**Aspergillus nidulans** Pmts form heterodimers in all pairwise combinations

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Eukaryotic protein O-mannosyltransferases (Pmts) are divided into three subfamilies (Pmt1, Pmt2, and Pmt4) and activity of Pmts in yeasts and animals requires assembly into complexes. In *Saccharomyces cerevisiae*, Pmt1 and Pmt2 form a heteromeric complex and Pmt4 forms a homomeric complex. The filamentous fungus *Aspergillus nidulans* has three Pmts: PmtA (subfamily 2), PmtB (subfamily 1), and PmtC (subfamily 4). In this study we show that *A. nidulans* Pmts form heteromeric complexes in all possible pairwise combinations and that PmtC forms homomeric complexes. We also show that MsbA, an ortholog of a Pmt4-modified protein, is not modified by PmtC.

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1. Introduction

Protein O-mannosylation is a type of protein glycosylation found in prokaryotes and eukaryotes [1–3]. In eukaryotes, protein O-mannosyltransferases (Pmts) are integral membrane proteins localized in the endoplasmic reticulum (ER) [4,5]. Pmts transfer a mannose residue from dolichyl phosphate mannose to the hydroxyl residue of serine or threonine in secreted proteins facing the luminal side of the ER [6,7]. Further elongation of O-mannosyl glycans takes place in the Golgi using a different set of enzymes and GDP-sugar [1]. O-mannosylation is important for stability, localization and function of secreted proteins [8–10] and a total lack of O-mannosylation is lethal in eukaryotes. Pmts in eukaryotes are grouped into 3 subfamilies with names based on the *Saccharomyces cerevisiae* enzymes Pmt1, Pmt2 and Pmt4 [11]. *S. cerevisiae* has 7 Pmts; *Candida albicans* has 5 [12]; and *Aspergillus nidulans* and other filamentous fungi have 3, one from each subfamily [2,13].

In *S. cerevisiae* Ser/Thr-rich domains of secreted proteins are likely to be mannosylated and membrane-associated proteins are mannosylated by Pmt4 [14,15]. But beyond these generalizations, the consensus sequence directing O-mannosylation is not known [3]. Nonetheless, some targets of Pmts have been found empirically and these exhibit specificity toward individual target proteins [8,16]. However, there is evidence of limited substrate overlap. For example, the *S. cerevisiae* Pmt4 and Pmt1/Pmt2 complex mannosylate different domains of Ccw5p [17].

O-mannosyltransferase activity requires a Pmt complex [18–20]. In *S. cerevisiae* and *S. pombe*, members of the Pmt1 subfamily form heteromeric complexes with members of the Pmt2 subfamily [13,21]. The Pmt4 subfamily of *S. cerevisiae* forms a homomeric complex [13].

In previous work, both our group and another group independently showed that the filamentous fungus *A. nidulans* has three pmts each representing a different subfamily: PmtA from subfamily 2, PmtB from subfamily 1, and PmtC from subfamily 4. Both groups also showed that ApmtA, ApmtB, ApmtC and the double ApmtA ApmtB were viable and that each null mutant had a distinctive phenotype [22,23]. These results strongly suggested that either PmtA and Pmt B do not form complexes in *A. nidulans* as the orthologous Pmt2 and Pmt1 do in *S. cerevisiae*, or that such subfamily 1/subfamily 2 complexes are not required for viability in *A. nidulans* as they are in *S. cerevisiae*. In this study we tested the three *A. nidulans* Pmts for the ability to form heteromeric and homomeric complexes. We also examined modifications of the *A. nidulans* ortholog of *S. cerevisiae* Msb2, a HOG pathway osmosensor modified by Pmt4 [24,25].
2. Materials and methods

2.1. Aspergillus strains and media

The A. nidulans strains used in this study (Table 1) were incubated in complete and minimal media (CM and MM) with supplements as previously described [22]. Genetic manipulations were carried out using standard A. nidulans protocols as previously described [22].

