Chapter 5

STUDIES ON THE REGULATION AND THE INTERACTION OF Dug1p, Dug2p AND Dug3p
5.1 Introduction:

In the previous chapter we described an attempt to understand how the DUG1, DUG2, and DUG3 genes participated in GSH degradation through a biochemical and genetic approach. Although Dug1p was sufficient for degradation of normal di- and tri-peptides (cys-gly and α-glu-cys-gly respectively), the presence of a γ-glu linkage either in di-peptides (γ-glu-cys) or tri-peptides (γ-glu-cys-gly) required the participation of all three gene products. To determine if the action of these proteins were sequential, dug1, dug2 and dug3 mutant extracts, grown in the presence of GSH were examined for the accumulation of intermediate thiols. However, no new or known thiol intermediates were observed to accumulate to any significant extent.

To obtain further insight into the pathway, we initiated studies on the regulation of the expression of these genes. We also investigated whether these proteins might be participating in a complex formation since WD40 domain containing proteins are known to be part of multi-protein complexes. The results of these investigations are described in the chapter.

Results:

5.2 DUG3, but not DUG1 or DUG2, is observed to be under sulphur regulation in both met15Δ and MET15 genetic backgrounds.

We examined the regulation of the DUG1, DUG2 and DUG3 genes using promoter-LacZ fusions. 600bp upstream region corresponding to the promoters of different DUG genes were amplified by PCR and fused in frame with the LacZ gene as described in materials and methods (Section 2.2.14). The respective promoter-LacZ constructs were transformed into the S. cerevisiae met15Δ (ABC 733) or MET15 (ABC 734) background. The effects of different sulphur sources on the expression of all of the three DUG genes were analyzed by growing the transformed cells in different medium conditions. The results revealed that the gene expressions of DUG1 and DUG2 remain similar under the various sulphur conditions, which we checked, that included no organic sulphur (-S), methionine (+Met), cysteine (+Cys) and GSH (+GSH) (Fig 5.1a & 5.1b). However, the DUG3 gene showed marked regulation under different sulphur conditions. DUG3 showed maximum repression under the condition when methionine was used as a
Fig 5.1a Effect of different sulphur sources on DUG1 promoter, DUG2 promoter and DUG3 promoter-β-galactosidase fusion expression in ABC 733 (met15d) strain (-S; NH₄SO₄, Met; Methionine, Cys; Cysteine, GSH; Glutathione)
Fig 5.1b Effect of different sulphur sources on DUG1 promoter, DUG2 promoter and DUG3 promoter-β-galactosidase fusion expression in (ABC 733) met15Δ strain (-S; NH₄SO₄, Met; Methionine, Cys; Cysteine, GSH; Glutathione)
sulphur source. Presence of cysteine as a sulphur source also repressed DUG3 gene expression strongly. GSH was found to repress DUG3 gene expression only mildly. The DUG3 gene was maximally expressed under sulphur limiting condition (Fig 5.1a & 5.1b). Furthermore, the DUG3 gene was observed to remain in repressed condition when methionine was added along with GSH (Fig 5.2).

The expressions of the DUG genes were also studied in the presence of inorganic sulphur (ammonium sulphate) and in the absence of inorganic sulphur (ammonium chloride) in a met15Δ strain background. We observed that the expressions of DUG1 and DUG2 gene were not affected by the presence or absence of sulphur. The expression of DUG3 gene in the met15Δ background was derepressed and not dependent in the presence or absence of inorganic sulphate in a met15Δ background (Fig 5.3).

Also, in the MET15 background, the transformants were grown under different sulphur conditions to check the effect of sulphur sources on the expression of DUG3. The results show that methionine strongly represses the expression of DUG3 gene whereas repression of DUG3 expression is milder in case of GSH compared to methionine. Maximum DUG3 gene expression was observed in the absence of any organic sulphur source in this background (Fig 5.4).

5.3 Nitrogen sources do not affect the expression of DUG genes.

All the three DUG promoter-LacZ constructs, transformed into MET15 S. cerevisiae strain (ABC 734) were grown under different nitrogen conditions to check the effect of repressing and non-repressing nitrogen sources on the expression of the DUG genes. We observed that the expression of DUG1 and DUG2 gene was similar irrespective of the presence of repressible nitrogen source (Ammonium Sulphate) or non-repressible nitrogen source (Glutamate). The expressions of DUG3 genes were observed to increase in the presence of glutamate compared to ammonium sulphate. This suggests that DUG3 may be under both sulphur and nitrogen regulation in the cell (Fig 5.5).
Fig 5.2 Effect of different sulphur sources on DUG3 promoter-β-galactosidase fusion expression in ABC 733 (met15d) strain. (S; NH₄SO₄, M; Methionine, GSH; Glutathione)
Fig 5.3 Effect of $+\text{NH}_4\text{SO}_4$ (Ammonium sulfate) and $+\text{NH}_4\text{Cl}$ (Ammonium chloride) sources on DUG1 promoter, DUG2 promoter and DUG3 promoter-β-galactosidase fusion expression in ABC733 ($\text{met15A}$) strain.
Fig 5.4 Effect of different sulphur sources on DUG3 promoter-\(\beta\)-galactosidase fusion expression in ABC 734 (MET15 Wild Type) strain. (\(\text{NH}_4\text{SO}_4\), in-organic sulphur; M; Methionine, GSH; Glutathione)
Fig 5.5 Effect of different Nitrogen sources on DUG1 promoter, DUG2 promoter and DUG3 promoter β-galactosidase fusion expression in ABC 734 (MET15 Wild Type) strain.
(Glu; Glutamate, NH₄⁺, Ammonia)
5.4 Phylogenetic footprinting of the DUG3 promoter reveals the presence of known sulphur cis-regulatory motif that is conserved among the orthologous promoters from the *Saccharomyces* spp.

