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Mammalian proapoptotic factor ChaC1 and its homologues function as γ-glutamyl cyclotransferases acting specifically on glutathione

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ChaC1 is a mammalian proapoptic protein of unknown function induced during endoplasmic reticulum stress. We show using *in vivo* studies and novel *in vitro* assays that the ChaC family of proteins function as γ-glutamyl cyclotransferases acting specifically to degrade glutathione but not other γ-glutamyl peptides. The overexpression of these proteins (but not the catalytically dead E>Q mutants) led to glutathione depletion and enhanced apoptosis in yeast. The ChaC family is conversed across all phyla and represents a new pathway for glutathione degradation in living cells, and the first cytosolic pathway for glutathione degradation in mammalian cells.

Keywords: apoptosis; ChaC1; γ-glutamyl cyclotransferases; glutathione; 5-oxoproline

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INTRODUCTION

Apoptosis, or programmed cell death, is an evolutionarily conserved process that is essential for normal development and senescence of living cells. A variety of different stimuli have been shown to trigger apoptosis. For many of these stimuli, glutathione (γ-glutamyl-cysteinyl-glycine, GSH), the redox buffer of living cells, and reactive oxygen species (ROS) have a crucial role [1]. Overexpression of glutathione biosynthetic enzymes (and higher intracellular glutathione levels) protect against apoptosis induced by different stimuli and have been associated with apoptotic-resistant phenotypes in several models of apoptosis and cancerous cells [2,3]. Conversely, glutathione depletion is found as an early hallmark of apoptosis [4]. Apoptosis execution by glutathione depletion occurs as a result of ROS generation and the consequent downstream cascade, but can also occur in an ROS-independent manner [5,6].

Endoplasmic reticulum (ER) stress is a cellular response to disturbances in ER function and is critical for cellular survival. However, chronic or persistent ER stress moves from a prosurvival phase to a propapoptotic phase, the latter leading to cell death via apoptosis, and is an important factor in many neurodegenerative diseases, as well as diabetes, atherosclerosis and renal disease. Although the exact mechanisms of ER stress-induced apoptosis are still not clear, it is found that both the IREI- and PERK (IRI/ASK1/JNK and PERK/ATF4/CHOP)-initiated signalling cascades of the unfolded protein response (UPR) are involved in apoptosis induction [7]. However, evidences suggest that upregulation of the transcription factor CHOP (C/EBP homologue protein) during ER stress (mediated by ATF4) is a main link between ER stress and apoptosis [8]. CHOP overexpression has been associated with upregulation of Bim (a proapoptotic protein) and downregulation of Bcl2, a major antiapoptotic protein [9,10]. CHOP overexpression also leads to ERO1 induction and consequently to hyperoxidation of the ER as well as release of calcium stores [11,12]. Cytoplasmic hyperoxidation and glutathione depletion have also been observed as a consequence of ER stress and is another potential trigger of apoptosis [10]. Glutathione depletion in the cell principally occurs by the oxidation of GSH to GSSG, the conjugation of GSH to electrophiles or by plasma membrane efflux of GSH [13–15]. However, the exact mechanism of CHOP-mediated glutathione depletion is not known [10]. Surprisingly, cytosolic glutathione degradation, a potential arm of glutathione depletion, has never been found to deplete glutathione.

Recently, a novel proapoptotic protein ChaC1, of unknown function, was identified in mammalian cells. ChaC1 localized to the cytosol, and was under the regulation of CHOP during the

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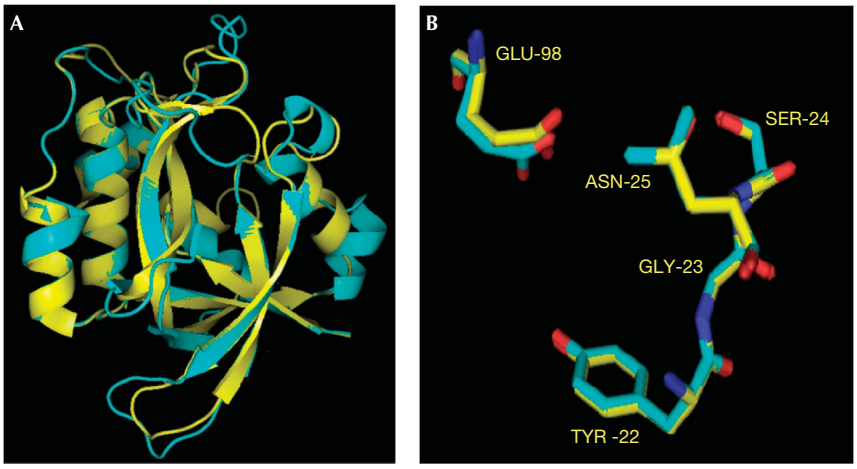

