Howard,R.J., Hou,Z. and Simmons,C.R. (2010) Cell number regulator1 affects plant and organ size in maize: implications for crop yield enhancement and heterosis. *Plant Cell*, **22**, 1057–1073.
26. Galaviz-Hernandez,C., Stagg,C., de Ridder,G., Tanaka,T.S., Ko,M.S.H., Schlessinger,D. and Nagaraja,R. (2003) Plac8 and Plac9, novel placental-enriched genes identified through microarray analysis. *Gene*, **309**, 81–89.
27. Rogulski,K., Li,Y., Rothermund,K., Pu,L., Watkins,S., Yi,F. and Prochownik,E.V. (2005) Onzin, a c-Myc-repressed target, promotes survival and transformation by modulating the Akt-Mdm2-p53 pathway. *Oncogene*, **24**, 7524–7541.
28. Li,Y., Rogulski,K., Zhou,Q., Sims,P.J. and Prochownik,E.V. (2006) The negative c-Myc target onzin affects proliferation and apoptosis via its obligate interaction with phospholipid scramblase I. *Mol. Cell. Biol.*, **26**, 3401–3413.
29. Ledford,J.G., Kovarova,M. and Koller,B.H. (2007) Impaired host defense in mice lacking onzin. *J. Immunol.*, **178**, 5132–5143.
30. Wu,S.F., Huang,Y., Hou,J.K., Yuan,T.T., Zhou,C.X., Zhang,J. and Chen,G.Q. (2010) The downregulation of onzin expression by PKCε-ERK2 signaling and its potential role in AML cell differentiation. *Leukemia*, **24**, 544–551.
31. Notredame,C., Higgins,D.G. and Heringa,J. (2000) T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.*, **302**, 205–217.
32. Rost,B. and Liu,J.F. (2003) The PredictProtein server. *Nucleic Acids Res.*, **31**, 3300–3304.
33. Horton,P., Park,K.J., Obayashi,T., Fujita,N., Harada,H., dams-Collier,C.J. and Nakai,K. (2007) WoLF PSORT: protein localization predictor. *Nucleic Acids Res.*, **35**, W585–W587.
34. Ceroni,A., Passerini,A., Vullo,A. and Frasconi,P. (2006) DISULFIND: a disulfide bonding state and cysteine connectivity prediction server. *Nucleic Acids Res.*, **34**, W177–W181.
35. Blom,N., Gammeltoft,S. and Brunak,S. (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J. Mol. Biol.*, **294**, 1351–1362.
36. Fomenko,D.E., Marino,S.M. and Gladyshev,V.N. (2008) Functional diversity of cysteine residues in proteins and unique features of catalytic redox-active cysteines in thiol oxidoreductases. *Mol. Cells*, **26**, 228–235.
37. Solioz,M. and Vulpe,C. (1996) CPx-type ATPases: a class of P-type ATPases that pump heavy metals. *Trends Biochem. Sci.*, **21**, 237–241.
38. Griffith,O.W. and Meister,A. (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). *J. Biol. Chem.*, **254**, 7558–7560.
39. Gomes,D.S., Riger,C.J., Pinto,M.L.C., Panek,A.D. and Eleutherio,E.C.A. (2005) Evaluation of the role of Ace1 and Yap1 in cadmium absorption using the eukaryotic cell model *Saccharomyces cerevisiae*. *Environ. Toxicol. Pharmacol.*, **20**, 383–389.
40. Flores-Rozas,H. and Kolodner,R.D. (1998) The *Saccharomyces cerevisiae* MLH3 gene functions in MSH3-dependent suppression of frameshift mutations. *Proc. Natl Acad. Sci. USA*, **95**, 12404–12409.
41. Witte,C., Jensen,R.E., Yaffe,M.P. and Schatz,G. (1988) MAS1, a gene essential for yeast mitochondrial assembly, encodes a subunit of the mitochondrial processing protease. *EMBO J.*, **7**, 1439–1447.
42. Vallino,M., Martino,E., Boella,F., Murat,C., Chiapello,M. and Perotto,S. (2009) Cu,Zn superoxide dismutase and zinc stress in the metal-tolerant ericoid mycorrhizal fungus *Oidiodendron maius* Zn. *FEMS Microbiol. Lett.*, **293**, 48–57.