As our promoter-LacZ fusion β-gal assays showed that only *DUG3* gene is under sulphur regulation, we analyzed the sequence of the *DUG3* promoters across the *Saccharomyces* spp. in order to find out the presence of conserved sulphur regulatory motifs. Sequence examination of the *DUG3* promoter revealed a putative S-regulatory motif, 5'-AAACTGTG-3'.

Phylogenetic footprinting is a bioinformatic approach to obtain insight into cis-regulatory elements in yeast (Lenhard *et al.*, 2003). The rationale of the approach is that the functionally important cis-elements are conserved among closely related organisms while non-functional elements diverged rapidly and thus this approach can be successfully used to identify such elements.

The Saccharomyces Genome Database (SGD) was used to retrieve promoter sequences of *DUG3* gene and corresponding orthologues from various *Saccharomyces* spp. We aligned these orthologous promoter sequences using CLUSTAL-X and output was re-formatted using CLOURE-C. Among the few conserved regions those could be detected, was the sulphur regulatory motif 5'-AAACTGTG-3' at -174 to -165 bp position upstream of the ATG of *S. cerevisiae DUG3* ORF (Fig 5.6) suggesting that it is likely to play a functional role. This motif has been studied extensively earlier. The Met4p has been observed to tether to this motif along with Met28p and either with Met31p or Met32p (Blaisseau *et al.* 1998). In the light of the sulphur regulation of *DUG3* that we observed experimentally, it is likely that *DUG3* regulation might proceed by the classical sulphur regulatory pathway, using this motif.

5.5 Yeast two hybrid analyses to determine probable protein-protein interaction among the *DUG* gene products.

To investigate if the 3 proteins encoded by *DUG1, DUG2, or DUG3* might be interacting with each other, we initiated investigations on possible interaction using the yeast two-hybrid system.
Fig 5.6 Multiple sequence alignment of promoter sequence of *YNL191w* of *S. cerevisiae* (Scer, 578b) with upstream sequences of orthologous promoters from *S. paradoxus* (Spar, 574b), *S. mikatae* (Smik, 540b), *S. bayanus* (Sbay, 589b). Shaded regions show the presence of conserved stretch of sequences, including the motif AAACTGTG, an important sulphur regulatory motif (Blaiseau and Thomas 1998).
To carry out these analyses DUG1, DUG2 and DUG3 fusions were created in yeast two hybrid vectors pEG202 and pJG4.5 (detail given in section 2.2.16) both as bait fusions and prey fusions as indicated below:

Construction of Bait: DUG1, DUG2 and DUG3 were independently cloned into pEG202 to create pBait-DUG1, pBait-DUG2, and pBait-DUG3 plasmid respectively.

Construction of Prey: DUG1, DUG2 and DUG3 were independently cloned into pJG4.5 to create pPrey-DUG1, pPrey-DUG2, and pPrey-DUG3 plasmids respectively. The WD40 domain of Dug2p (1-420aa) and peptidase domain of Dug2p (480-878aa) were also independently cloned into pJG4.5 to construct pPrey-DUG2WD40 and pPrey-DUG2Pep respectively, as described in materials and methods (Section 2.1.3.1).

Reporter system: two reporter systems were used; (1) growth on -Leu and (2) colour on +X-Gal. For growth on -Leu, the strain EGY48 was used which contained 6LexA operators upstream of LEU2 gene. In X-Gal colour, the plasmid pSH18-34 was transformed into the EGY48 strain. This pSH18-34 plasmid contained 8LexA operators upstream of LacZ.

Positive control: pSH17-4, encoding LexA fused with Gal4p activation domain when transformed into EGY48 cells and induced with galactose, activates transcription and allow the transformants to grow on CM-Leu plate and EGY48 that contains pSH18-34 will turn deep blue on plates containing CM+X-Gal.

