

Etp1 confers arsenite resistance by affecting ACR3 expression

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One sentence summary: Etp1 confers arsenite resistance in *Saccharomyces cerevisiae* by affecting ACR3 expression in a Yap8-independent fashion.

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Abstract

In a high-throughput yeast two-hybrid screen of predicted coiled-coil motif interactions in the *Saccharomyces cerevisiae* proteome, the protein Etp1 was found to interact with the yeast AP-1-like transcription factors Yap8, Yap1 and Yap6. Yap8 plays a crucial role during arsenic stress since it regulates expression of the resistance genes ACR2 and ACR3. The function of Etp1 is not well understood but the protein has been implicated in transcription and protein turnover during ethanol stress, and the *etp1*Δ mutant is sensitive to ethanol. In this current study, we investigated whether Etp1 is implicated in Yap8-dependent functions. We show that Etp1 is required for optimal growth in the presence of trivalent arsenite and for optimal expression of the arsenite export protein encoded by ACR3. Since Yap8 is the only known transcription factor that regulates ACR3 expression, we investigated whether Etp1 regulates Yap8. Yap8 ubiquitination, stability, nuclear localization and ACR3 promoter association were unaffected in *etp1*Δ cells, indicating that Etp1 affects ACR3 expression independently of Yap8. Thus, Etp1 impacts gene expression under arsenic and other stress conditions but the mechanistic details remain to be elucidated.

Keywords: arsenic, metalloid, Yap8, Etp1, Acr3

Introduction

Cells continuously monitor and respond to changes in their internal and external environment. For instance, the yeast *Saccharomyces cerevisiae* rapidly regulates gene expression, protein synthesis and metabolism in response to stress conditions, including exposure to toxic metals, oxidative stress, high temperature and fluctuations in osmolarity. These responses are important for survival in harsh conditions as well as for growth and proliferation in suboptimal environments (Hohmann and Mager 1997).

The metalloid arsenic is highly toxic and abundant in the environment. In humans, chronic arsenic exposure causes cancers of the skin, lung, bladder, kidney and liver, and is associated with various neurodegenerative disorders, cardiovascular disease, hypertension and diabetes. The molecular mechanisms underlying the toxic and carcinogenic effects of arsenic are not well understood but may involve binding to and inactivation of specific enzymes, induction of oxidative and proteotoxic stress, inhibition of DNA repair systems, deregulation of cell proliferation, changes to the epigenome and interference with signal transduction pathways (Shen et al. 2013; Tamás et al. 2014; Zhou and Xi 2018). Microorganisms are also exposed to arsenic and past studies in *S. cerevisiae* uncovered several toxicity and resistance mechanisms, including transport proteins that mediate arsenic influx or catalyze its export, intracellular and extracellular chelation mechanisms that prevent arsenic influx or protect the intracellular envi-

ronment from toxic arsenic interactions, and protein quality control systems that safeguard the integrity of the proteome during exposure. In several cases, similar mechanisms have been shown to operate in higher eukaryotes (Rosen and Tamás 2010; Wysocki and Tamás 2010, 2011; Maciaszczyk-Dziubinska, Wawrzycka and Wysocki 2012).

Arsenic can enter cells in form of pentavalent arsenate [As(V)] through phosphate transporters (Bun-ya et al. 1996; Yompakdee et al. 1996; Shen et al. 2012), or in form of trivalent arsenite [As(III)] through aquaglyceroporins (Wysocki et al. 2001) and hexose transporters (Liu, Boles and Rosen 2004). The arsenate reductase Acr2 (also called Arr2) converts intracellular As(V) to As(III) (Mukhopadhyay and Rosen 1998; Mukhopadhyay, Shi and Rosen 2000) and intracellular As(III) can be exported out of cells by the plasma membrane protein Acr3 (also called Arr3) (Wysocki, Bobrowicz and Ulaszewski 1997) or into vacuoles via the ABC transporter Ycf1 (Ghosh, Shen and Rosen 1999). *Saccharomyces cerevisiae* responds to As(III) by increasing the production of the tripeptide glutathione for intracellular and extracellular chelation and detoxification (Thorsen et al. 2007, 2012; Talemi et al. 2014). Additionally, intracellular As(III) can be methylated by the methyltransferase Mtq2 and the presence of methylarsenite appears to elicit distinct protective responses (Lee and Levin 2018, 2019, 2022). The systems above are regulated at transcriptional and posttranslational levels. For example, transcriptional activation

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of detoxification genes involves Yap8 (also called Acr1 and Arr1) that controls expression of ACR2 and ACR3 (Menezes et al. 2004; Wysocki et al. 2004; Kumar et al. 2016), Yap1 that regulates YCF1 expression (Wysocki et al. 2004) and Met4 that together with Yap1 regulates expression of glutathione biosynthesis-related genes (Wysocki et al. 2004; Thorsen et al. 2007). Similarly, posttranslational regulation of arsenic transporters affects intracellular arsenic concentration and resistance (Thorsen et al. 2006; Beese, Negishi and Levin 2009; Ahmadvpour et al. 2016; Lee and Levin 2018; Jochem et al. 2019; Wawrzycka et al. 2019; Lee and Levin 2022). Widespread protein misfolding and aggregation contributes to the toxicity of As(III) (Jacobson et al. 2012; Andersson et al. 2021) and As(III)-exposed cells decrease global protein synthesis and increase the protein degradation capacity to enhance resistance (Jacobson et al. 2012; Guerra-Moreno et al. 2015; Andersson et al. 2021). The broad range of responses launched by yeast cells to arsenic stress is probably a reflection of the pleiotropic nature of the toxicity of this metalloid.