43. Langlands,K., Anand,G., Yin,X. and Prochownik,E.V. (1997) Differential interactions of Id proteins with basic-helix-loop-helix transcription factors. *J. Biol. Chem.*, **272**, 19785–19793.
44. Chen,S.h., Smolka,M.B. and Zhou,H. (2007) Mechanism of Dun1 activation by Rad53 phosphorylation in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **282**, 986–995.
45. Jaroszewski,L., Li,Z., Krishna,S.S., Bakolitsa,C., Wooley,J., Deacon,A.M., Wilson,I.A. and Godzik,A. (2009) Exploration of uncharted regions of the protein universe. *PLoS Biol.*, **7**, e1000205.
46. Galperin,M.Y. and Koonin,E.V. (2010) From complete genome sequence to 'complete' understanding? *Trends Biotechnol.*, **28**, 398–406.
47. Finn,R.D., Mistry,J., Tate,J., Coggill,P., Heger,A., Pollington,J.E., Gavin,O.L., Gunasekaran,P., Ceric,G., Forslund,K. *et al*. (2010) The Pfam protein families database. *Nucleic Acids Res.*, **38**, D211–D222.
48. Galperin,M.Y. and Koonin,E.V. (2004) 'Conserved hypothetical' proteins: prioritization of targets for experimental study. *Nucleic Acids Res.*, **32**, 5452–5463.
49. Fomenko,D.E. and Gladyshev,V.N. (2003) Identity and Functions of CxxC-derived motifs. *Biochemistry*, **42**, 11214–11225.
50. Argüello,J.M. (2003) Identification of ion-selectivity determinants in heavy-metal transport P1B-type ATPases. *J. Membrane Biol.*, **195**, 93–108.
51. Wemmie,J.A., Szczypka,M.S., Thiele,D.J. and Moye-Rowley,W.S. (1994) Cadmium tolerance mediated by the yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, YCF1. *J. Biol. Chem.*, **269**, 32592–32597.
52. Lee,J., Godon,C., Lagniel,G., Spector,D., Garin,J., Labarre,J. and Toledano,M.B. (1999) Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *J. Biol. Chem.*, **274**, 16040–16046.
53. Kolodner,R.D. and Marsischky,G.T. (1999) Eukaryotic DNA mismatch repair. *Curr. Opin. Genetics Develop.*, **9**, 89–96.
54. Kolas,N.K. and Cohen,P.E. (2004) Novel and diverse functions of the DNA mismatch repair family in mammalian meiosis and recombination. *Cytogenet. Genome Res.*, **107**, 216–231.
55. Hoffmann,E.R. and Borts,R.H. (2004) Meiotic recombination intermediates and mismatch repair proteins. *Cytogenet. Genome Res.*, **107**, 232–248.
56. Pang,Q., Prolla,T.A. and Liskay,R.M. (1997) Functional domains of the *Saccharomyces cerevisiae* Mlh1p and Pms1p DNA mismatch repair proteins and their relevance to human hereditary nonpolyposis colorectal cancer-associated mutations. *Mol. Cell. Biol.*, **17**, 4465–4473.
57. Lipkin,S.M., Wang,V., Jacoby,R., Banerjee-Basu,S., Baxevanis,A.D., Lynch,H.T., Elliott,R.M. and Collins,F.S. (2000) MLH3: a DNA mismatch repair gene associated with mammalian microsatellite instability. *Nat. Genet.*, **24**, 27–35.
58. Kondo,E., Horii,A. and Fukushige,S. (2001) The interacting domains of three MutL heterodimers in man: hMLH1 interacts with 36 homologous amino acid residues within hMLH3, hPMS1 and hPMS2. *Nucleic Acids Res.*, **29**, 1695–1702.
59. Harrington,J.M. and Kolodner,R.D. (2007) *Saccharomyces cerevisiae* Msh2-Msh3 acts in repair of base-base mispairs. *Mol. Cell. Biol.*, **27**, 6546–6554.