Negative control: pRFHM1, encoding LexA fused with C-terminus of Drosophila sp. Bicoid protein (LexA-Bicoid fusion protein does not bind DNA), when transformed into EGY48 cells and induced with galactose, does not activate transcription and thus does not allow the transformants to grow on CM-Leu plate and EGY48 that contains pSH18-34 will not turn deep blue on plates containing CM+X-Gal.

To examine the possible auto-activation potential of Bait-protein fusion; each of the pBait-DUG construct, positive control bait, pSH17-4 and negative control bait, pRFHM1 were transformed along with the pSH18-34 LacZ reporter in S. cerevisiae EGY48. Transformants were patched on CM-Leu and CM+X-Gal plate and the growth was checked. An ideal Bait-protein fusion construct should not grow on CM-Leu and should not develop any colour on CM+X-Gal plate. The pBait-DUG1 and pBait-DUG3
fusions behaved as true baits, however the pBait-DUG2 protein showed some auto-activations. Yeast two-hybrid results were interpreted accordingly using these controls.

5.6 Yeast two hybrid analyses suggest that Dug1p possibly interacts with Dug2p.

The pBait-DUG1, pPrey-DUG2 along with the pSH18-34 were transformed into the yeast strain EGY48, and transformants were patched on CM-Leu and CM+X-Gal plates and checked for growth or colour development respectively. The yeast two hybrid experimental results show that EGY48 transformed with pBait-DUG1, pPrey-DUG2 and pSH18-34 grew very well on CM-Leu plates and but did not produce deep blue colour on CM+X-Gal plates. The results indicate that Dug1p could interact with Dug2p to form heterodimer (Fig 5.7a).

The pBait-DUG2, pPrey-DUG1 along with the pSH18-34 were transformed into the yeast strain EGY48, and transformants were patched on CM-Leu and CM+X-Gal plates and checked for growth or colour development respectively. The results show that EGY48 transformed with pBait-DUG2, pPrey-DUG1 and the LacZ containing vector pSH18-34 grew very well on CM-Leu plates and but did not produce deep blue colour on CM+X-Gal plates. However, the pBait-DUG2 alone also activated the transcription (auto-activation), which was almost comparable to the test transformants in relative to growth on CM-Leu plate. Mild auto-activation of pBait-DUG2 could be observed using the X-Gal assay. We therefore used this for determining the interaction between pBait-DUG2 and pPrey-DUG1 and found increased colour development in comparison to the small amount of colour seen with pBait-DUG2 alone and led us to conclude that possibly Dug2p interacts with Dug1p (Fig 5.7b).

5.7 Yeast two hybrid analyses suggest that Dug2p possibly interacts with Dug3p.

The pBait-DUG3, pPrey-DUG2 along with the pSH18-34 were transformed into the yeast strain EGY48, and transformants were patched on CM-Leu and CM+X-Gal plates and checked for growth or colour development respectively. The results show that EGY48 transformed with pBait-DUG3, pPrey-DUG2 and the LacZ containing vector pSH18-34 grew very well on CM-Leu plates and produced deep blue colour on CM+X-
Fig 5.7 Yeast two-hybrid assay to study Dug1p-Dug2p interaction.
Gal plates, which were comparable to the positive control. This result showed that Dug2p might have a propensity to interact with Dug3p to form heterodimer (Fig 5.8a).

The reverse interaction with the pBait-DUG2, pPrey-DUG3 along with the pSH18-34 was also evaluated. The plasmids were transformed into the yeast strain EGY48, and transformants were patched on CM-Leu and CM+X-Gal plates and checked for the growth or colour development respectively. The results show that EGY48 transformed with pBait-DUG2 and pPrey-DUG1 grew very well on CM-Leu plates and produced deep blue colour on CM+X-Gal plates, which were comparable to the positive control. The pBait-DUG2 alone (Bait control) also activated the transcription, in which the growth on CM-Leu was comparable to the test transformants. Mild activation by pBait-DUG2 could be observed using the X-Gal assay. We therefore used this for determining the interaction between pBait-DUG2 and pPrey-DUG3 and found increased colour development in comparison to the small amount of colour seen with the pBait-DUG2 alone and led us to conclude that possibly Dug2p interacts with Dug3p (Fig 5.8b).

5.8 Yeast two hybrid analyses suggest that Dug1p does not interact with Dug3p.

The pBait-DUG1, pPrey-DUG3 along with pSH18-34 were transformed into the yeast strain EGY48, and transformants were patched on CM-Leu and CM+X-Gal plates and checked for growth or colour development respectively. The results show that EGY48 transformed with pBait-DUG1, pPrey-DUG3 and the LacZ containing vector pSH18-34 did not grow on CM-Leu plates and also did not produce deep blue colour on CM+X-Gal plates. This result suggested that Dug1p might not have any propensity to interact with Dug3p (Fig 5.9a).