2.2. Construction of tagged Pmts

Strains bearing single copy epitope tagged Pmts were constructed by fusion PCR [26] using primers listed in Table 2. Primer names in Table 2 indicate tag identity and whether primers are upstream or downstream of the designated pmt gene. Amplicons were purified and transformed into ATK45. Homologous integration resulting in strains bearing a single tagged Pmt replacing the original Pmt was verified by PCR and Southern. All strains constructed along with detailed genotypes are shown in Table 1. All primer sequences are shown in Table 2. Primer names in Table 2 indicate tag identity and whether primers are upstream or downstream of the designated pmt gene.

2.4. Membrane fraction preparation

1 × 10⁸ conidia/ml of the specified tagged-PMT strain were inoculated to CM (50 mL for target protein extraction and 1 L for immunoprecipitation) and shaken at 200 rpm and 30°C for 8 h. Mycelia were filtered, washed with cold stop buffer (0.9% NaCl, 1 mM Na₂HPO₄, 10 mM EDTA, 50 mM NaF, pH 7.0), and ground in liquid nitrogen. Two milliliters of cold extraction buffer (50 mM Tris–HCl, pH 7.5, 0.3 mM MgCl₂ plus protease Inhibitors (Complete, Mini, EDTA-free; Protease Inhibitor Cocktail Tablets, Roche) were added to 1 g ground mycelia and vortexed for 10 min at 4°C. The cell suspension was centrifuged at 500 × g for 10 min at 4°C. The supernatant was collected and centrifuged for 30 min at 20,000 rpm at 4°C (Sorvall SS34 rotor). One mL buffer containing 50 mM Tris–HCl, pH 7.5, 7.5 mM MgCl₂, and 15% glycerol was added per 1 mL of pellet and stored at -80°C. Protein was quantified with DC Protein Assay Kit (Bio-Rad Laboratories, CA) using bovine serum albumin as a standard.

2.5. Immunoprecipitation

Immunoprecipitation methods were adapted from Girrbach and colleagues [7]. Twenty milligrams of membrane fraction was solubilized in 4 mL of lysis buffer (20 mM Tris–HCl, pH 7.5, 140 mM NaCl, 0.3 mM MgCl₂, 10% glycerol, 0.35% sodium deoxycholate, 0.5% Triton X-100 plus Protease Inhibitor (Complete, Mini, EDTA-free; Protease Inhibitor Cocktail Tablets, Roche). One hundred µL of agarose immobilized anti-epitope tag antibody slurry was added per 20 mg of protein. Agarose immobilized rabbit anti-S tag or