60. McMurray,C.T. and Tainer,J.A. (2003) Cancer, cadmium and genome integrity. *Nat. Genet.*, **34**, 239–241.
61. Lützen,A., Liberti,S.E. and Rasmussen,L.J. (2004) Cadmium inhibits human DNA mismatch repair in vivo. *Biochem. Biophys. Res. Commun.*, **321**, 21–25.
62. Liu,W., Yang,Y.S., Francis,D., Rogers,H.J., Li,P. and Zhang,Q. (2008) Cadmium stress alters gene expression of DNA mismatch repair related genes in *Arabidopsis* seedlings. *Chemosphere*, **73**, 1138–1144.
63. Liu,W., Zhou,Q., Li,P., Gao,H., Han,Y.P., Li,X.J., Yang,Y.S. and Li,Y. (2009) DNA mismatch repair related gene expression

as potential biomarkers to assess cadmium exposure in *Arabidopsis* seedlings. *J. Hazardous Mater.*, **167**, 1007–1013.
64. Fujs,S., Gazdag,Z., Poljsak,B., Stibilj,V., Milacic,R., Pesti,M., Raspor,P. and Batic,M. (2005) The oxidative stress response of the yeast *Candida intermedia* to copper, zinc, and selenium exposure. *J. Basic Microbiol.*, **45**, 125–135.
65. Samikkannu,T., Chen,C.H., Yih,L.H., Wang,A.S.S., Lin,S.Y., Chen,T.C. and Jan,K.Y. (2003) Reactive oxygen species are involved in arsenic trioxide inhibition of pyruvate dehydrogenase activity. *Chem. Res. Toxicol.*, **16**, 409–414.
66. Chiou,T.J. and Tzeng,W.F. (2000) The roles of glutathione and antioxidant enzymes in menadione-induced oxidative stress. *Toxicology*, **154**, 75–84.
67. Davidson,J.F., Whyte,B., Bissinger,P.H. and Schiestl,R.H. (1996) Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **93**, 5116–5121.
68. Prakash,S. and Prakash,L. (2000) Nucleotide excision repair in yeast. *Mutation Res/Fundamental and Mol. Mech. Mutagenesis*, **451**, 13–24.
69. Cortes-Ledesma,F. and Aguilera,A. (2006) Double-strand breaks arising by replication through a nick are repaired by cohesin-dependent sister-chromatid exchange. *EMBO Rep.*, **7**, 919–926.
70. van Peer,A.F., de Bekker,C., Vinck,A., Wosten,H.A.B. and Lugones,L.G. (2009) Phleomycin increases transformation efficiency and promotes single integrations in *Schizophyllum commune*. *Appl. Environ. Microbiol.*, **75**, 1243–1247.
71. O'Brien,V. and Brown,R. (2006) Signalling cell cycle arrest and cell death through the MMR system. *Carcinogenesis*, **27**, 682–692.
72. Wang,J.Y.J. and Edelmann,W. (2006) Mismatch repair proteins as sensors of alkylation DNA damage. *Cancer Cell*, **9**, 417–418.
73. Polosina,Y.Y. and Cupples,C.G. (2010) Wot the [']L–Does MutL do? *Mutat. Res./Rev. Mut. Res.*, **705**, 228–238.
74. Frary,A., Nesbitt,T.C., Frary,A., Grandillo,S., Knaap,E.v.d., Cong,B., Liu,J., Meller,J., Elber,R., Alpert,K.B. *et al.* (2000) fw2.2: a quantitative trait locus key to the evolution of tomato fruit size. *Science*, **289**, 85–88.
75. Cong,B. and Tanksley,S.D. (2006) FW2.2 and cell cycle control in developing tomato fruit: a possible example of gene co-option in the evolution of a novel organ. *Plant Mol. Biol.*, **62**, 867–880.
76. Libault,M., Zhang,X.C., Govindarajulu,M., Qiu,J., Ong,Y.T., Brechenmacher,L., Berg,R.H., Hurley-Sommer,A., Taylor,C.G. and Stacey,G. (2010) A member of the highly conserved FWL (tomato FW2.2-like) gene family is essential for soybean nodule organogenesis. *Plant J.*, **62**, 852–864.
77. Libault,M. and Stacey,G. (2010) Evolution of FW2.2-like (FWL) and PLAC8 genes in eukaryotes. *Plant Signal. Behavior*, **5**, 1226–1228.