The pBait-DUG3, pPrey-DUG1 along with the pSH18-34 were transformed into the yeast strain EGY48, and transformants were patched on CM-Leu and CM+X-Gal plates and checked for growth or colour development respectively. The results show that EGY48 transformed with pBait-DUG1 and pPrey-DUG3 did not grow on CM-Leu plates and also did not produce deep blue colour on CM+X-Gal plates. This result further confirmed that the Dug1p does not interact with Dug3p (Fig 5.9b).
Fig 5.8 Yeast two hybrid assay to study Dug3p-Dug2p interaction.
Fig 5.9 Yeast two hybrid assay to study Duglp-Dug3p interaction.
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5.9 Yeast two hybrid analysis reveals that Dug1p possibly does not interact with Dug2pWD40 domain or Dug2pPep domain independently.

The Dug2p protein contains two distinct domains, an N-terminal WD40 domain and a C-terminal M20 peptidase domain. Since yeast two-hybrid analysis suggested that Dug2p interacts with both Dug1p and Dug3p, we attempted to determine if any one of the Dug2p domains was involved in the interaction. The WD40 domain (1-420aa) and the peptidase domain (480-878aa) were separately amplified by PCR and fused to the bait and prey plasmid as described in materials and methods (Section 2.1.3.1).

The pBait-DUG1, pPrey-DUG2WD40 along with the LacZ containing vector pSH18-34 was transformed into the yeast strain EGY48, and transformants were patched on CM-Leu and CM+X-Gal plates and checked for growth or colour development respectively. The yeast two hybrid experimental results show that EGY48 transformed with pBait-DUG1 and pPrey-DUG2WD40 did not grow on CM-Leu plates and also did not produce deep blue colour on CM+X-Gal plates. This result suggested that Dug1p might not interact with Dug2pWD40 domain (Fig 5.10a).

The pBait-DUG1, pPrey-DUG2Pep along with the pSH18-34 was also transformed into the yeast strain EGY48, and transformants were patched on CM-Leu+Gal+Raf and CM+Gal+Raf+X-Gal plates and checked for growth or colour development respectively. The yeast two hybrid experimental results show that EGY48 transformed with pBait-DUG1 and pPrey-DUGPep did not grow on CM-Leu plates and also did not produce deep blue colour on CM+X-Gal plates. This result did not indicate any interaction of Dug1p with the Dug2pPep domain (Fig 5.10b).

5.10 Yeast two hybrid analysis reveals that Dug3p possibly interacts with Dug2pWD40 domain but not with Dug2pPep domain independently.

To determine if the interaction of Dug3p with Dug2p might be domain specific, interactions of Dug3p with the different domains of Dug2p (WD40 and peptidase) were examined as described in the previous section 5.9.

The pBait-DUG3, pPrey-DUG2WD40 along with the pSH18-34 was transformed into the yeast strain EGY48, and transformants were patched on CM-Leu and CM+X-Gal plates and checked for growth or colour development respectively. The yeast two hybrid
Prey - D
Bait - DUG I
Positive control
Negative control
CM+Raf

Bait-DUG1
Prey-DUG2WD40

Bait-DUG1
Prey-DUG2WD40

Fig 5.10a Yeast two-hybrid assay to study interaction between Dug1p and Dug2pWD40

Bait-DUG1
Prey-DUG2Pep

Bait-DUG1
Prey-DUG2Pep

Fig 5.10b Yeast two-hybrid assay to study interaction between Dug1p and Dug2pPep

Bait-DUG1
Positive control
Negative control
CM-LEU

Bait-DUG1
Positive control
Negative control
CM+Xgal
experimental results show that EGY48 transformed with pBait-DUG3, pPrey-DUGWD40 along with the pSH18-34 grew on CM-Leu plates and also produced mild blue colour on CM+X-Gal plates. This result showed that Dug3p might interact with Dug2pWD40 domain (Fig 5.11a).

Similarly, the pBait-DUG3, pPrey-DUG2Pep and along with the pSH18-34 was transformed into the yeast strain EGY48, and transformants were patched on CM-Leu and CM+X-Gal plates and checked for growth or colour development respectively. The experimental results show that EGY48 transformed with pBait-DUG3, pPrey-DUG2Pep along with the pSH18-34 did not grow on CM-Leu plates and also did not produce any blue colour on CM+X-Gal plates. The result indicated that Dug3p did not interact with the Dug2p peptidase domain (Fig 5.11b). These results specifically suggest that Dug3p specifically interacts with the WD40 domain of Dug2p but not with the peptidase domain.

5.11 Yeast two hybrid analyses reveal that Dug1p possibly interacts with itself.

The pBait-DUG1, pPrey-DUG1 along with the pSH18-34 was transformed into the yeast strain EGY48, and transformants were patched on CM-Leu and CM+X-Gal plates and checked for growth or colour development respectively. Auto-activation control, positive control and negative control have been described earlier in section (5.2.1). The yeast two-hybrid experimental results show that EGY48 transformed with pBait-DUG1, pPrey-DUG1 and the LacZ containing pSH18-34 grew very well on CM-Leu plates and produced deep blue colour on CM+X-Gal plates which was comparable to positive control. This result showed that Dug1p possibly could interact with itself (Fig 5.12).