Table 1

| Strain | Genotype/phenotype | Source or reference |
|--------|--------------------|---------------------|
| A850   | argB2::trpC_B metG | FGSC                |
| ATK08  | pyrG89 argB2::trpC_B pyroA4 ΔpmtA::ApypG | [22] |
| ATK16  | pyrG89 ΔpmtB::ApypG argB2 pyroA4 | [22] |
| ATK38  | pyrG89 wA3 argB2 pyroA4 ΔpmtC::ApypG | [22] |
| ATK42  | pyrG89 wA3 argB2::trpC_B pyroA4 | This study |
| ATK45  | pyrG89 argB::trpC_B ΔpmtB::ApypG pyroA4 | [22] |
| ATK89  | pyrG89 argB::trpC_B ΔpmtB::ApypG pyroA4 pmtA::S-tag-AfpyG | This study |
| ATK95  | pyrG89 argB2::trpC_B pyroA4 ΔpmtA::ApypG | This study |
| ATK103 | ATK38:Afarg-Af-gpd(P)::ANID_01359-S-tag | This study |
| ATK104 | pyrG89 wA3 ΔpmtB::ApypG argB2 pyroA4 | [22] |
| ATK154 | pyrG89 argB::trpC_B ΔpmtB::ApypG pyroA4 pmtC::HA-tag-AfargB | This study |
| ATK165 | pyrG89 argB::trpC_B pyroA4 pmtA::S-tag-AfpyG pmtC::HA-tag-AfargB | This study |
| ATK168 | pyrG89 ΔpmtB::Afpyro argB::trpC_B pyroA4 pmtA::S-tag-AfpyG pmtC::HA-tag-AfargB | This study |
| ATK172 | A850:ΔpmtB-gpd(P)::ANID_07041(MsbA)::S-tag | This study |
| ATK177 | ATK16:Afarg-Af-gpd(P)::ANID_07041(MsbA)::S-tag | This study |
| ATK179 | ATK08:Afarg-Af-gpd(P)::ANID_07041(MsbA)::S-tag | This study |
| ATK184 | ATK38:Afarg-Af-gpd(P)::ANID_07041(MsbA)::S-tag | This study |
| ATK187 | pyrG89 pmtB::HA-tag-AfargB argB::trpC_B ΔpmtB::ApypG pyroA4 | This study |
| ATK192 | pyrG89 pmtB::HA-tag-AfargB argB::trpC_B pyroA4 pmtA::S-tag-AfpyG | This study |
| ATK193 | pyrG89 pmtB::HA-tag-AfargB argB::trpC_B pyroA4 pmtC::S-tag-AfpyG | This study |
| ATK195 | ATK149 X ATK104 | This study |
| ATK200 | pyrG89 pmtB::HA-tag-AfargB argB::trpC_B pyroA4 pmtA::ApypG pmtC::S-tag-AfpyG | This study |
| ATK208 | pyrG89 argB::trpC_B pyroA4 pmtC::S-tag-AfpyG | This study |
| ATK211 | ATK177 X ATK05 | This study |
| ATK217 | pyrG89/pyrG89 argB::trpC_B argB::trpC_B pyroA4/pyroA4 pmtA::S-tag-AfpyG pmtC::HA-tag-AfargB | This study |

Plasmids

pAfargB2 AmpR argB2 | G. S. May |
| pAO81 | G418-Tag ApypG | [26] |
| pDV2 | AmpR argB-gpd(p)-ccdB-sgfp | [28] |
| pMT-3xHA | AmpR argB-alcA(p)-ccdB-3xHA | [28] |
| pFN03 | KanR GAS-GFP ApypG | [26] |
| pTK74 | pDV2::ANID_07041-S-tag | This study |

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rabbit anti-HA antibody was used for immunoprecipitation (Immunology Consultants Laboratory, Inc., Newberg, OR). Incubation with the solubilized membrane fraction was carried out at 4°C on a rocker for 2 h followed by 5 washes at 4°C with equal volume of cold lysis buffer and one wash with 1 mL cold Tris-buffered saline. Agarose beads were resuspended in 50 μL cold Tris-buffered saline. The bound proteins were eluted with 3 × SDS loading dye by 5 min at 95°C.

2.6. Western blot analyses

20 μL per lane of epitope-tagged Pmt eluent was loaded into 2 gels to be further probed with anti-S tag or anti-HA antibody. The proteins were resolved on 4–20% gradient Tris–HEPES SDS gels to be further probed with anti-S tag or anti-HA antibody.

3. Results and discussion

To investigate Pmt complex formation in A. nidulans, we constructed the following strains with C-terminal purification tags: ATK89 (PmtAΔ), ATK187 (PmtBΔ) and ATK154 (PmtCΔ). Because we were concerned that over-expression of individual Pmts might alter interactions within complexes, we constructed these strains by replacing native Pmts with the corresponding tagged protein using homologous integration. In these strains, the tagged Pmt fusion constructs were integrated at native Pmt loci behind native