Yap8 is a member of the yeast AP-1-like (yAP) transcription factor family (Rodrigues-Pousada et al. 2019) and it plays a crucial role during arsenic stress by regulating expression of the arsenic resistance genes ACR2 and ACR3 (Menezes et al. 2004, 2008; Wysocki et al. 2004; Kumar et al. 2016). These genes are transcribed in opposite directions from a shared promoter that contains a Yap8-binding element (Wysocki et al. 2004; Ilina et al. 2008; Maciaszczyk-Dziubinska et al. 2020). Yap8 is bound to the ACR2-ACR3 promoter both in the absence and presence of As(III). Direct binding of As(III) to specific cysteine residues within Yap8 induces a conformational change that converts inactive Yap8 into an active transcriptional regulator (Wysocki et al. 2004; Di and Tamás 2007; Kumar et al. 2016). Additionally, Yap8 is phosphorylated by the Hog1 kinase during As(III) stress and this phosphorylation contributes to efficient induction of ACR2 and ACR3 expression by an unknown mechanism (Guerra-Moreno et al. 2019). Full induction of ACR2 and ACR3 expression also involves coactivator complexes and chromatin remodeling factors (Menezes et al. 2017; West et al. 2019). Although Yap8 and ACR3 regulation have been intensely studied, several questions remain unanswered regarding how Yap8 couples arsenic sensing to transcriptional activation of ACR2 and ACR3, and the role of additional proteins in ACR2 and ACR3 regulation.

In a high-throughput yeast two-hybrid screen of predicted coiled-coil motif interactions in the *S. cerevisiae* proteome, the protein Etp1 (encoded by *YHL010c*) was found to interact physically with the AP-1-like transcription factors Yap8, Yap1 and Yap6 (Wang et al. 2012). Yap1 controls expression of genes encoding antioxidant and protective functions in response to oxidative and metal stress (Kuge and Jones 1994; Wu and Moye-Rowley 1994; Gasch et al. 2000; Wysocki et al. 2004; Thorsen et al. 2007), whereas Yap6 overexpression confers resistance to cisplatin (Furuchi et al. 2001) and to high concentrations of NaCl and LiCl (Mendizabal et al. 1998). Etp1 is a 67 kDa large cytoplasmic protein of unknown function. A number of amino acid residues within Etp1 are phosphorylated or ubiquitinated but the functional relevance of these modifications is unknown. Expression of the *ETP1* gene is induced during amino acid starvation conditions and during the transition from fermentative growth to glycerol-based respiratory growth (Cherry et al. 2012). It has been shown that Etp1 affects transcription of certain genes (*ENA1*, *HSP12*, *HSP26*) and turnover of specific proteins (Nha1, Hxt3) during ethanol stress and that the *etp1Δ* mutant is sensitive to ethanol (Snowdon et al. 2009). The human Etp1 homolog BRAP2 is an E3 ubiquitin ligase (Matheny et al. 2004) that binds to nuclear localization sequences and BRAP2 may func-

tion in retaining proteins in the cytoplasm (Li et al. 1998; Asada et al. 2004). Etp1 has a zinc finger ubiquitin-binding domain, which is also present in BRAP2, and both proteins can bind ubiquitin (Reyes-Turcu et al. 2006). Whether Etp1 functions as an E3 ubiquitin ligase and/or in the regulation of protein localization has not been demonstrated. In this current study, we show that Etp1 protects cells during As(III) stress by affecting ACR3 expression in a Yap8-independent fashion.

Materials and methods

Yeast strains, plasmids and growth conditions

The *S. cerevisiae* strains used in this study are based on BY4742 (MAT α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) (Brachmann et al. 1998) and the following mutants from yeast deletion collection (Winzeler et al. 1999): *etp1Δ* (BY4742 *etp1Δ::kanMX*) and *yap8Δ* (BY4742 *yap8Δ::kanMX*). The *etp1Δ yap8Δ* double mutant (BY4742 *etp1Δ::kanMX yap8Δ::kanMX*) was generated by crossing haploid single mutants using standard procedures. Yeast cells were routinely grown at 30°C on minimal SC (synthetic complete) medium (0.67% yeast nitrogen base; YNB) supplemented with auxotrophic requirements and 2% glucose as a carbon source. Sodium arsenite (NaAsO₂) (Sigma-Aldrich, St. Louis, MO, USA), sodium arsenate (Na₂HAsO₄) (Sigma-Aldrich) and cycloheximide (Sigma-Aldrich) were added directly to the growth medium. Plate growth assays were performed as previously described (Wysocki et al. 2001).

The plasmids used in this study include Yap8-HA under the control of the constitutive *TPI1* promoter in pYX122 (CEN, *HIS3*) (Di and Tamás 2007), GFP-Yap8 controlled by the endogenous YAP8 promoter in YEplac195 (2 μ , *URA3*) (Wysocki et al. 2004), p415TEF1-10 \times His-Ub-LEU2 (pGR295; kindly provided by Gwenaël Rabut), pES15 ACR3-*lacZ* (CEN, *URA3*) (Wysocki et al. 2004) and pA103 ACR3 (2 μ , *URA3*) (Bobrowicz et al. 1997).

β -Galactosidase activity

Yeast cells expressing the ACR3-*lacZ* gene fusion were either untreated or exposed to 0.25 mM As(III) for 6 h. The β -galactosidase activity was measured at least three times in triplicates on permeabilized cells as described previously (Guarente 1983).

RNA analysis

Cells were grown in synthetic medium during 4 h, then 0.25 mM As(III) was added to the culture and cells collected at 0, 30, 60 and 120 min. RNA extractions were performed with the phenol: chloroform method. Reverse transcription and real-time qPCR (RT-qPCR) were performed as previously described (Sanvisens et al. 2014). Real-time PCR was performed under the following conditions: 95°C for 10 s, followed by 40 cycles of 10 s at 95°C and 15 s at 55°C. At the end, a melting-curve analysis was conducted to verify the specificity of the reaction. *IPP1* was used as a reference gene for normalization. The primers used were ACR3-F CGCA CCGATATACTGACTACGA, ACR3-R ACGGGAAGAAGGCACATAGA, *IPP1*-F TTACACTGGTCAAGTCAAG and *IPP1*-R ATCGTTAATATC AATGGCAATA. The comparative threshold cycle (CT) method for relative quantification ($\Delta\Delta$ CT method) was used to analyze the data. The data and error bars represent the relative average and standard deviations of three independent biological samples.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as previously described (Litwin et al. 2018; Maciaszczyk-Dziubinska et al. 2020) in *yap8Δ* and *etp1Δ yap8Δ* cells transformed with

