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To finish things well: cysteine methylation ensures selective GTPase membrane localization and signalling.

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Abstract

Isoprenylcysteine-O-Carboxyl Methyltransferase (ICMT) catalyzes the final step in the prenylation process of different proteins including members of the Ras superfamily of GTPases. While cysteine methylation is essential in mammalian cells for growth, membrane association, and signaling by Ras and Rho GTPases, its role during signal transduction events in simple eukaryotes like yeasts appears irrelevant. By using a multidisciplinary approach our group has recently shown that, contrary to this initial assumption, in the fission yeast *Schizosaccharomyces pombe* ICMT activity encoded by the Mam4 gene is not only important to promote selective plasma membrane targeting of Ras and specific Rho GTPases, but also to allow precise downstream signalling to the Mitogen Activated Protein Kinase (MAPK) and Target of Rapamycin (TOR) pathways in response to diverse environmental cues. Thus, the dynamic regulation of *in vivo* methylation as a modulator of GTPase localization and function is an evolutionary conserved mechanism, making fission yeast an appealing model organism to study the regulation of this process.

Keywords

Cysteine methylation; ICMT; GTPase; Membrane targeting; Signalling.

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Ras and Rho GTPases, which bind guanine nucleotides (GDP or GTP) and harbour intrinsic GTPase activity to hydrolyze the bound GTP, are molecular switches that play pivotal roles in eukaryotic organisms to regulate multiple cellular functions including proliferation, cytoskeletal dynamics and polarity (Ahearn et al. 2012, Boulter et al. 2012). Given the large number of functionally relevant Ras and Rho GTPase family members present in eukaryotic cells, their activity must be strictly regulated in time and space to efficiently accomplish their biological roles. Guanine Nucleotide Exchange Factors (GEFs) promote dissociation and substitution of GDP by GTP and control timely GTPase activation. In turn, GTPase downregulation is executed by GTPase Activating Proteins (GAPs) that enhance hydrolysis of GTP to GDP, and by GDP Dissociation Inhibitors (GDIs), which sequester GTPase to the cytosol in an inactive state (Tcherkezian and Lamarche-Vane 2007). Another major regulatory layer for spatial control of Ras and Rho GTPases activity relies upon proper targeting to cellular membranes to constitute platforms for localized downstream signalling (Henis et al. 2009, Ahearn et al. 2012). This goal is mainly achieved through prenylation (i.e. modification by isoprenoid lipids), which involves the constitutive and covalent linkage of either farnesyl or geranylgeranyl lipids to a cysteine residue located at a conserved GTPase C-terminal tetrapeptide motif named the CaaX box (Wang and Casey 2016). Next, the –aaX tripeptide is proteolytically cleavaged removed from the prenylated -CaaX box at the endoplasmic reticulum by mediated by a conserved RAS-converting CaaX endopeptidase 1 (RCE1). The prenylation process finishes when the free carboxyl group of the isoprenylated cysteine becomes methylated by an evolutionary conserved specific isoprenylcysteine-O-carboxyl methyltransferase (ICMT) also resident at the ER (Wang and Casey 2016) (Fig. 1a).

ICMT activity is essential in metazoans for cell growth and development, and ICMT deficient mice are embryonic lethal (Bergo et al. 2001). Cysteine methylation is critical for proper localization and membrane association of Ras, and either pharmacological or conditional inhibition of ICMT elicit Ras mislocalization and defective downstream signalling and activation of kinases such as Akt an ERK (Winter-Vann et al. 2003). In addition to Ras, ICMT function also regulates membrane tethering, stability, and activity of Rho GTPases (RhoA, Rac1) during the control of the integrity of the actin cytoskeleton and migration (Wang and Casey 2016). Indeed, the last decade has seen the development of several inhibitors of ICMT activity that constitute a potential new class of anti-cancer drugs to target Ras-related cancers (Yang et al. 2017).