5.12 Evaluating Dug protein interactions through Co-Immunoprecipitation analyses:

The extensive yeast two-hybrid analyses that we carried out, demonstrated protein-protein interaction among the Dug proteins. However yeast two hybrid is sometimes artifactual. So it is necessary to substantiate the result through an alternative
Fig 5.11a Yeast two hybrid assay to study interaction between Dug3p and Dug2pWD40

Fig 5.11b Yeast two hybrid assay to study interaction between Dug3p and Dug2pPep
Fig 5.12 Yeast two hybrid assay to study Dug1p-Dug1p Interaction.
method. We decided to substantiate the important findings on protein-protein interaction that we obtained using yeast two hybrid, by Co-immunoprecipitation studies.

In immunoprecipitation experiment, essentially, the cell is lysed under the native condition and protein Y is immunoprecipitated by using antiY antibody. Under native condition, protein X may maintain a stable interaction with protein Y in vitro as well. Thus protein X may eventually get precipitated as well along with protein Y. Thus the precipitation of X along with Y is termed as Co-immunoprecipitation (CoIP).

For the CoIP assay, we tagged the C-terminus of all the three Dug proteins differently. Tags were generated by PCR based method. The Dug1p was C-terminally tagged with either His₈ or single HA epitope and cloned into p416TEF (URA3 Marker) (and pRS315 (LEU2 Marker)) or p414TEF (TRP1 Marker). The Dug2p was C-terminally tagged with the single cMyc epitope and was cloned into p414TEF. The Dug3p was C-terminally tagged with HA epitope and was cloned into p416TEF. All the tagged proteins were checked for their functionality using plate based growth phenotype complementation assays and were found to be functional. The methodology of cell extract preparation and CoIP has been described in detail in chapter 2 (Section 2.2.17).

All the proteins were expressed in a major proteases deficient S. cerevisiae strain (ABC710). This allowed us to limit degradation of proteins by peptidase activity considerably during protein preparation for CoIP assay. This strain lacks the master proteases, e.g. Protease A (Pra1p or Pep4p), Protease B (Prbp) and the carboxypeptidase (Prc1p). The Pep4p is a vacuolar aspartyl protease (Proteinase A), which is involved in the posttranslation precursor maturation of vacuolar proteinases. The Prbp is a kind of serine protease (vacuolar proteinase B) belonging to the subtilisin family, which is involved in the vacuolar protein degradation. The Prc1p is a vacuolar carboxypeptidase Y, involved in protein degradation in vacuole.

5.13 Co-IP analysis reveals that Dug3pHA interacts with Dug2pcMyc.

Dug3pHA and Dug2pcMyc, both were expressed in S. cerevisiae strain ABC 710. The cell extract was prepared and immunoprecipitation done by using Anti-cMyc antibody. As a control, only Dug2pcMyc was expressed in S.cerevisiae strain ABC 710 and cell extracts were immunoprecipitated using Anti-cMyc antibody. Subsequently, the
immunoblot of all the samples, both total cell extract and IP samples were detected by using Anti-HA antibody. We could detect (~40kDa) Dug3pHA in total cell extract of Test sample (Fig 5.13 Lane 6) and in its corresponding IP sample (Fig 5.13 Lane 4 & 5) as well. We could not detect Dug3pHA in the total cell extract of the control sample (Fig 5.13 Lane3) and in its corresponding IP sample (Fig 5.13 Lane2) as well. The result indicates that Dug3pHA is co-immunoprecipitated along with Dug2pcMyc, and thus suggests that Dug3pHA possibly interacts with Dug2pcMyc.

5.14 Co-IP analysis reveals that Dug1pHis$_8$ interacts with Dug2pcMyc.

Dug1pHis$_8$ and Dug2pcMyc, both were over-expressed in $S.cerevisiae$ strain (ABC 710). The cell extract was prepared and immunoprecipitation done by using anti-His antibody. As a control, only Dug1His$_8$p was expressed in $S.cerevisiae$ strain ABC 710 and the cell extracts were immunoprecipitated using Anti-His antibody. Subsequently, the immunoblot of all the samples, both total cell extract and IP samples were detected by using Anti-cMyc antibody. We could detect (~97.7kDa) Dug2pcMyc in total cell extract of Test sample (Fig 5.14 Lane 5) and its corresponding IP sample (Fig 5.14 Lane 4) as well. But we could not detect Dug2pcMyc in the total cell extract of control sample (Fig 5.14 Lane3) and in its corresponding IP sample (Fig 5.14 Lane2) as well. The result indicates that Dug2pcMyc co-immunoprecipitated along with Dug1pHis$_8$, and suggests that Dug1pHis$_8$ possibly interacts with Dug2pcMyc.