Fig 1 | The ChaC family proteins have structure (BtrG/γ-GCT fold) and active site similar to γ-GCT/γ-GACT. The protein ChaC1 was modelled using PHYRE server. The homology model obtained was superimposed on the crystal structure of γ-GCT (C7orf24, PDB code 2PN7 and 2RBH) with the help of PyMOL molecular graphics software. (**A**) Superimposition of homology-modelled ChaC1 structure (cyan colour) on γ-GCT structure (yellow colour). (**B**) Putative active site residues of ChaC1 (^{39}YGSL^{42} and E^{116}, cyan in colour) were superimposed on corresponding active site residues of γ-GCT (^{22}YGSN^{25} and E^{98}, yellow in colour). γ-GCT residues are labelled in yellow colour. γ-GACT, γ-glutamylamine cyclotransferase; γ-GCT, γ-glutamyl cyclotransferase.

UPR of ER stress [16]. The ChaC1 protein was also identified as a prognostic marker in certain types of cancer although in these cells a role in apoptosis could not be established [17]. Homologues of the protein are found across phyla. In *Escherichia coli*, *chaC* is a gene of the cha operon (Ca^{++}/H^{+} antiporter) and is assumed to be a possible regulator of this operon [18]. The homologue in *Saccharomyces cerevisiae*, YER163c, is also listed as a protein of unknown function.

In this manuscript, we demonstrate that members of the ChaC family contain the BtrG/γ-glutamyl cyclotransferase (γ-GCT) fold and belong to the family of γ-GCTs. Using *in vivo* growth assays in yeast, and novel *in vitro* assays developed to investigate glutathione degradation, the preferential activity of ChaC proteins towards glutathione was revealed, which involved cleaving of the γ-glutamyl bond of glutathione to yield 5-oxoproline and cys-gly. Overpexpression of mouse wild-type (WT) ChaC1 but not its catalytically inactive mutant (E>Q) led to glutathione depletion and enhanced apoptosis in yeasts. The studies delineate the molecular function of ChaC1, and explain the proapoptotic nature of these proteins. The findings also reveal a novel pathway for glutathione degradation in living cells, and the first cytosolic pathway for glutathione degradation in mammalian systems.

RESULTS AND DISCUSSION

The ChaC proteins belong to the γ-GCT superfamily

The ChaC1 protein and its yeast homologue YER63c that were modelled by the PHYRE server indicated the presence of a BtrG/γ-GCT fold (as also indicated in the Conserved Domain Database, supplementary Fig S1 online) that is characterized by unique beta barrels surrounded by alpha helices in these proteins [19]. When the homology models of these proteins were superimposed on the γ-GCT (C7orf24) structure they revealed very high structural similarity, despite no sequence similarity in their primary sequences (Fig 1A; supplementary Fig S2A online). Moreover, ^{39}YGSL^{42} and E^{116} residues of ChaC1, and ^{13}YGSL^{16} and E^{115} residues of YER163c are equivalent to the critical ^{22}YGSN^{25} and E^{98} residues of γ-GCT that are involved in substrate binding and catalysis and superimposed very well on the corresponding residues of γ-GCT (Fig 1B; supplementary Fig S2B online).

Multiple sequence alignment of the ChaC1 homologues from different phyla revealed that all the members contained the signature motif and catalytic glutamate residue confirming that the ChaC family of proteins belongs to the family of γ-GCTs (supplementary Fig S3 online). This family includes γ-GCT that acts on various γ-glutamyl amino acids to yield 5-oxoproline and amino acids [19]. γ-glutamylamine cyclotransferase (γ-GACT), another enzyme found in mammalian cells, acts on γ-glutamyl amine to yield 5-oxoproline and the amine [20] (Table 1). BtrG, a bacterial protein, acts on γ-glutamyl butirosin, an intermediate in butirosin biosynthesis, and cleaves the γ-glutamyl moiety to yield 5-oxoproline and butirosin [21]. These different members do not show any significant sequence similarity among themselves despite having a common BtrG/γ-GCT fold. However, they all produce 5-oxoproline from different γ-glutamyl compounds.

ChaC proteins allow utilization of glutathione in yeasts

To examine if a possible γ-GCT activity might be associated with ChaC family proteins, it was necessary to identify the γ-glutamyl compounds that might be potential substrates against which this activity would be displayed. Towards this goal, we cloned ChaC1 (mouse), YER163c (*S.cerevisiae*) and *E. coli* chaC genes along with known BtrG/γ-GCT-fold proteins, *γ-GCT* (*C7orf24*) and *γ-GACT* genes of mouse in the yeast expression vector p426GPD. These constructs were transformed into the *S.cerevisiae* strain *met15*Δ *ecm38*Δ *dug3*Δ. This strain is an organic sulphur auxotroph owing to a *met15*Δ. The strain also carries a deletion in *ECM38* (which encodes for γ-glutamyl transpeptidase, γ-GT) and also carries a deletion in *DUG3* (which degrades glutathione by the alternative