| Primer name | Sequence |
|-------------|----------|
| Pmt2FWent   | CACCATGCGTGAATATTGCGTTTG |
| Pmt2RNstop  | GTCAGCGATCCGCAACCCG |
| pm2-GA4Fw   | GAGCCGTTGCGGACATCGTAAAGCCGTCGTCGAGCCC |
| Stag-pmt2R2V| GAAGCTGATCACGCTGCTCGTCTGGTACAGGAGAGAGCAAAGATG |
| pm2DNFW     | GCGCCTCCATCGTTCTCAGTCTCAGATC |
| pm2DNRV     | CCAATGCGGTTCGTAAGCGAGTCATCCAGTCC |
| PMT2F301    | GCTTCGTGCAATCGCCCAAGAGAGAGG |
| PMT2B311    | TGGGACGTCGAGGAAAGGCGAGAG |
| up177PMT2F  | TCGGAGCGTCGCGCGGAGATGAAAG |
| StagR       | GCGGACATTGCTGCTGAGGAGAGAG |
| PMT11093F   | GTGTCGTGCAATCGCCCAAGAGAGAGG |
| Pmt1RNstop  | ACCGCGGCTCTGCTGAGT |
| Pmt1GASF    | GCAACGCGCCCGCGTGAGGAGAGAGG |
| argBAP-MPT1DNR | CAACAGACGGCGATATATTCAGGAGAGAG |
| Pmt1dn-FW   | CATATAATGCTGCTGAGGAGAGAG |
| Pmt1dnR2    | CTCGAGGGAGATGAGAGGAGAGAG |
| 463Pmtn4F   | GTCCTCCATGCGTGAG |
| Pmt4RNstop  | TTTCGACGAGGCGATCTACAG |
| Pmt4GAF4    | CTAGAACCTGACGTCCGCGAAAGAGCGCTGTCGAG |
| pmy4AFRV-Pm4SPRV | AGAACGAGACGACACAGCATCATCTCTGCTGAGGAGGACATGATG |
| Pmt4dRNv    | CTACACGCTGGCTTTCTG |
| argBpm4Rv   | AGAACGAGATCGATGACTCATCATTCGCTGAGGAGGACATGATG |
| StagR       | GCCACATGTCAGTTCTGAGGAGAG |
| MSB2entF    | CACCATGCGGCTTCGAGGAG |
| MSB2-StagR  | GCGGACATGCGGCTTCGAGGAG |
| StagF       | GGACGTTGTCGAGGAGGAG |
| StagR       | GCGGCAATGCGTGTGAGGAG |

Pmt promoters resulting in expression levels as close to wildtype as possible. Correct integrations were confirmed by PCR and Southern hybridization. All tagged Pmt strains showed wildtype phenotypes at 30°C and 42°C (data not shown). The S and HA tags are both small, with deduced molecular mass of 2.26 kDa and 3.98 kDa, respectively. The predicted molecular mass of PmtAΔ is 86.86 kDa, of PmtBΔ is 108.98 kDa, and of PmtCΔ is 92.28 kDa. Attempts to detect tagged Pmts by western blot of total membrane fractions were unsuccessful, suggesting low levels of fusion protein. Therefore, immunoprecipitation of solubilized membrane fractions with agarose-immobilized anti-HA or anti-S tag antibodies was used, followed by SDS–PAGE and immunoblot (Fig. 1). The PmtAΔ strain yielded two bands with apparent molecular mass of approximately 68 kDa and 165 kDa. The PmtBΔ strain yielded two bands with apparent molecular mass of approximately 120 kDa and 180 kDa. The PmtCΔ strain yielded two bands with apparent molecular mass of approximately 80 kDa and 160 kDa. In all cases, the lower band is likely the Pmt based on predicted molecular mass, strength of the signal and reproducibility. Pmts isolated from yeasts and animals frequently show apparent molecular masses that are smaller or larger than predicted [13,18]. Giri-bach and Strahl reported similar double band patterns in S. cerevisiae blue native PAGE experiments with the higher bands resulting from heteromeric Pmt complex formation [13]. However, our experiments included SDS so that proteins are expected to be denatured. Further these bands always appear at the same molecular mass for specific Pmts and their presence in strains carrying a Pmt deletion along with a tagged Pmt suggests that they are not heterodimers. A similar double band pattern was reported when the human PMT ortholog Pmt1 was expressed in HEK293T cells. In this case the authors speculated that the upper band resulted from protein aggregation due to Pmt1 over-expression [18]. Though the Pmts in our study were all expressed from endogenous promoters rather than over-expressed, it is possible that the higher bands seen in our immunoblots resulted from aggregation of these hydrophobic proteins during processing. It is also possible that the upper bands represent homodimers of tagged Pmts.
3.1. PmtA (subfamily 2) forms heteromeric complexes with PmtB (subfamily 1)