5.15 Co-IP analysis reveals that Dug1pHis$_8$ interacts with Dug1pHA.

As a test sample, Dug1pHis$_8$ and Dug1pHA, both were expressed in $S.cerevisiae$ strain (ABC 710). The cell extracts were prepared and immunoprecipitation was done using Anti-His antibody. As a control, only Dug1His$_8$p was expressed in $S.cerevisiae$ (ABC 710) and cell extract was immunoprecipitated by using Anti-His antibody. Subsequently, the immunoblot of all the samples, both total cell extract and IP samples were detected by using Anti-HA antibody. We could detect (~54kDa) Dug1pHA in total cell extract of Test sample (Fig 5.15 Lane 6) and its corresponding IP sample (Fig 5.15 Lane 5) as well. But we could not detect Dug1pHA in the total cell extract of control sample (Fig 5.15 Lane 2) and in its corresponding IP sample (Fig 5.15 Lane 1) as well.
Fig 5.13 CoIP analysis showing Dug2p & Dug3p interaction. Total cell extract of *S. cerevisiae* strain ABC710 expressing Dug3pHA and Dug2pcMyc as well as control cell extract expressing only Dug2pcMyc. Immunoprecipitated with anti-cMyc antibody and detection with anti-HA antibody.

Lane 1: cell extract made from cells expressing Dug3pHA.
Lane 2: cell extract made from cells expressing Dug2pcMyc & immunoprecipitated with anti-cMyc.
Lane 3: cell extract made from cells expressing Dug2pcMyc.
Lane 4 & 5: cell extract made from cells expressing Dug2pcMyc & Dug3pHA and immunoprecipitated with anti-cMyc.
Lane 6: cell extract made from cells expressing Dug2pcMyc & Dug3p HA.
Fig 5.14 CoIP analysis showing Dug1p & Dug2p interaction. 
Total cell extract of *S. cerevisiae* strain ABC710 expressing Dug1pHis8 and Dug2pMyc as well as control cell extract expressing only Dug1pHis8 or vector alone. 
Immunoprecipitated with anti-His antibody and detection with anti-cMyc antibody. 

Lane 1: cell extract made from cells transformed with vector alone. 
Lane 2: cell extract made from cells expressing Dug1pHis8 and immunoprecipitated with anti-His antibody. 
Lane 3: cell extract made from cells expressing Dug1pHis8. 
Lane 4: cell extract made from cells expressing Dug1pHis8 & Dug2pMyc and immunoprecipitated with anti-His antibody. 
Lane 5: cell extract made from cells expressing Dug1pHis8 & Dug2pMyc.
Fig 5.15 CoIP analysis showing Dug1p interaction with itself.
Total cell extract of *S. cerevisiae* ABC710 strain expressing Dug1pHis₈ and Dug1pHA and control cell extract expressing Dug1pHis₈ or Dug1pHA or vector alone. Immunoprecipitated with anti-His antibody and detection with anti-HA antibody.

Lane 1: cell extract made from cells expressing Dug1pHis₈ and immunoprecipitated with anti-His antibody.
Lane 2: cell extract made from cells expressing Dug1pHis₈.
Lane 3: cell extract made from cells transformed with vectors only.
Lane 4: cell extract made from cells expressing Dug1pHA.
Lane 5: cell extract made from cells expressing Dug1pHis₈ & Dug1pHA and immunoprecipitated with anti-His antibody.
Lane 6: cell extract made from cells expressing Dug1pHis₈ & Dug1pHA.
The result indicates that Dug1pHA co-immunoprecipitated along with Dug1pHis8 and suggests that Dug1pHis8 possibly interacts with Dug1pHA.

5.16 Double Co-IP analyses reveal that Dug1pHis8 along with Dug2pcMyc and Dug3pHA can possibly form heterotrimeric complex.

The Dug1pHis8, Dug2pcMyc and Dug3pHA, bearing plasmids were transformed into \textit{S.cerevisiae} strain ABC 710. The cell extracts were prepared and immunoprecipitations done using an Anti-His antibody. As a control, Dug1pHis8 along with either Dug2pcMyc or Dug3pHA was expressed in \textit{S.cerevisiae} strain ABC 710 and the cell extract was immunoprecipitated using Anti-His antibody. Subsequently, the immunoblot of all the samples, both total cell extract and IP samples were detected using either Anti-HA or Anti-cMyc antibody.

We could detect (~ 97.7kDa) Dug2pcMyc in the total cell extract of the sample where all the three Dug proteins were co-expressed (Fig 5.16 Lane 6) and it’s corresponding IP sample (Fig 5.16 Lane 5) as well. But we could not detect Dug2pcMyc in the total cell extract of control sample (Fig 5.16 Lane 4) and in its corresponding IP sample (Fig 5.16 Lane 3) in which Dug1pHis8 was expressed along with Dug3pHA.