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Table 1 | Comparison of the BtrG/γ-GCT-fold proteins

Protein	Enzyme name	Structure known/ catalytic residue	Substrate	Product	Reference
C7orf24	γ-GCT	Known E98	γ-Glu-aa	5-OP, amino acid	[19]
1VKB	γ -GACT	Known	γ-Glu-amine	5-OP, amine	[20]
BtrG		Known	γ-Glu-butirosin	5-OP, butirosin	[21]
ChaC1	γ-GCG	Unknown E116	γ-Glu-cys-gly	5-OP,cys-gly	(This study)

γ-GACT, γ-glutamylamine cyclotransferase; γ-GCG, γ-glutamylcyclotransferase-glutathione-specific; γ-GCT, γ-glutamyl cyclotransferase.

DUG pathway [22]). As a consequence of the absence of the two enzymes capable of cleaving the γ-glutamyl bond, the strain is deficient in glutathione and γ-glutamyl amino-acid utilization. This strain was used for *in vivo* assays to evaluate the γ-glutamyl degrading ability of the ChaC family proteins. We examined two different sulphur-containing γ-glutamyl amino acids, γ-glu-cys and γ-glu-met. As γ-GCT (C7orf24) is a known γ-GCT, transformants carrying this open reading frame could grow on both γ-glu-cys and γ glu-met. In contrast, γ-GACT failed to act on either of the γ-glutamyl dipeptides as it is specific for γ-glutamylamine (expression was confirmed by an haemagglutinin-tagged protein, data not shown), while members of the ChaC family proteins cleaved γ-glu-cys but were completely inactive against γ-glu-met (Fig 2A). Glutathione is a γ-glutamyl-tripeptide (γ-glu-cys-gly) and we examined whether glutathione was a substrate of the ChaC family using this assay. Interestingly, it was observed that strains expressing ChaC family proteins were able to grow very well on glutathione (Fig 2B). Furthermore, this was also dependant on the cys-gly peptidase (Dug1p) suggesting also that the release of cys-gly from glutathione by the action of ChaC family proteins (Fig 2C). In contrast, γ-GCT expression (C7orf24) did not lead to growth when glutathione was used as the sulphur source, (Fig 2B). The result suggests that the ChaC family proteins alone display γ-GCT activity towards glutathione.

To further confirm this γ-GCT activity towards glutathione, we mutated the putative catalytic glutamate residue of YER163c (E^{115}) and ChaC1 (E^{116}) to glutamine (E > Q). These mutations led to loss in activity against glutathione (Fig 2D) although protein expression was not altered (data not shown), demonstrating the involvement of this residue in catalysis. This suggests that the γ-GCT, γ-GACT and ChaC family proteins share similar cyclotransferase reaction mechanisms and structure, despite the differences in their protein sequences and substrate preferences.

Purified ChaC proteins have γ-GCT activity *in vitro*

To confirm the *in vivo* activities of the ChaC family proteins, recombinant ChaC1 and YER163c proteins were purified from *E. coli* using His-tags and Ni-NTA affinity chromatography (supplementary Fig S4 online). To identify the products of these ChaC family proteins on different γ-glutamyl amino acids (γ-glu-ala, γ-glu-cys, γ-glu-met, γ-glu-his and γ-glu-lys) and glutathione, products of the reaction mixture were analyzed by high-performance liquid chromatography (HPLC) as described in methods. The results showed that although YER163c showed some activity towards γ-glu-ala, none of the other γ-glu-amino acids examined were substrates as no new peak corresponding to 5-oxoproline appeared with these γ-glu-amino acids (supplementary Fig S5A,B online). However, when these proteins were incubated with glutathione, the peak corresponding to glutathione disappeared and new peaks corresponding to 5-oxoproline and cys-gly appeared (Fig 3). The generation of 5-oxoproline from glutathione confirmed the γ-GCT activity of the ChaC family proteins. These results revealed that ChaC proteins cleave glutathione according to the following reaction scheme (supplementary Fig S6 online),

$$\text{Glutathione} \xrightarrow{\text{ChaC proteins}} \text{5-oxoproline} + \text{Cys-gly}$$

To quantitate the relative activities of the ChaC proteins towards different γ-glu-peptides and glutathione, we measured their γ-GCT activity by 5-oxoproline (a common product of BtrG/γ-GCT-fold proteins acting on various γ-glutamyl compounds, Table 1) release. In a novel assay, the 5-oxoproline released was acted on by 5-oxoprolinase (purified from yeast) to yield glutamate that was estimated by the sensitive Amplex Red fluorometric method [23]. We observed that ChaC1 and YER163c showed barely any activity against γ-glu-dipeptides, while, in contrast, glutathione was the most preferred substrate for both the ChaC family proteins (Fig 3D). The absence of any activity on γ-glu-cys *in vitro* (unlike what we observed *in vivo*) could be owing to the fact that the growth experiments are measured over a long time and might pick up residual activity.