Similar to other signaling cascades (Ho and Gasch 2015), many Ras/Rho GTPases and the prenylation machinery are strongly conserved in the eukaryotic lineage including *Ascomycota* and the genetically amenable model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, where ICMT activity is encoded by the *ste14*$^{+}$ and *mam4*$^{+}$ genes, respectively

(Michaelis and Barrowman 2012). While *ste14Δ* and *mam4Δ* mutants are viable, strains of the a (*S. cerevisiae*) and h^- (*S. pombe*) mating types display a sterile phenotype due to abrogated cysteine carboxylmethylation and maturation of the corresponding farnesylated **a-** and M-factors (Michaelis and Barrowman 2012). Intriguingly, *ste14Δ* and *mam4Δ* cells growth normally and do not show obvious phenotypes. This may suggest that in lower eukaryotes cysteine methylation has no functional impact and plays a dispensable role in GTPase localization and signalling. Another possibility is that GTPase methylation might still take place in these organisms through an alternative ICMT-independent mechanism.

In our recent work we sought to answer these questions (Franco et al. 2017). We focussed our study in the role of fission yeast Mam4 on membrane localization and function of the single Ras ortholog Ras1, and Rho GTPases Rho1 (RhoA ortholog), Rho2 (RhoB ortholog) and Cdc42. They were selected given their role as major regulators of morphogenesis, polarity, and sexual differentiation in *S. pombe* (Rho1, Cdc42, and Ras1), and as the core upstream activators of the cell integrity MAPK pathway (Rho2 and Rho1; Perez and Cansado 2010). By employing isoelectric focusing (IEF) coupled to Western blotting we found that the assayed GTPases are present in wild type cells as a ~1:1 ratio of methylated versus unmethylated isoforms, and that methylated GTPases were absent in *mam4Δ* cells. These results were consistent with earlier work showing that the *mam4Δ* mutant lacks detectable methyltransferase activity (Imai et al. 1997). Although the number of prenylated targets assayed is limited (4 out of a total of 35 in fission yeast proteome showing *in vivo* prenylatable motifs) our findings strongly suggest that, like in mammalian cells, Mam4 encodes the only ICMT present in fission yeast.

Quantitative analysis of fluorescence microscopy images from control versus *mam4Δ* cells expressing GFP-tagged GTPases revealed the existence of a differential effect of ICMT activity on GTPase membrane targeting. Indeed, whereas localization of Rho1 and Cdc42 was not significantly altered in Mam4-less cells as compared to control cells, plasma membrane localization of Rho2 and Ras1 was consistently reduced from ~30% (Rho2) to ~50% (Ras1) in the *mam4Δ* mutant (Franco et al. 2017) (Fig. 1b). Which is/are the reason/s for these differences? Besides the prenylated cysteine, both Ras1 and Rho2 harbor an additional cysteine residue upstream the –CaaX box that is also palmitoylated in vivo. Palmitoylation is a dynamic and reversible process that enhances and stabilizes the membrane association of some prenylated Ras and Rho GTPases, allowing for compartmentalization of GTPase targeting and signalling (Salaun et al. 2010) (Fig. 1a). However, defective palmitoylation is not behind the reduced plasma membrane tethering in absence of ICMT activity, as Ras1 and Rho2 palmitoylation levels remained unchanged in control and *mam4Δ* cells. The possibility that ICMT activity favours proper membrane localization of farnesylated-, but not

geranylgeranylated- GTPases in mammalian cells has found different support (Roberts et al. 2008). Our results suggest that this is likely the case, as plasma membrane tethering of a geranylgeranylated Rho2 chimera was reduced minimally in *mam4Δ* cells as compared to the wild type farnesylated GTPase. Therefore, increased hydrophobicity provided by 4 (geranylgeranyl) versus 3 (farnesyl) isoprenyl units attenuates the need for cysteine methylation to optimize GTPase membrane localization (Fig. 1c). Importantly, the existence of a polybasic motif next to the CaaX box that is present in Rho1 and Cdc42 and absent in Ras1 and Rho2 is a major structural constraint for the differential requirement of ICMT activity to elicit proper GTPase plasma membrane tethering. Indeed, in contrast to the wild type GTPase, plasma membrane localization of a Rho2 chimera including the Rho1 polybasic motif was not reduced in *mam4Δ* cells. Conversely, plasma membrane localization of a Rho1 chimera fused to the palmitoylated and farnesylated CaaX box of Rho2 was significantly reduced in absence of ICMT activity (Franco et al. 2017).