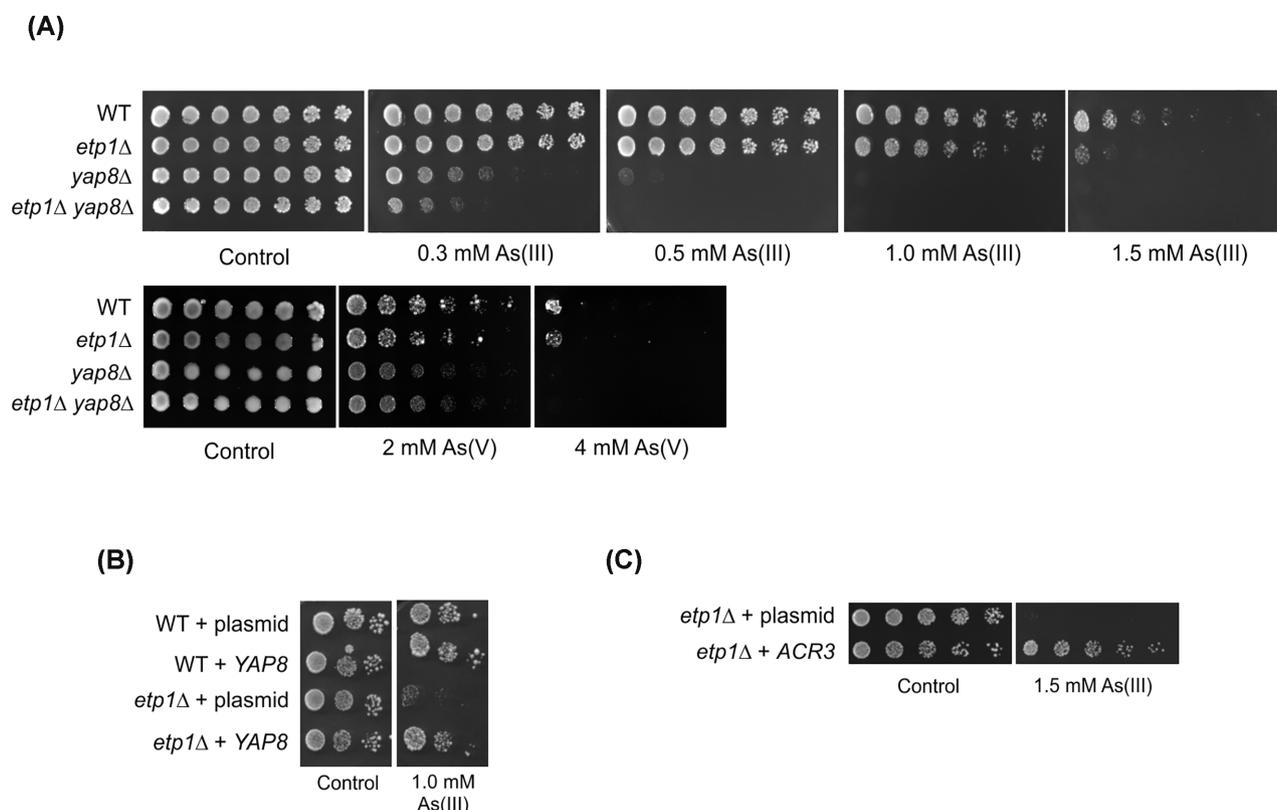


Figure 1. The *etp1Δ* mutant is sensitive to As(III). **(A)** Ten-fold serial dilutions of the indicated strains were plated onto YNB agar plates with or without As(III) and As(V) at the indicated concentrations. Growth was monitored after 2–3 days at 30°C. Growth assays were performed with at least two biological replicates and a representative image is shown. **(B)** YAP8 overexpression improves As(III) resistance in the *etp1Δ* mutant. An empty plasmid or a plasmid carrying GFP-tagged YAP8 (endogenous YAP8 promoter, episomal plasmid) was transformed into the indicated strains and growth assays performed as above. **(C)** ACR3 overexpression improves As(III) resistance in the *etp1Δ* mutant. Growth assays were performed as above with the indicated strains transformed with an empty plasmid or a plasmid carrying ACR3 behind its endogenous promoter on a multicopy plasmid.

HA-tagged YAP8 behind the *TPI1* promoter (Di and Tamás 2007) or the empty plasmid. Cells were either untreated or exposed to 0.5 mM As(III) for 30 min, and sheared chromatin was immunoprecipitated using anti-HA antibody (H6908, Sigma-Aldrich, 1:2500 dilution) overnight followed by incubation with sepharose protein G beads (Dynabeads Protein G, Life Technologies, Carlsbad, CA, USA). Quantitative PCR (qPCR) was performed using both immunoprecipitates and input samples as templates, a 2× PCR Master Mix SYBR Kit (A&A Biotechnology, Gdansk, Poland) and the CFX Connect Real-Time PCR System (Bio-Rad, Hercules, CA, USA) in a total volume of 15 μ l. Primers used for qPCR are PRACR3-for TTACGCTTGCTGGATTGTCA and PRACR3-rev CGTTGCCGCTAAAGTTGATT for the ACR3 promoter and IPP1-for CT TTATTGGATGAAGGTGA and IPP1-rev TTAATTGTTTCCAGGAGT C for the IPP1 reference gene. Amplification conditions were as follows: 1 min at 95°C; 40 cycles of 10 s at 95°C; 15 s at 60°C; and 20 s at 72°C. The percentage (% input) value for each sample was calculated according to the formula: Δ CT [normalized ChIP] = CT [ChIP] – [CT [Input] – log₂ (dilution factor)] and Input % = 100/2 Δ CT [normalized ChIP]. The % input value represents the enrichment of protein at the specific locus and is normalized to the IPP1 reference gene. ChIP experiment were performed at least three times. qPCRs were performed two times for each sample and error bars indicate \pm standard deviations.