We also developed an alternative assay that could determine the kinetic parameters with glutathione as a substrate. This assay exploits the cys-gly generation capability of ChaC family proteins from glutathione. The cys-gly generated from glutathione by ChaC family proteins is converted into free cysteine and glycine with the help of Dug1p (a cys-gly peptidase from *S.cerevisiae*), followed by estimation of cysteine [24]. Using this method, we again found that glutathione but not γ-glu-cys was acted on by ChaC family proteins (supplementary Fig S7 online). The kinetic parameters revealed similar K_m for both the proteins ChaC1 (3.13 + 0.40 mM) and YER163c (1.52 + 0.18 mM) although the V_{max} were significantly different, ChaC1, 980 + 50, and YER163c, 110 + 7 μmoles h^{-1} mg per protein, respectively (supplementary Fig S8 online). More importantly, the K_m for glutathione in the range between 1.5 and 3 mM reveals that the enzymatic action on glutathione is physiologically relevant.

ChaC overexpression leads to glutathione depletion

Our results revealed that glutathione is the preferred substrate of ChaC proteins. Glutathione depletion is an important factor for apoptosis initiation and execution. Moreover, ChaC1 has been shown as a proapototic factor in mammals [1,16]. To examine if the ChaC family proteins caused glutathione depletion *in vivo* and

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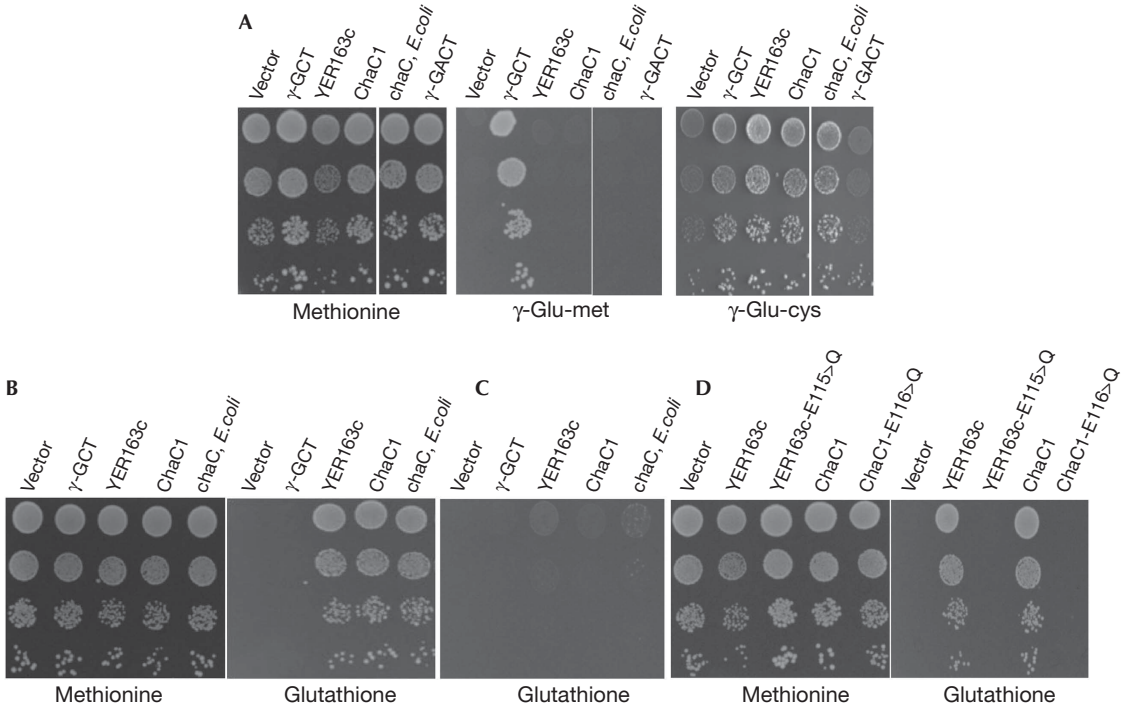