To investigate whether A. nidulans PmtA and PmtB form a heteromeric complex like their S. cerevisiae orthologs, Pmt2 and Pmt1 [13], we derived a PmtA S PmtB HA strain (ATK192) from sexual crosses. Immunoprecipitation was performed with agarose-immobilized anti-HA antibodies and agarose-immobilized anti-S tag antibody. The immunoprecipitate was divided into two aliquots. One aliquot was probed with anti-S tag antibody and the other was probed with anti-HA antibody. When probed with anti-S tag antibody, the same bands that were seen in the PmtA strain were visible (approximately 68 kDa and 165 kDa) (Fig. 2). When probed with anti-HA antibody, the same bands that were seen in the PmtB strain were visible (approximately 120 kDa and 180 kDa) (Fig. 2). Reciprocal experiments performed using anti-HA antibodies for immunoprecipitation before immunoblotting showed very high background, though the results were consistent with heteromeric complex formation by PmtA and PmtB. Our results show that PmtA and PmtB form heteromeric complexes in A. nidulans.

3.2. PmtA (subfamily 2) forms heteromeric complexes with PmtC (subfamily 4)

In S. cerevisiae, Pmt2 and Pmt4 do not form complexes [13]. To investigate the interactions between the A. nidulans orthologs, strains carrying PmtA S and PmtC HA in the presence of PmtB (pmtA::S tag, pmtC::HA, ATK165) and absence of PmtB (pmtA::S tag, pmtC::HA, ΔpmtB; ATK168) were derived from sexual crosses. Immunoprecipitations were performed with agarose-immobilized anti-S tag antibody as described above. When probed with anti-HA antibody, Western blots of immunoprecipitates from both strains showed a band of approximately 80 kDa whether or not PmtB was present (Fig. 3). Reciprocal experiments performed using anti-HA antibodies for immunoprecipitation before immunoblotting were consistent with heteromeric complex formation by PmtA and PmtC (Fig. S1). Our results show that A. nidulans PmtA forms a heteromeric complex with PmtC in the presence and absence of PmtB.

3.3. PmtB (subfamily 1) forms heteromeric complexes with PmtC (subfamily 4)

To investigate the interactions between PmtB and PmtC, a strain bearing PmtB HA and PmtC S in the presence of PmtA (pmtB::HA, pmtC::S tag; ATK193) and absence of PmtA (pmtB::HA, pmtC::S tag, ΔpmtA; ATK200) were derived from sexual crosses. Immunoprecipitation and Western blots were performed as described above. Immunoblots probed with anti-HA antibody showed a band of approximately 120 kDa whether or not PmtA was present (Fig. 4). Reciprocal experiments performed using anti-HA antibodies for immunoprecipitation before immunoblotting showed very high background, though the results were consistent with heteromeric complex formation by PmtB and PmtC.