Therefore, neutralization of the negatively charged prenylated cysteines by methylation is particularly relevant to increase hydrophobicity and efficient interaction with acidic plasma membrane lipids of palmitoylated and farnesylated GTPases lacking polybasic residues surrounding the CaaX box (Fig. 1c). If this hypothesis is correct, then downstream signalling functions within this specific group should be compromised in absence of ICMT function. In agreement with this prediction, induced activation of the pheromone signalling MAP kinase Spk1 and conjugation, which are specifically regulated by palmitoylated and plasma membrane bound Ras1 (Onken et al. 2006), were partially abrogated in *mam4Δ* cells in response to nitrogen deprivation. Similarly, activation of the cell integrity MAP kinase Pmk1 in response to stress, which mostly relies on palmitoylated and plasma membrane bound Rho2 (Sanchez-Mir et al. 2014), was also reduced in the *mam4Δ* mutant. Conversely, the negative effect of Mam4 deletion on signalling by GTPases containing polybasic motifs near the CaaX box was either undetectable (Cdc42), or very moderate (Rho1) (Franco et al. 2017). The biological relevance of this control was further confirmed as the recently described Pmk1 cross-inhibition of TORC2 (Madrid et al. 2016), one of the two TOR complexes present in fission yeast, was partially relieved in *mam4Δ* cells. Mammalian ICMT also methylates the -CxC class of mammalian isoprenylated Rab proteins (Bergo et al. 2001), but this does not appear to be the case in *S. pombe*. The number of GTPase substrates whose biologically activity is subjected to ICMT control might be limited to a specific subset of proteins in fission yeast as compared to higher eukaryots. Besides, only 4 out of 25 fission yeast CaaX proteins are essential for cell growth, thus providing a reasonable explanation to the observation that, in contrast to the mammalian counterparts, ICMT activity is dispensable for yeast growth.

An important feature of GTPase methylation is its dynamic and reversible nature, which

might allow for fine tuning of membrane targeting and output signal (Fig. 1a). This regulatory mechanism has been tightly conserved during evolution, as evidenced by our observation that, like in mammalian cells, fission yeast GTPases naturally coexist as both methylated and unmethylated isoforms. Mammalian carboxylesterase CES1 has been shown to negatively control RhoA methylation, and upregulated RhoA activity in response to CES1 silencing induced important morphological and cytoskeletal changes in breast cells that were phenocopied by ICMT overexpression. However, other Rho GTPases like Cdc42 are not subjected to negative control by CES1, suggesting the existence of additional non-redundant carboxylesterases that perform this role (Cushman et al. 2013). In fission yeast Mam4 overexpression is lethal, but this is likely due to interference with normal endoplasmic reticulum organization and/or function, since the toxic phenotype is also observed in cells overexpressing a catalytically-dead version of the enzyme (Franco et al. 2017). This issue should be considered when employing multicopy ICMT constructs to analyze cellular phenotypes in other model organisms. The identification and functional characterization of novel negative regulators of GTPase methylation, together with the study of the biological significance of GTPase methylation dynamics and signalling under different physiological contexts (growth, differentiation, response to stress), remain important questions in the field. In this context, we have recently identified several candidates in the fission yeast proteome that might negatively regulate either Ras and/or Rho methylation (unpublished results). Yeasts are important models for the study of fundamental cellular processes due to the significant functional homology of their intracellular transduction pathways with those of higher cells (Ho and Gash 2015; Sarto-Jackson and Tomaska 2016). A simple eukaryote like fission yeast rises again as complementary and reliable model organism to elucidate mechanisms that regulate essential cellular functions of general significance.