Fig 2 | The ChaC proteins expressed in yeast act on glutathione to enable glutathione utilization in a cys-gly peptidase (Dug1p)-dependent manner. Other γ-GCTs are inactive against glutathione. ChaC family proteins encoding gene from *S.cerevisiae* (YER163c), mouse (ChaC1) and *E. coli* (chaC) along with mouse γ-GCT (C7orf24) and mouse γ-GACT were cloned in the yeast expression vector p426GPD and transformed in yeast strain ABC1723 (*met15*Δ *ecm38*Δ *dug3*Δ, that is organic sulphur auxotroph and deficient in glutathione and γ-glu-amino-acids utilization). The transformants were overnight grown and harvested, washed and resuspended in water and serially diluted to give 0.2, 0.02, 0.002 and 0.0002 OD_{600} nm of cells. A 10-μl aliquot of each dilution was spotted on minimal medium containing (**A**) 0.07 mg/ml methionine, 300 μM γ-glu-met and 300 μM γ-glu-cys (methionine is used as a positive control), (**B**) 300 μM Glutathione. (**C**) To examine Dug1p dependence, transformants of yeast strain ABC2295 (*met15*Δ, *ecm38*Δ *dug3*Δ *dug1*Δ that cannot cleave cys-gly as they carry a *dug1*Δ) were spotted on 300 μM glutathione, and failed to grow on glutathione control methionine plates not shown. (**D**) Putative catalytic glutamate residues of YER163c (E^{115}) and ChaC1 (E^{116}) were changed into Glutamine (Q) using site-directed mutagenesis. Functionality of the mutant genes was checked using glutathione as a substrate, as done above. Methionine plates are used as growth assay controls. (Photographs were taken after 3 days of growth.) γ-GACT, γ-glutamylamine cyclotransferase; γ-GCT, γ-glutamyl cyclotransferase.

lead to apoptosis, the WT and E>Q mutants were expressed in the *S.cerevisiae* strain *met15*Δ, *ecm38*Δ and *dug3*Δ. Cells expressing WT ChaC1 and YER163c led to significant depletion in glutathione despite ongoing glutathione synthesis in these cells. However, cells expressing the catalytically dead mutants ChaC1-E>Q and YER163c-E>Q did not show any glutathione depletion (Fig 4A). As these strains had ongoing glutathione synthesis, we also examined the effects in the yeast strain *met15*Δ, *ecm38*Δ, *dug3*Δ, *gsh1*Δ that is unable to synthesize glutathione owing to a deletion in *GSH1*. Only the mutants ChaC1-E>Q or YER163c-E>Q expressing cells of the above strain were able to grow on the plate containing 10 μM glutathione, while WT ChaC1 and YER163c expression showed highly compromised growth (Fig 4B). Addition of extracellular glutathione rescued these cells from this effect (Fig 4C). These data confirm the ability of these proteins to deplete glutathione. We subsequently evaluated the ability of ChaC1 WT and E>Q mutant to induce apoptosis in these cells. We observed a significant increase in apoptosis caused by the WT clone as compared with the mutant as seen both by Annexin V assays (Fig 4D) and TdT-mediated dUTP nick end labelling assays (Fig 4E). Furthermore, the enhanced apoptosis in yeasts could be reversed by addition of glutathione (Fig 4D,E).

In summary, we have characterized a new group of γ-GCTs that function specifically to degrade glutathione. The yeast homologue has been renamed as GCG1 (γ-GCT acting on glutathione). The mammalian homologue, ChaC1, is a known proapoptotic protein that is highly induced in UPR stress under the CHOP cascade [16]. As CHOP overexpression also leads to glutathione depletion and ROS generation [10], our finding fills an important gap in this cascade as it provides an explanation how expression of CHOP leads to glutathione depletion, and assists in bringing about the permissive condition for apoptosis execution. The studies described here represent a novel pathway for glutathione degradation, and the only known pathway of cytosolic glutathione degradation in higher eukaryotes, that is conserved from bacteria to mammals (supplementary Fig S9 online). As the ChaC1 protein is also significantly induced in certain cancers and have been suggested as a prognostic marker [17], they are likely to have a significant role in the physiology and redox biology of both normal and malignant cells. These findings,