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**Fig. 1.** Immunoprecipitation of Epitope-tagged Pmts. Western blot analyses of immunoprecipitates from ATK89 (PmtA S), ATK187 (PmtB HA) and ATK154 (PmtC HA). Proteins were expressed from their native loci under the control of their endogenous promoters. Solubilized membrane-enriched fractions were immunoprecipitated with the corresponding agarose immobilized antibody against the epitope tag. Immunoprecipitates were resuspended in SDS loading dye and resolved on 4–20% gradient Tris–HEPES–SDS–polyacrylamide gels, transferred to membranes, and probed with anti-S tag antibody (left) or anti-HA antibody (two blots on right). Arrows indicate Pmts.

**Fig. 2.** Immunoprecipitation of PmtA-PmtB heteromeric complexes. Solubilized membrane-enriched fractions from ATK192 (PmtA S, PmtB HA) were immunoprecipitated with agarose immobilized anti-S tag antibody. Co-immunoprecipitates were resuspended in SDS loading dye and resolved on 4–20% gradient Tris–HEPES–SDS–polyacrylamide gels, transferred to membranes, and probed with anti-S tag antibody (left) or anti-HA antibody (right). Arrows indicate Pmts.

**Fig. 3.** Immunoprecipitation of PmtA-PmtC heteromeric complexes. Solubilized membrane-enriched fractions from ATK168 (PmtA S, PmtC HA, ΔpmtB; lane 1) and ATK165 (PmtA S, PmtC::HA, ΔpmtB; lane 2) were immunoprecipitated with agarose immobilized anti-S tag antibody. Co-immunoprecipitates were resuspended in SDS loading dye and resolved on 4–20% gradient Tris–HEPES–SDS–polyacrylamide gels, transferred to membranes, and probed with anti-S tag antibody (left) or anti-HA antibody (right). Arrows indicate Pmts.
3.4. PmtC (subfamily 4) forms homomeric complexes

In *S. cerevisiae*, Pmt4 functions as a dimer, not a monomer [13]. To investigate whether the *A. nidulans* ortholog, PmtC, is a monomer or forms homomeric complexes in *A. nidulans*, a stable diploid PmtC<sup>Δ</sup> PmtC<sup>Δ</sup> strain (*pmtC::S tag/pmtC::HA; ATK217*) was constructed. Immunoprecipitation and Westerns were performed as described above and probed with anti-S tag antibody or anti-HA antibody. In both cases the same bands that were seen in the PmtC<sup>Δ</sup> strain were visible (approximately 80 kDa and 160 kDa) (Fig. 5). Reciprocal experiments performed using anti-HA antibodies for immunoprecipitation before immunoblotting were consistent with homomeric complex formation by PmtC (Fig. S1). Our results show that *A. nidulans* forms a PmtC homomeric complex as is true for the orthologous *S. cerevisiae* Pmt4 [13].

Genetic evidence from our previous work suggests that this PmtC–PmtC homomeric complex is sufficient to support growth because the ΔpmtA ΔpmtB strain is viable [22]. Despite repeated attempts, we were unable to recover appropriately marked strains to investigate PmtA–PmtA and PmtB–PmtB homomeric complex formation. However, if PmtA–PmtA and PmtB–PmtB homomeric complexes exist, neither appears to be sufficient to support *A. nidulans* growth in the absence of the other Pmts since both the ΔpmtA ΔpmtC and ΔpmtB ΔpmtC double mutants are inviable.

3.5. Pmt complexes are formed in vivo

To eliminate the possibility that the *A. nidulans* Pmt complexes we detected might result from nonspecific aggregation of Pmts in solubilized membrane fractions, we performed the following control experiment. Membrane fractions were isolated from strains in which S tagged and HA tagged Pmts were co-expressed, and solubilized. Membrane fractions were also isolated separately from strains in which S tagged Pmts were expressed, and from strains in which HA tagged Pmts were expressed. Isolated membrane fractions were combined and solubilized. Agarose-immobilized anti-S tag antibody was used to immunoprecipitate protein from solubilized membranes of the strains co-expressing S tagged and HA tagged Pmts or from the combined and solubilized membranes of the strains separately expressing tagged Pmts. Identical aliquots were probed with anti-S tag antibody or anti-HA antibody. The co-expressed Pmts co-precipitated, but the separately expressed and combined Pmts did not, suggesting that the Pmt complexes we detected in earlier experiments were formed in vivo and not the result of nonspecific protein aggregation (Fig. 6).