We could also detect (~ 40kDa) Dug3pHA in the total cell extract of the sample where all three Dug proteins were co-expressed (Fig 5.16 Lane 11) and in it’s corresponding IP sample (Fig 5.16 Lane 10) as well. But we could not detect Dug3pHA in the total cell extract of control sample (Fig 5.16 Lane 9) and in its corresponding IP sample (Fig 5.16 Lane 8) as well in, which Dug1pHis8 was expressed along with Dug2pcMyc.

The experiment was repeated using a sample in which, Dug1pHis8, Dug2pcMyc and Dug3pHA were co-expressed in \textit{S.cerevisiae} strain ABC 710. The cell extract was prepared and immunoprecipitation done using Anti-HA antibody. As a control, Dug3pHA along with either Dug2pcMyc or Dug1pHis8 were expressed in \textit{S.cerevisiae} strain ABC 710 and the cell extract was immunoprecipitated by using Anti-His antibody. Subsequently immunoblot of all the samples, both the total cell extract and IP samples were detected by using either Anti-His or Anti-cMyc antibody.
Fig 5.16 CoIP analysis showing Dug1p, Dug2p and Dug3p forming ternary complex.

Total cell extract of S. cerevisiae ABC710 strain expressing Dug1pHis$_8$, Dug2pcMyc and Dug3pHA and control cell extract expressing Dug1pHis$_8$ & Dug3pHA or Dug2pcMyc or Dug1pHis$_8$ & Dug2pcMyc or Dug3pHA or vectors only. Immunoprecipitated with anti-His antibody and detection with either anti-cMyc antibody (Lane 1-6) or anti-HA antibody (Lane 7-11).

Lane 1: cell extract made from cells transformed with vectors only.
Lane 2: cell extract made from cells expressing Dug2pcMyc.
Lane 3: cell extract made from cells expressing Dug1pHis$_8$ & Dug3pHA and immunoprecipitated with anti-His antibody.
Lane 4: cell extract made from cells expressing Dug1pHis$_8$ & Dug3pHA.
Lane 5: cell extract made from cells expressing Dug1pHis$_8$, Dug3pHA & Dug2pcMyc and immunoprecipitated with anti-His antibody.
Lane 6: cell extract made from cells expressing Dug1pHis$_8$, Dug3pHA & Dug2pcMyc.
Lane 7: cell extract made from cells expressing Dug3pHA.
Lane 8: cell extract made from cells expressing Dug1pHis$_8$ & Dug2pcMyc and immunoprecipitated with anti-His antibody.
Lane 9: cell extract made from cells expressing Dug1pHis$_8$ & Dug2pcMyc.
Lane 10: cell extract made from cells expressing Dug1pHis$_8$, Dug2pcMyc & Dug3pHA and immunoprecipitated with anti-His antibody.
Lane 11: cell extract made from cells expressing Dug1pHis$_8$, Dug2pcMyc & Dug3pHA.
We could detect (~ 97.7kDa) Dug2pcMyc in the total cell extract of the sample, where all the three proteins were co-expressed (Fig 5.17 Lane 6) and in its corresponding IP sample (Fig 5.17 Lane 5) as well. But we could not detect Dug2pcMyc in the total cell extract of control sample (Fig 5.17 Lane 4) and in its corresponding IP sample (Fig 5.17 Lane 3) in which Dug3pHA was expressed along with Dug1pHis8.

We could detect (~ 54kDa) Dug1pHis8p in total cell extract of the sample where the three proteins were co-expressed (Fig 5.17 Lane 12) and in its corresponding IP sample (Fig 5.16 Lane 11) as well. But we could not detect Dug1pHis8 in the total cell extract of control sample (Fig 5.17 Lane 10) and in its corresponding IP sample (Fig 5.17 Lane 9) as well, in which Dug3pHA along with Dug2pcMyc was expressed.

The result of the first Co-IP shows that Dug3pHA and Dug2pcMyc co-immunoprecipitated along with Dug1pHis8. The result of the second Co-IP shows that Dug1pHis8 and Dug2pcMyc co-immunoprecipitated along with Dug3pHA. This suggests that Dug1pHis8, Dug2pcMyc and Dug3pHA possibly interact with each other to form a hetero-trimeric complex.

5.17 Conclusion:

In the present chapter, we described the results of our efforts to obtain further insights into the regulation of expression of the DUG genes and formation of complex by the Dug proteins.

The promoter-LacZ fusion assays revealed that the expressions of DUG1 and DUG2 genes were not regulated by either sulphur or nitrogen sources in contrast to DUG3, which showed strong de-repression of expression under sulphur starvation. Glutathione, as a sole sulphur source was found to repress DUG3 expression mildly whereas cysteine and methionine were strong repressors. This pattern is typical of the sulphur regulatory circuit where methionine and cysteine were strong repressors. The expression of DUG3 gene was also found to be up-regulated in the presence of glutamate (non-repressible nitrogen source) compared to ammonium sulphite. This suggests that DUG3 gene expression might be regulated by the nitrogen regulatory circuit as well. Phylogenetic foot-printing of DUG3 promoter revealed the presence of a known sulphur cis-regulatory motif 5'-AAACTGTG-3' at -174 to -165 bp position up-stream of the
Fig 5.17 CoIP analysis showing Dug1p, Dug2 and Dug3p forming ternary complex.