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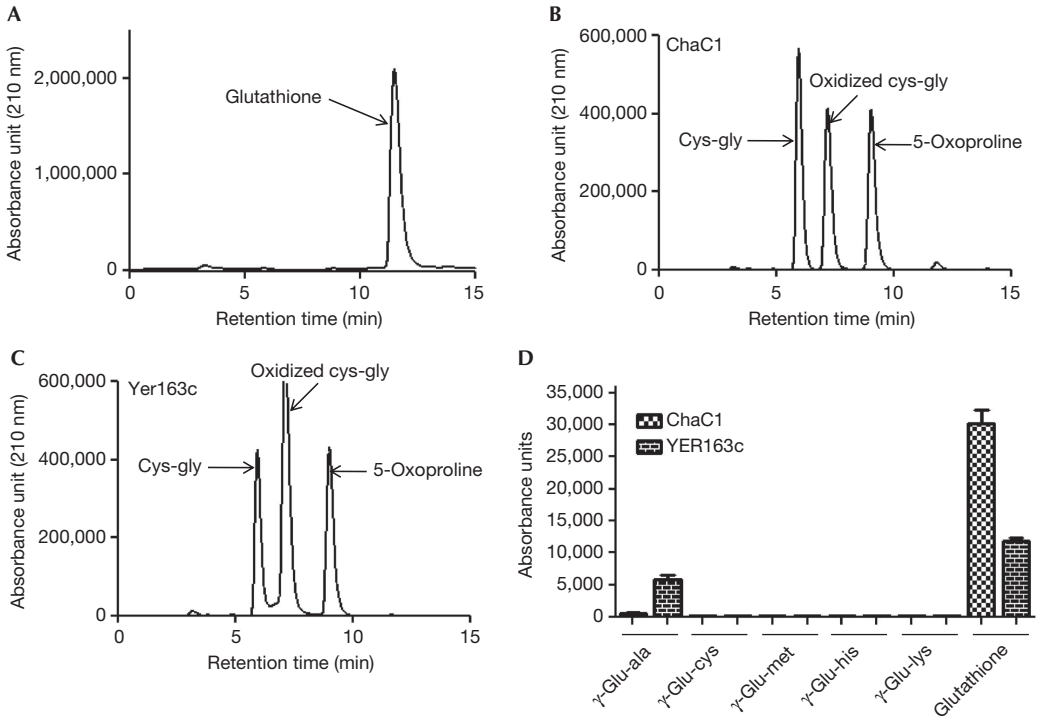

Fig 3 | The ChaC proteins exhibit γ-glutamyl cyclotransferase activity specifically towards glutathione producing 5-oxoproline and cys-gly, but do not act on γ-glutamyl amino acids to any significant extent. Ten micrograms of proteins (**B**) ChaC1 and (**C**) YER163c were incubated with 10 mM glutathione and different γ-glutamyl amino acids (γ-glu-ala, γ-glu-cys, γ-glu-met, γ-glu-his and γ-glu-lys) for 60 min. Twenty microlitres of the terminated reaction mix was analyzed using high-performance liquid chromatography system on a C18 column (250 × 4.6 mm^2, Phenomenex) and a mobile phase of 2% (v/v) aqueous perchloric acid at 1.0 ml/min. Data are shown for glutathione only and data for γ-glutamyl amino acids are shown in supplementary Fig 5 online. Substrate and products of reaction mix were monitored at 210 nm. Cys-gly was detected at 5.9 min, 5-oxoproline was detected at 9 min and glutathione was detected at 11.6 min (oxidized cys-gly was detected at 7.1 min). Substrate and products were identified with authentic standards. (**D**) ChaC activity against glutathione and different γ-glutamyl amino acids as seen by the 5-oxoprolinase-coupled Amplex Red-based glutamate detection method. ChaC proteins of 2.5 μg were incubated with 10 mM of different γ-glu-amino acids or glutathione for 60 min. 5-oxoprolinase enzyme of 5 μgwas then added to the heat-inactivated, above reaction mix and incubated for a further 60 more minutes. This was followed by 50 μl of Amplex Red-based glutamate/glutamate oxidase assay kit solution and a further incubation for 30 more minutes. Fluorescence was measured using 544 and 584 nm excitation and emission wavelengths, respectively. Experiments were performed in triplicates. Data are means ± s.e.m. Panel A refers to the Control sample (without added ChaC proteins) with glutathione as substrate.

and the sensitive and convenient assays developed for assaying these activities will greatly facilitate our understanding of these processes in living organisms.

METHODS

HPLC analysis. To study the γ-GCT activity of the ChaC proteins, 10 μg of either YER163c or ChaC1 was incubated with 10 mM γ-glutamyl-containing dipeptide (γ-glu-ala, γ-glu-cys, γ-glu-met, γ-glu-his or γ-glu-lys) or glutathione in an 100-μl reaction mix containing 50 mM Tris–HCl buffer (pH 8). The reaction mix was incubated for 1 h at 37 °C. The reactions were terminated by heat denaturation at 95 °C for 5 min. Samples were centrifuged for 30 min to remove the inactivated proteins before HPLC analysis. To analyze the products of the reaction, 20 μl of the reaction mixes were subjected to HPLC analysis using C18 HPLC column (250 × 4.6 mm, Phenomenex) with a 2% (v/v) aqueous solution of perchloric acid used as the mobile phase with 1 ml/min flow rate. Peaks of different γ-glu-dipeptides, cys-gly, glutathione and 5-oxoproline were monitored at 210 nM. Substrate and products were identified with authentic standards. Reaction product peaks corresponding to 5-oxoproline and cys-gly appeared at 9 min and 5.9 min, respectively. A third peak (between 5-oxoproline and cys-gly peaks), at 7.1 min appeared in reaction mix, was identified as oxidized cys-gly, and appeared even if standard cys-gly was incubated in Tris buffer (pH 8) for prolonged periods.