Yap8 stability

To determine Yap8 protein stability, yeast cells were either untreated or exposed to 0.5 mM As(III). Then, protein translation

was stopped by the addition of cycloheximide (CHX) to a final concentration of 50 μ g/ml. Cells were harvested at the indicated time points and protein extracts were obtained using the alkali method. Similar amounts of protein were resolved on SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The primary antibodies used include anti-HA (H9658, Sigma-Aldrich, dilution 1:5000) and anti-Pgk1 (22C5D8, Thermo Fisher Scientific, Waltham, MA, USA, dilution 1:10 000) and their appropriate HRP-conjugated secondary antibodies. Immunoblots were scanned using LAS-100 image reader (Fujifilm, Minato, Japan).

Yeast chromatin fractionation and Yap8 localization

Yeast chromatin fractionation was carried out as previously described (Keogh et al. 2006; Oh et al. 2018), with slight modifications. One hundred milliliter (OD₆₀₀ = 0.8) yeast cells (*yap8Δ* and *etp1Δ yap8Δ*) expressing Yap8-HA (pYX122-YAP8) or the empty plasmid (pYX122) either untreated or exposed to 0.5 mM As(III) for 30 min were collected, then washed successively with 10 ml distilled water, 10 ml SB (1 M Sorbitol, 20 mM Tris–Cl pH 7.5), 1.5 ml PSB (20 mM Tris–Cl pH 7.5, 2 mM EDTA, 100 mM NaCl, 10 mM β -Mercaptoethanol) and 1.5 ml SB, then resuspended with 1 ml SB. Yeast cell walls were digested by the addition of 125 μ l Zymolase (10 mg/ml, Seikagaku, Japan) for 1 h at room temperature. After the digestion, 1 ml ice-cold SB was added, then spheroplasts were collected by gentle centrifugation (2K, 5 min, 4°C) and washed once with 1 ml ice-cold SB. The spheroplasts were resuspended

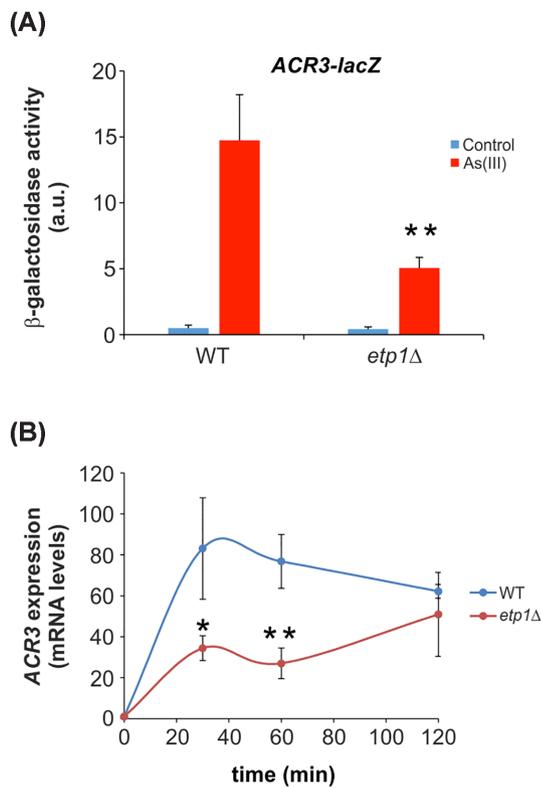


Figure 2. ACR3 expression is lower in As(III)-exposed *etp1Δ* cells. **(A)** β -Galactosidase activity driven by the ACR3-promoter-*lacZ* fusion construct was measured in the indicated strains. Cells were exposed to 0.25 mM As(III) for 6 h or left untreated for the control. The values are the means of three biological replicates performed in triplicate \pm standard deviation (SD). * $P < 0.05$ and ** $P < 0.005$. **(B)** Cells were exposed to 0.25 mM As(III) and samples for RNA extraction were taken at the indicated time points followed by qPCR as described in the 'Materials and Methods' section. ACR3 expression was normalized to *IPP1* expression. Values are the means of three biological replicates \pm SD. * $P < 0.05$ and ** $P < 0.005$.

in 500 μ l EBX (20 mM Tris–Cl pH 7.5, 100 mM NaCl, 0.25% Triton X-100, 15 mM β -Mercaptoethanol), and then 2.8 μ l 100% Triton X-100 was added to lyse the outer cell membrane. Cells were placed on ice for 10 min with occasional mixing. 50 μ l of cells were taken and aliquoted as 'Total'. 1 ml NIB (20 mM Tris–Cl pH 8.0, 100 mM NaCl, 1.2 M Sucrose, 15 mM β -Mercaptoethanol) was layered over the remainder, then centrifuged for 15 min at 12K, 4°C. The upper layer (50 μ l) was taken as the 'Cytoplasmic' fraction. Glassy, white nuclear pellets were resuspended in 500 μ l EBX, then 5.6 μ l 100% Triton X-100 was added to the resuspended nuclear pellets. The resuspended nuclear pellets were incubated on ice for 10 min with gentle mixing every few minutes. Chromatin was pelleted by centrifugation (15 K, 10 min, 4°C). The supernatant was stored as the 'Chromatin' fraction. The chromatin fraction was resuspended by 100 μ l Laemmli sample buffer then boiled (5 min) for western blot experiments. All fractions were resolved on 12% SDS–PAGE, blotted on nitrocellulose membranes and probed with anti-HA (6908, Merck, Darmstadt, Germany, 1:5000 dilution), anti-H2A (07-146, Merck, Darmstadt, Germany, 1:1000 dilution) or anti-glucose-6-phosphate dehydrogenase (G6PDH; A9521-1VL, Merck, Darmstadt, Germany, 1:10 000 dilution) antibodies. Chemiluminescence signal detection was performed using the Bio-Rad ChemiDoc MP System and Image Lab software (Bio-Rad, Hercules, CA, USA).