Total cell extract made from *S. cerevisiae* ABC710 strain Dug1pHis$_8$, Dug2pcMyc and Dug3pHA and control cell extract expressing Dug1pHis$_8$ & Dug3pHA or Dug2pcMyc or Dug3pHA & Dug2pcMyc or Dug1pHis$_8$ or vectors only. Immunoprecipitated with anti-HA antibody and detection with either anti-cMyc antibody (Lanes 1-6) or anti-His antibody (Lanes 7-12).

Lane 1: cell extract made from cells transformed with vectors only.
Lane 2: cell extract made from cells expressing Dug2pcMyc.
Lane 3: cell extract made from cells expressing Dug1pHis$_8$ & Dug3pHA and immunoprecipitated with anti-HA antibody.
Lane 4: cell extract made from cells expressing Dug1pHis$_8$ & Dug3pHA.
Lane 5: cell extract made from cells expressing Dug1pHis$_8$, Dug2pcMyc & Dug3pHA and immunoprecipitated with anti-HA antibody.
Lane 6: cell extract made from cells expressing Dug1pHis$_8$, Dug2pcMyc & Dug3pHA.
Lane 7: cell extract made from cells transformed with vectors only.
Lane 8: cell extract made from cells expressing Dug1pHis$_8$.
Lane 9: cell extract made from cells expressing Dug2pcMyc & Dug3pHA and immunoprecipitated with anti-His antibody.
Lane 10: cell extract made from cells expressing Dug2pcMyc & Dug3pHA.
Lane 11: cell extract made from cells expressing Dug1pHis$_8$, Dug2pcMyc & Dug3pHA and immunoprecipitated with anti-His antibody.
Lane 12: cell extract made from cells expressing Dug1pHis$_8$, Dug2pcMyc & Dug3pHA.
ATG of the *S. cerevisiae* DUG3 ORF that is conserved in the promoters of orthologous proteins of other *Saccharomyces* spp. and indicates it to be functionally significant in the regulation of DUG3.

The results of the exhaustive yeast two hybrid analyses, as summarized in Table 5.1, suggest that Dug1p, Dug2p and Dug3p form a heterotrimeric complex where Dug1p and Dug2p, and Dug2p and Dug3p interact. The results of the yeast two hybrid studies were also substantiated with immunoprecipitation experiment in which the different proteins were tagged with epitope, co-transformed into yeast and subjected to immunoprecipitation and co-immunoprecipitation studies. Dug1p was also found to be able to interact with itself. As this peptidase was found to be sufficient for degradation of normal di- and tri-peptides, it possibly forms a homodimer like many other metallopeptidases belonging to the M20A sub-family (Rowsell *et al.*, 1997; Jozic *et al.*, 2002). Our results also suggest that the WD40 domain of Dug2p may be important for the interaction with Dug3p.
Table 5.1: Summary: Yeast Two Hybrid Results

<table>
<thead>
<tr>
<th></th>
<th>Growth on -LEU</th>
<th>COLOR on X-Gal</th>
<th>Auto-activation (Bait-fusion)</th>
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<tbody>
<tr>
<td>Bait- <em>DUG1</em></td>
<td>+++++</td>
<td>+++++</td>
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<tr>
<td>Prey- <em>DUG1</em></td>
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<td></td>
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<tr>
<td>Bait- <em>DUG1</em></td>
<td>+++++</td>
<td>+++++</td>
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<tr>
<td>Prey- <em>DUG2</em></td>
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<td></td>
<td></td>
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<tr>
<td>Bait- <em>DUG2</em></td>
<td>+++++</td>
<td>+++++</td>
<td>+++</td>
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<tr>
<td>Prey- <em>DUG1</em></td>
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<td></td>
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<tr>
<td>Bait- <em>DUG3</em></td>
<td>+++++</td>
<td>+++++</td>
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<tr>
<td>Prey- <em>DUG2</em></td>
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<td></td>
<td></td>
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<tr>
<td>Bait- <em>DUG2</em></td>
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<td>+++++</td>
<td>+++</td>
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<tr>
<td>Prey- <em>DUG3</em></td>
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<td>Bait- <em>DUG1</em></td>
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<tr>
<td>Bait- <em>DUG1</em></td>
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</tr>
<tr>
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<tr>
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<tr>
<td>Prey- <em>DUG2Pep</em></td>
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<td>Bait- <em>DUG3</em></td>
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<td>+++</td>
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</tr>
<tr>
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<td>Bait- <em>DUG3</em></td>
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<tr>
<td>Prey- <em>DUG2Pep</em></td>
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