5-oxoprolinase-coupled γ-GCT assay for ChaC family proteins. As 5-oxoproline appeared as a common product of the ChaC protein's action on γ-glutamyl compounds, a 5-oxoprolinase-coupled, extremely sensitive, 5-oxoproline detection-based γ-GCT assay was developed. Ten millimolar of γ-glutamyl-containing dipeptide (γ-glu-ala, γ-glu-cys, γ-glu-met, γ-glu-his or γ-glu-lys) or glutathione was incubated with 2.5 μg of YER163c or ChaC1 protein for 60 min at 37 °C in 50 μl of reaction mixture containing 50 mM Tris–HCl (pH 8). The reaction mixture was then placed at 95 °C for 5 min to inactivate the enzyme. After inactivation, 10 μl of the 5-oxoprolinase reaction mix was added. This mix typically consists of 5 mM ATP, 10 mM $MgCl_2$, 150 mM KCl and 5 μg of yeast 5-oxoprolinase, and enables the conversion

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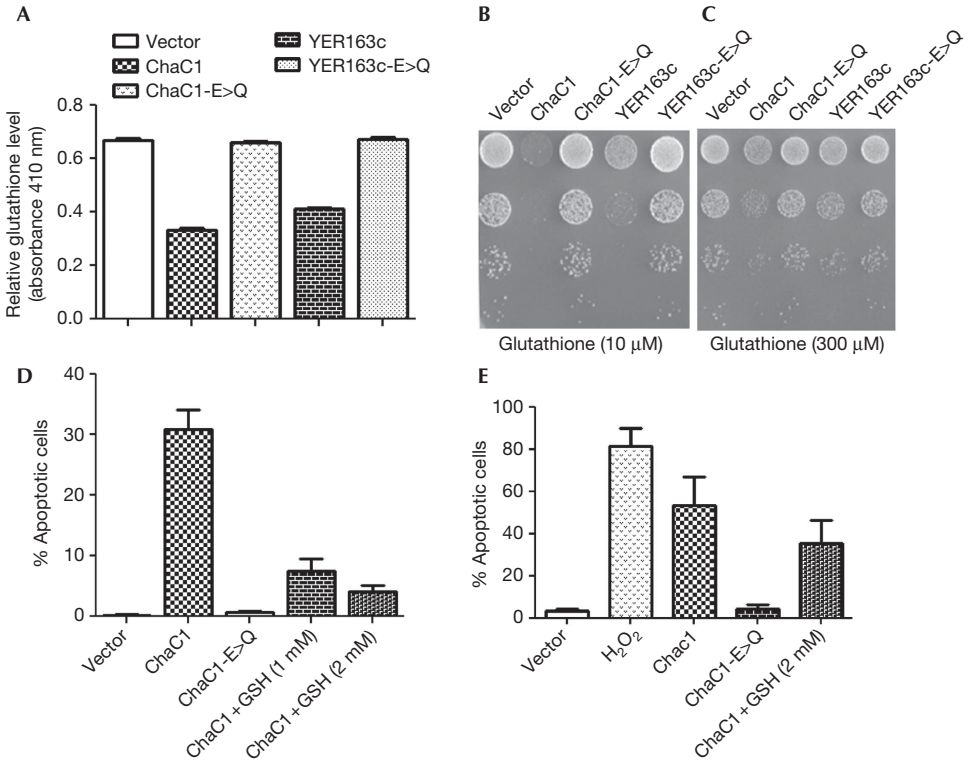

Fig 4 | The ChaC proteins, but not the catalytically dead E>Q mutants, cause glutathione depletion and enhance apoptosis in yeast. (**A**) Glutathione was estimated in the yeast strain ABC1723 expressing ChaC family proteins (ChaC1 and YER163c) and their catalytically dead mutants (ChaC1-E>Q and YER163c-E>Q). Glutathione estimation was performed using the DTNB-glutathione reductase method. The experiment was done in triplicates. Data are mean ± s.e.m. Expression of ChaC1 and YER163c leads to ~50% and ~40% glutathione depletion, respectively, in comparison with vector control. (**B,C**) The ChaC proteins cause growth retardation in a *gsh1Δ* background that can be reversed by addition of glutathione. Chac and their mutants were expressed in yeast strain ABC2712 (*met15Δ ecm38Δ dug3Δ gsh1Δ* that is a glutathione auxotroph because of the deletion in *GSH1*). The transformants were spotted on minimal medium containing (**B**) 10 μM glutathione and (**C**) 300 μM glutathione. (**D,E**) The ChaC proteins but not the E>Q mutants cause enhanced apoptosis. Yeast strain ABC2712 expressing ChaC1 and mutant ChaC1-E>Q were grown in minimal medium, with and without glutathione as indicated in the figure. Cells showing apoptosis were stained with (**D**) FITC-labelled annexin V for detection of exposed phosphatidylserine and analysed by flow cytometry or (**E**) FITC-labelled anti-5-bromodeoxyuridine Antibody Solution (TdT-mediated dUTP nick end labelling assay) in the dark for 120 min at room temperature as described in methods. Hydrogen peroxide treatment at 100 mM for 1 h was used as a positive control. FITC, fluorescein isothiocyanate.