Ubiquitin pulldown assay

Ubiquitinated proteins were purified from yeast cells (*yap8Δ* and *etp1Δ yap8Δ*) expressing Yap8-HA (pYX122-YAP8) and N-terminally 10 \times His-tagged ubiquitin (p415TEF1-10 \times His-Ub-LEU2 (pGR295) derived from pGR140 (Rabut et al. 2011; kindly provided by Gwenaël Rabut) using a protocol adapted from (Hovsepian et al. 2016). Yeast untreated or exposed to 0.5 mM As(III) for 30 min were grown in SC-leu⁻, his⁻ to OD₆₀₀ = 0.7. One hundred milliliter cells were collected, washed with cold 10 ml distilled water and then resuspended with 500 μ l 10% trichloroacetic acid (TCA), incubated for 10 min on ice and then harvested by centrifugation. Pellet was resuspended in 200 μ l 10% TCA and lysed using glass beads (Sigma-Aldrich) in a FastPrep FP120 (Thermo Fischer Scientific) for 2 \times 30 s at 6K then harvested by centrifugation (13K, 10 min, 4°C). To neutralize the residual TCA present in the pellet 30 μ l of 1 M Tris (non-buffered) was added, then pellet was resuspended in 1 ml buffer A (6 M GdnHCl, 20 mM Tris–HCl pH 8, 100 mM NaCl, 20 mM imidazole, 0.1% (v/v) Triton X-100, 100 mM K₂HPO₄) and lysates were solubilized at room temperature for 10 min with overhead rotation and harvested by centrifugation (13K, 5 min, RT). Twenty-five microliters of the lysates were taken as the 'Input' fraction, diluted with 1.35 ml water, precipitated with 150 μ l 100% trichloroacetic acid and resuspended in 25 μ l Sample buffer (250 mM Tris–HCl pH 6.8, 500 mM dithiothreitol, 10% SDS, 0.01% bromophenol blue, 50% glycerol). Remaining samples were incubated with overhead rotation for 2 h at room temperature with Ni-NTA beads (Qiagen, Hilden, Germany) pre-equilibrated 2 \times with buffer A. The beads were transferred to a Costar (Thermo Fisher Scientific) chromatography column then washed with 2 \times buffer A, 3 \times with wash 1 buffer (20 mM Tris–HCl pH 8, 1 M NaCl, 20 mM imidazole, 0.1% (v/v) Triton X-100, 100 mM K₂HPO₄) and 3 \times with wash 2 buffer (20 mM Tris–HCl pH 8, 1 M NaCl, 10 mM imidazole, 0.1% (v/v) Triton X-100, 20 mM K₂HPO₄). 10 \times His-ubiquitin conjugates were finally eluted with 100 μ l elution buffer (20 mM Tris–HCl pH 8, 100 mM NaCl, 500 mM imidazole, 100 mM K₂HPO₄). Seventeen microliters of eluate was taken as the 'Eluate' fraction and resuspended in Sample buffer. All fractions ('Input' and 'Eluate') were denatured at 95°C for 10 min. Five microliters of the 'Input' fraction and 10 μ l of the 'Eluate' fraction were analyzed by SDS–PAGE (in 4–20% Mini-PROTEAN[®] TGX Precast Protein Gels, Bio-Rad), transferred on nitrocellulose membrane using Trans-Blot[®] Turbo Transfer System (Bio-Rad) and immunoblotting with antibodies against the HA-tag (6908, Merck, Darmstadt, Germany, 1:5000 dilution). The level of ubiquitin conjugates was assessed with anti-ubiquitin (P4D1 HRP conjugate, sc-8017, lot #B0817, Santa Cruz Biotechnology, Dallas, TX, USA, 1:1000 dilution) antibodies. Chemiluminescence signal detection was performed using the Bio-Rad ChemiDoc MP System and Image Lab software (Bio-Rad, Hercules, CA, USA).

Results

Cells lacking ETP1 are sensitive to As(III) and As(V)

Since Etp1 was shown to interact with Yap8 in a yeast two-hybrid screen of predicted coiled-coil motif interactions (Wang et al. 2012), we tested whether deletion of ETP1 is needed for growth in the presence of arsenic, i.e. a condition that requires Yap8 function. As demonstrated previously, *yap8Δ* cells were sensitive to As(III) and As(V) (Menezes et al. 2004; Wysocki et al. 2004). The *etp1Δ* mutant showed sensitivity to high concentrations of As(III) (Fig. 1A). The *etp1Δ* mutant was also somewhat sensitive to As(V)