3.6. MsbA, an ortholog of a Pmt4-modified protein, is not modified by PmtC

In *S. cerevisiae*, Msb2, an osmosensor in the HOG pathway, is modified by Pmt4 [24,25]. Using the *S. cerevisiae* Msb2 protein sequence to query the *A. nidulans* genome database at the Broad Institute (http://www.broad.mit.edu) yielded ANID_07041.1, which we named “MsbA”. MsbA<sup>Δ</sup> was expressed from the gpd promoter in wildtype and Δpmt mutants, membrane fractions were isolated and probed with anti-S tag antibodies in Westerns (Fig. 7). The apparent molecular mass of MsbA in the ΔpmtA and ΔpmtB mutants was 160 and 170 kDa respectively, while the molecular mass of MsbA<sup>Δ</sup> in ΔpmtAΔpmtB mutant appeared lower at 150 kDa. The apparent molecular mass of MsbA in the ΔpmtC mutant was approximately 240 kDa, which was the same as that in the wildtype. MsbA possesses a putative N-glycosylation site. The molecular mass of PNGase F treated MsbA<sup>Δ</sup> in ΔpmtA, ΔpmtB and ΔpmtC was unchanged (data not shown). Our results suggest that MsbA is modified by PmtA and PmtB, but not by PmtC, and that PmtA and PmtB make separate modifications to MsbA.

In *S. cerevisiae* both Pmt subfamily 1 and Pmt subfamily 2 have two members (Fig. 8). In immunoprecipitation experiments, the predominant interaction partner for Pmt1 is Pmt2, though low levels of Pmt3 (from subfamily 2) are also reproducibly precipitated. Similarly, the predominant interaction partner for Pmt 5 (from subfamily 1) is Pmt3, though low levels of Pmt2 are also precipitated [13]. The Pmt 4 subfamily has only one member and it only forms homodimers. Thus in *S. cerevisiae* five Pmt complexes exist and low level complexes can substitute if the predominant complexes are lost.

In contrast *A. nidulans* contains only 3 Pmts, one from each subfamily (Fig. 8). Not surprisingly, we found that PmtA (subfamily 2) interacts with Pmt B (subfamily 1) and PmtC (subfamily 4) interacts with itself. More surprisingly, we also found that both PmtA and Pmt B interact with Pmt C (subfamily 4). Because we were unable to generate appropriately marked diploid strains, we do not know if PmtA and Pmt B can also form homodimers and because single copy Pmts driven by native promoters were not produced in large quantities, we were unable to address quantitative issues such as which complexes predominate. However, it is clear that in *A. nidulans* at least four Pmt complexes exist, though it is not clear which complexes predominate. *A. nidulans* is the first fungus reported to form heteromeric complexes involving the Pmt4 subfamily, though such complexes have been reported in animals where POMT1 (subfamily 4) forms a heteromeric complex with Pmt4.
It is possible that the novel heterodimers we observed with Pmt C might be low level forms that can substitute when other complexes are lost allowing *A. nidulans* to survive with its reduced complement of single Pmts from each subfamily [22].

### 4. Conclusions

In this study we show that the three *A. nidulans* Pmts (PmtA from subfamily 2, PmtB from subfamily 1, and PmtC from subfamily 4) form heteromeric complexes in all pairwise combinations and that PmtC forms a homomeric complex. We also show that MsbA, the *A. nidulans* ortholog of *S. cerevisiae* Msb2, is modified by PmtA and PmtB, but not by PmtC.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2014.03.006.

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