of released 5-oxoproline to glutamate. Incubation was carried out for 1 h at 37 °C. After this, 50 μl of the glutamate/glutamate oxidase Amplex Red assay kit solution (Invitrogen) was added to the reaction mixture and incubated for 30 more minutes at 37 °C. The reaction mixture was then transferred to 96-well plates and the fluorescence was measured using a fluorescence microplate reader with an excitation at 544 nm and an emission at 584 nm. Controls lacking the ChaC proteins were treated similarly. The assay was performed in triplicates and data are means ± s.e.m.

Cys-gly peptidase (Dug1p)-coupled γ-GCT assay for ChaC family proteins. Cys-gly is a product when glutathione is acted on by the ChaC proteins. As 5-oxoprolinase purified from yeast is very labile, a cys-gly peptidase-coupled, sensitive and convenient spectrophotometric assay was also developed to study the kinetic parameters of the ChaC proteins. In this assay, 2.5 μg of YER163c or ChaC1 protein was incubated with glutathione for 30 min at 37 °C in 50 μl of reaction mixture containing 50 mM Tris–HCl (pH 8). The reaction mixture was then placed at 95 °C for 5 min to inactivate the enzyme. After inactivation, 10 μl of cys-gly peptidase reaction mixture, that will convert cys-gly dipeptide into free cysteine and glycine, was added. The reaction mixture consisted of 20 μM $MnCl_2$ and 5 μg of Dug1p, a cys-gly peptidase. This reaction mixture was further incubated for 1 h at 37 °C. The free cysteine generated was measured by acidic ninhydrin. In brief, after the incubation, 40 μl of water was added to the above reaction mixture to make up the volume to 100 μl. Thereafter, 100 μl acetic acid and 100 μl of acidic ninhydrin solution (20 g ninhydrin dissolved in acetic acid and HCl that are mixed in 3:2 ratio) were added to the reaction mixture and the reaction mixture boiled in a water bath for 10 min to develop the pink colour. Two hundred microlitres coloured solutions were transferred in 96-well enzyme-linked immunosorbent assay (ELISA) plates and the OD was taken at 560 nm using ELISA reader. Controls containing everything except the ChaC protein were treated similarly. Cysteine liberated from the γ-glu-cys by

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ChaC family proteins activity was measured by acidic ninhydrin-based cysteine detection method and do not need to be treated by cys-gly peptidase (Dug1p). The assay is performed in triplicates and data are means ± s.e.m.. To study the kinetic parameters of the ChaC proteins towards glutathione, the cys-gly peptidase (Dug1p)-coupled method was used. For the kinetic experiments, concentration of glutathione ranging from 0.25 to 15 mM were incubated with protein YER163c (1 μg), and ChaC1 (300 ng) was used in 50 μl of reaction mixture, and reactions performed as described above. The K_m and V_{max} data are means ± s.e.m. ($n = 5$ independent experiments)

Total glutathione estimation. ChaC1, YER163c and their mutants ChaC1-E>Q and YER163c-E>Q were expressed in the yeast strain ABC1723, using the yeast expression vector p426GPD. Cells were grown overnight, reinoculated in fresh medium and further grown for 8 h. 10 OD_{600} of cells expressing each construct were lysed in 800 μl of 5% sulphosalicylic acid using glass bead lysis. Cells were vortexed for 20 min with intervals and centrifuged for 30 min at 4 °C. Supernatant was taken for glutathione estimation. Glutathione estimation was done as described earlier [25]. In brief, 10 μl of supernatant was mixed with 60 μl of DTNB (2 mg/3ml) and 60 μl of glutathione reductase (2.5 unit/ml) in 96-well ELISA plates. Sixty microlitres of NADPH (2 mg/3ml) was added in the above mixtures to start the reaction. Absorbance was taken at every 15 s at 410 nm. Absorbance at 2 min was used to compare the glutathione levels in the different samples.

Supplementary information is available at EMBO *reports* online (http://www.emboreports.org).

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Author contributions: A.K. and A.K.B. conceived the project, planned the experiments, analysed the results and wrote the paper. A.K. performed all the experiments relating to the cloning, assays and enzymology. Amandeep K. performed the haemagglutinin tagging of the proteins and the western blots; S.M., Shantanu S., Sagar S. and S.T. planned the experiments on apoptosis; and S.M. and S.T. executed the experiments on apoptosis.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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