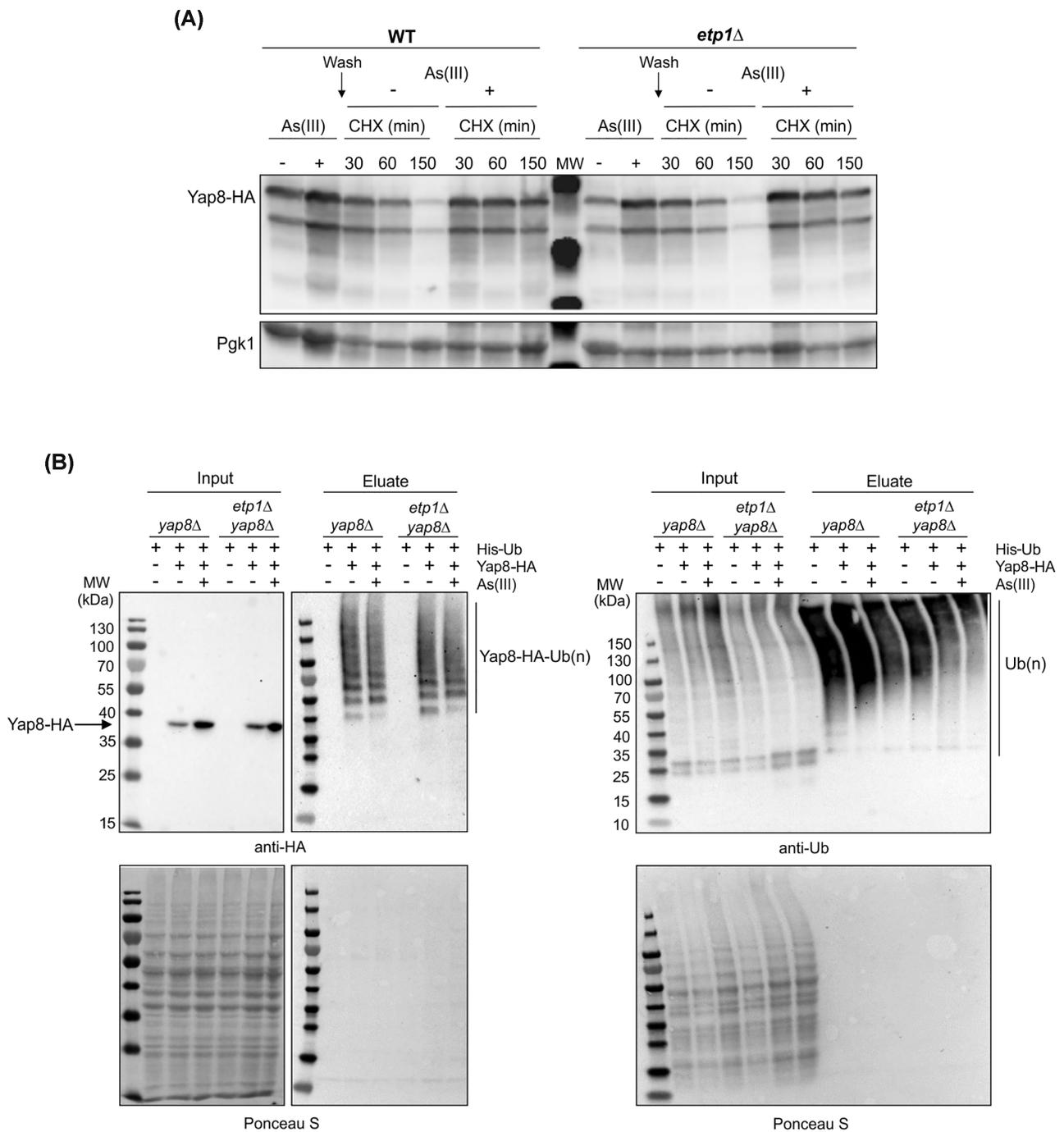


Figure 3. Etp1 does not regulate Yap8 ubiquitination and stability. **(A)** Yap8 stability is unaffected in *etp1* Δ cells. Yap8-HA was expressed from the constitutive *TP11* promoter in the wild type and the *etp1* Δ mutant and samples were taken for SDS-PAGE at the indicated time points. Cells were exposed to 0.5 mM As(III) for 1 h, then washed and placed in growth medium with or without As(III) as indicated. Cycloheximide (50 μ g/ml CHX) was added as indicated to inhibit *de novo* protein synthesis. Immunoblotting was performed with anti-HA and anti-Pgc1 as loading control. The assay was performed with at least two biological replicates and a representative image is shown. **(B)** Yap8 ubiquitination is unaffected in *etp1* Δ cells. Ubiquitination of Yap8-HA was monitored in the indicated cells expressing 10 \times histidine (His)-tagged ubiquitin. Total cell extracts and ubiquitin conjugates eluted after immobilized-nickel affinity chromatography were separated by SDS-PAGE followed by immunoblotting with anti-HA and anti-ubiquitin antibodies. The ubiquitin pull-down assay was performed with at least two biological replicates and a representative image is shown.

but the growth defect was modest (Fig. 1A). We conclude that Etp1 is required for optimal growth in the presence of As(III).

ACR3 expression is affected in *etp1* Δ cells

As(III) is exported via the plasma membrane transporter Acr3 and a failure to appropriately induce ACR3 expression leads to arsenic sensitivity (Wysocki, Bobrowicz and Ulaszewski 1997;

Wysocki et al. 2004). We addressed whether ACR3 expression is affected in the *etp1* Δ mutant using an ACR3-*lacZ* reporter assay. Indeed, As(III)-induced β -galactosidase activity was nearly 3-fold lower in *etp1* Δ cells compared to the wild type (Fig. 2A). To substantiate this, we measured ACR3 mRNA levels during As(III) exposure using qPCR. Induction of ACR3 expression occurred with a lower rate in *etp1* Δ cells and reached only ~60% of the

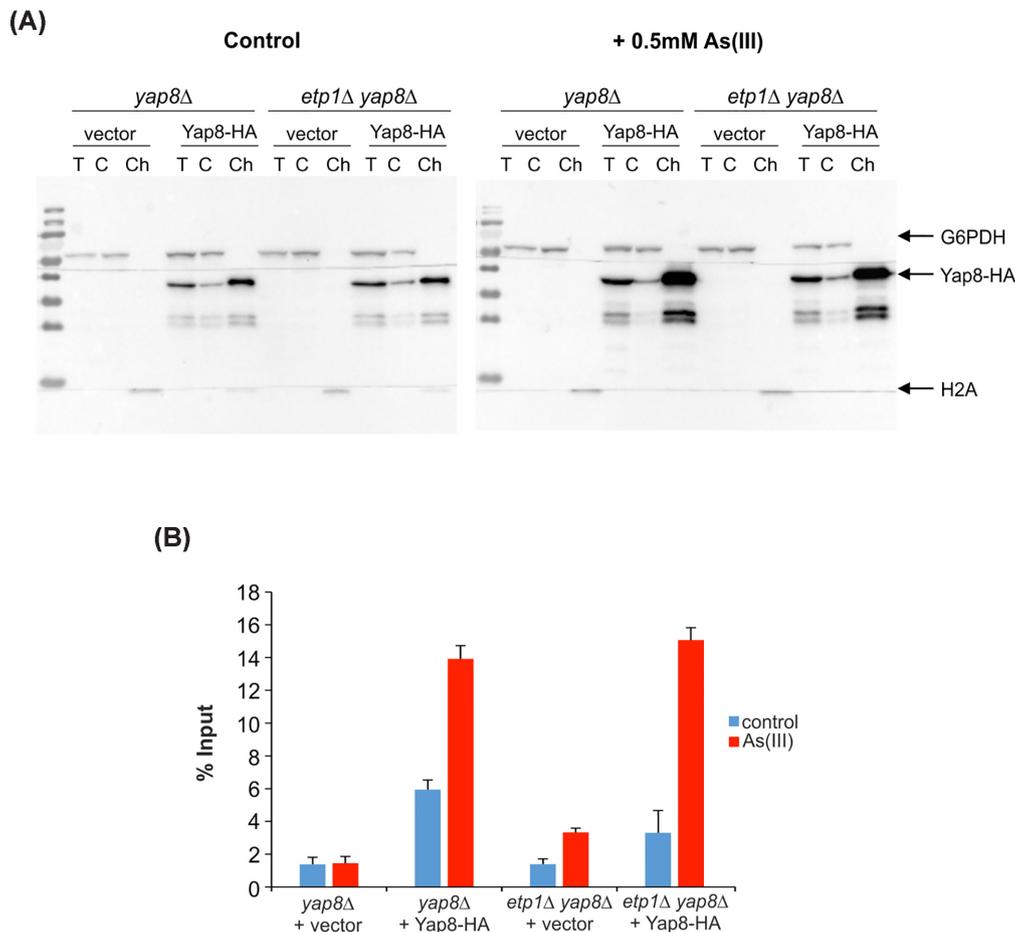


Figure 4. Etp1 does not regulate Yap8 nuclear localization and ACR3 promoter association. **(A)** Nuclear localization of Yap8 is unaffected in *etp1Δ* cells. Yeast chromatin was fractionated as described in the 'Materials and Methods' section and the presence of Yap8-HA was monitored by immunoblotting. T, total extract; C, cytosolic fraction; Ch, chromatin fraction. Immunoblotting was performed with antibodies against the HA-tag, histone H2A as a marker for the chromatin fraction and glucose-6-phosphate dehydrogenase (G6PDH) as a marker for the cytosolic fraction. The assay was performed with at least two biological replicates and a representative image is shown. **(B)** *In vivo* occupancy of Yap8 on the ACR3 promoter is unaffected in *etp1Δ* cells as determined by ChIP. The indicated cells carrying Yap8-HA or the empty vector were either untreated (control) or exposed to 0.5 mM As(III) for 30 min, and qPCR was performed on chromatin fragments isolated after immunoprecipitation using an anti-HA antibody as described in the 'Materials and Methods' section. Data from three biological replicates are shown, and the error bars represent standard deviations.

maximal induction level in wild-type cells after 2 h of exposure (Fig. 2B). Thus, ACR3 expression is clearly compromised in *etp1Δ* cells. A lower amount of Acr3 in the plasma membrane likely explains the observed As(III) sensitivity of *etp1Δ*. To corroborate this, we overexpressed YAP8 since overexpression of YAP8 has been shown to result in increased ACR3 expression (Di and Tamás 2007). As predicted, overexpression of YAP8 enhanced As(III) resistance of the *etp1Δ* mutant (Fig. 1B). Likewise, overexpression of ACR3 mitigated the As(III) sensitivity of *etp1Δ* (Fig. 1C). Taken together, these results support the notion that ACR3 expression is affected in *etp1Δ* cells and that reduced ACR3 expression accounts for the As(III) sensitivity of *etp1Δ*.

Yap8 ubiquitination and stability are unaffected by ETP1 deletion

Since Yap8 is the only transcription factor known to regulate ACR2 and ACR3 expression (Ilina et al. 2008), we asked whether Etp1 affects ACR3 expression by regulating Yap8. Previous studies showed that Yap8 turnover is regulated at the posttranslational level (Di and Tamás 2007; Ferreira, Menezes and Rodrigues-Pousada 2015). Yap8 levels are low in untreated cells due to

degradation via the ubiquitin-proteasome pathway (Di and Tamás 2007) involving the E2 ubiquitin-conjugating enzyme Ubc4 (Di and Tamás 2007) and the E4 ubiquitin ligase Ufd2 (Ferreira, Menezes and Rodrigues-Pousada 2015). Yap8 is stabilized during As(III) exposure resulting in elevated Yap8 protein levels and ACR3 expression (Di and Tamás 2007). We first analyzed Yap8 stabilization by monitoring the levels of an HA-tagged version of Yap8. Yap8-HA is fully functional and complements the As(III) sensitivity of *yap8Δ* and restores the ability to induce ACR3 expression in the *yap8Δ* mutant (Di and Tamás 2007). Yap8 protein levels increased similarly during As(III) exposure in wild-type and *etp1Δ* cells (Fig. 3A). We next monitored Yap8 half-life after transferring back the cells to As(III)-free medium in the presence of the protein synthesis inhibitor cycloheximide. The half-life of Yap8 appeared unaffected in *etp1Δ* cells compared to that in wild-type cells, both in the absence and presence of As(III) (Fig. 3A). Since Etp1 possesses a zinc finger ubiquitin-binding domain and binds ubiquitin (Reyes-Turcu et al. 2006), we reasoned that Etp1 might affect Yap8 ubiquitination. To address this, we immunoprecipitated Yap8-HA from untreated and As(III) exposed cells and analyzed the presence of ubiquitin conjugates

by western blot. Yap8 was ubiquitinated both in the absence and presence of As(III), manifested by the occurrence of several slow-migrating bands on the western blot (Fig. 3B). Whether the slow-migrating forms of Yap8 represent polyubiquitination of specific lysine residue(s) or whether Yap8 is monoubiquitinated on several lysine residues is unknown. Notably, Yap8 ubiquitination appeared unaffected in cells lacking *ETP1* (Fig. 3B). Hence, Yap8 ubiquitination and stability are not regulated by Etp1.

Yap8 nuclear localization and promoter association are unaffected by *ETP1* deletion

We previously demonstrated that Yap8 resides in the nucleus where it constitutively binds to the ACR2-ACR3 promoter as a homodimer, and that the presence of As(III) does not impact Yap8 homodimerization, nuclear localization or promoter association (Wysocki et al. 2004; Di and Tamás 2007; Kumar et al. 2016). In contrast, another group reported that As(III) exposure results in the translocation of Yap8 from the cytoplasm to the nucleus (Menezes et al. 2004). The reason for this discrepancy is not clear. Nevertheless, the fact that the human Etp1 homolog BRAP2 binds to nuclear localization sequences (Li et al. 1998; Asada et al. 2004) raised the possibility that Etp1 might affect Yap8 localization. To address this, we analyzed Yap8-HA localization by biochemically isolating yeast nuclei followed by western blotting. In agreement with our earlier results (Wysocki et al. 2004; Kumar et al. 2016), Yap8-HA localization was predominantly nuclear and unaffected by As(III) (Fig. 4A). Importantly, the absence of Etp1 did not affect Yap8 nuclear localization, neither in the absence or presence of As(III) (Fig. 4A). We next addressed the *in vivo* association of Yap8 with the ACR3 promoter by ChIP assays. The ChIP indicated no major impact of *ETP1* deletion on Yap8-HA occupancy on the ACR3 promoter (Fig. 4B), neither in the absence or presence of As(III). We conclude that nuclear localization and promoter association of Yap8 *in vivo* are unaffected by *ETP1* deletion.

Etp1 confers As(III) resistance independently of Yap8

The results above indicated that Etp1 does not regulate Yap8. To test whether Etp1 confers As(III) resistance independently of Yap8, we compared growth of the *etp1Δ yap8Δ* double mutant to that of the single mutants. The *yap8Δ* single mutant was highly As(III) sensitive as expected, and the *etp1Δ yap8Δ* double mutant was somewhat more As(III) sensitive than the *yap8Δ* single mutant (Fig. 1A). The additive As(III) sensitivity of *ETP1* deletion in *yap8Δ* cells was similar to that of *ETP1* deletion in wild-type cells, suggesting that Etp1 confers As(III) resistance independently of Yap8. Additional deletion of *ETP1* did not further sensitize *yap8Δ* cells to As(V) (Fig. 1A), supporting the notion that Etp1 function is important primarily during As(III) stress.

Discussion

Previous work implicated Etp1 in transcriptional activation of *ENA1*, encoding an Na⁺ and Li⁺ efflux protein, during salt and ethanol stress as well as ethanol stress-dependent induction of the heat shock genes *HSP12* and *HSP26* (Snowdon et al. 2009). Etp1 also affected the turnover of the Na⁺/H⁺ symporter *Nha1* and the hexose transporter *Hxt3* during ethanol stress as more *Nha1* and *Hxt3* were present in cells lacking *ETP1*. Additionally, Etp1 is required for optimal growth on ethanol-containing medium whereas it is dispensable for resistance to salt stress (LiCl and NaCl) (Snowdon et al. 2009). Here, we showed that Etp1 contributes

to As(III)-induced ACR3 expression and confers As(III) resistance. Reduced ACR3 expression is likely to account for the As(III) sensitivity of *etp1Δ* since As(III) export via *Acr3* is critical for resistance (Bobrowicz et al. 1997; Wysocki, Bobrowicz and Ulaszewski 1997; Wysocki et al. 2004). Thus, Etp1 appears to impact gene expression under various stress conditions. How does Etp1 affect transcription? Etp1 has a cytosolic localization and no obvious DNA-binding domain (Snowdon et al. 2009). Hence, Etp1 is not likely to directly bind DNA and act as a transcription factor. Instead, Etp1 might affect transcription indirectly. The human Etp1 homolog BRAP2 binds to nuclear localization sequences and regulates the localization of some nuclear proteins (Li et al. 1998; Asada et al. 2004; Fulcher et al. 2010). Hence, Etp1 might affect the localization of transcriptional regulators in yeast in analogy to BRAP2. Moreover, BRAP2 is an E3 ubiquitin ligase (Matheny et al. 2004) and like BRAP2, Etp1 possess a zinc finger ubiquitin-binding domain and can bind ubiquitin (Reyes-Turcu et al. 2006). The presence of polyubiquitin chains dictates the fate of proteins, such as the degradation of proteins with K48-linked ubiquitin chains attached, by the proteasome (Swatek and Komander 2016; Yau and Rape 2016). Whilst Etp1 affected turnover of specific proteins (Snowdon et al. 2009), it remains to be demonstrated whether Etp1 functions as an E3 ligase and whether transcriptional regulators are among its substrates. In this current study, we showed that Yap8 ubiquitination, stability, nuclear localization and ACR3 promoter association were unaffected in *etp1Δ* cells. Thus, Etp1 affects ACR3 expression independently of Yap8. This was corroborated by genetic data indicating that Etp1 confers As(III) resistance independently of Yap8. Moreover, we found no interaction in a dedicated yeast two-hybrid assay between full-length Yap8 (bait) and full-length Etp1 (prey). Thus, it appears that the interaction between the predicted coiled-coil regions in Etp1 (residues 487–552) and Yap8 (residues 20–75) (Wang et al. 2012) does not occur with the full-length proteins. How can Etp1 affect ACR3 expression independently of Yap8 given that Yap8 is the only transcription factor known to control ACR2 and ACR3 expression (Wysocki et al. 2004; Iliina et al. 2008)? Recent studies have shown that coactivator complexes and chromatin remodeling factors are implicated in proper induction of ACR3 expression during As(III) stress (Menezes et al. 2017; West et al. 2019). It is tempting to speculate that Etp1 affects ACR3 mRNA levels by regulating localization and/or turnover of component(s) of these coactivator complexes and chromatin remodeling factors.

To sum up, our data indicate that Etp1 affects ACR3 expression and confers As(III) resistance in a Yap8-independent fashion. Thus, Etp1 is a novel arsenic resistance factor that impacts gene expression under various stress conditions; the mechanistic details remain to be elucidated. Since Etp1 is a putative E3 ligase, future efforts should be directed toward demonstrating its ubiquitin ligase activity and to identify its physiological substrates.

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Author contributions

Study conception and design: MJT, EM-D and RW; data collection and analysis: AMR, EM-D, EL, MM, EA and MJT; and funding acquisition: MJT, AMR, EM-D and RW. First draft was written by AMR, EM-D and MJT. All authors commented on previous versions and read and approved the final manuscript.

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