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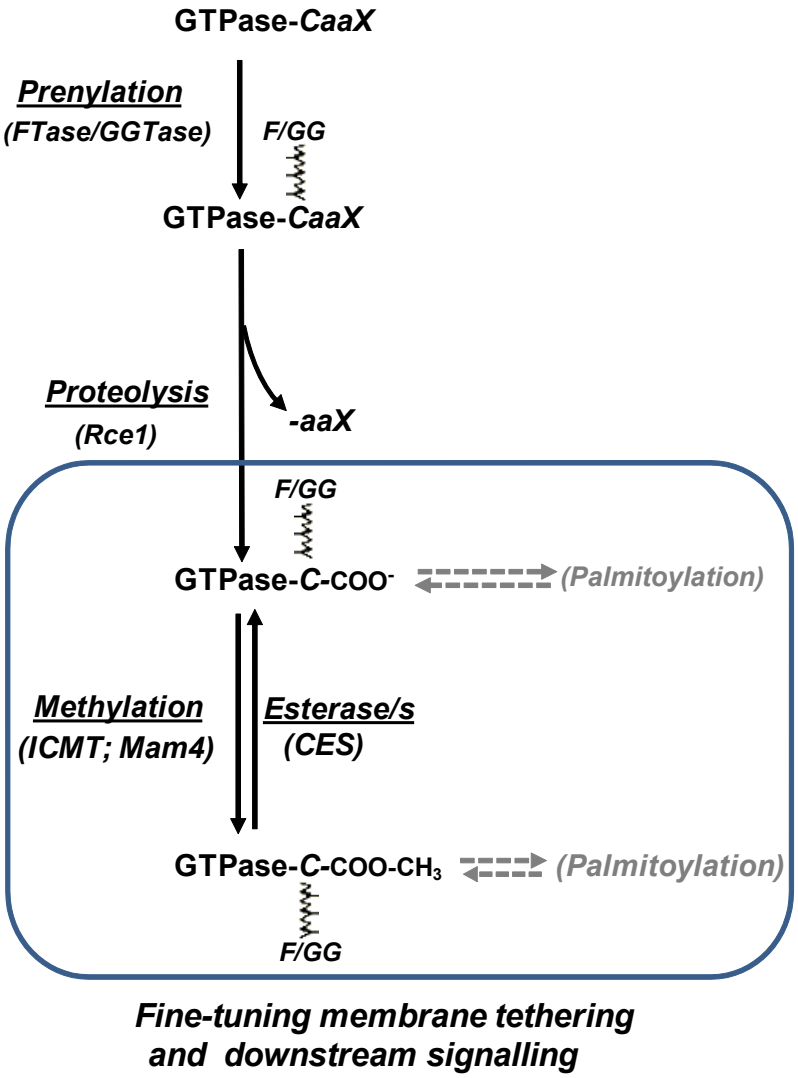
Figure Legends

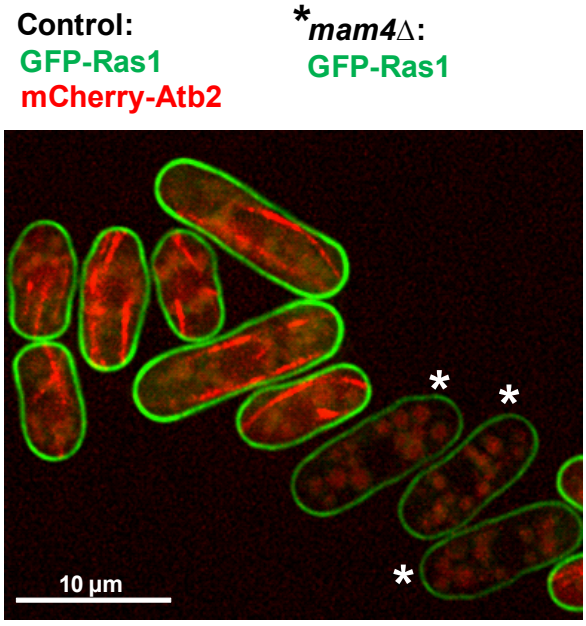
Fig. 1 a Main stages in the prenylation process of -CaaX proteins within the Ras family of GTPases. For specific details please see text. F: farnesyl; GG: geranylgeranyl. **b** Deconvolved images of mixed control (GFP-Ras1, mCherry-Atb2 (alpha-tubulin)) and *mam4*Δ (GFP-Ras1) fission yeast cells were observed by fluorescence microscopy. Asterisks indicate *mam4*Δ cells with reduced GFP-Ras1 plasma membrane tethering as compared to control cells. **c** The dependence on Mam4 function for membrane targeting is minimal in geranylgeranylated GTPases harbouring polybasic motifs and maximal in those which are farnesylated and palmitoylated. P: palmitoylation.

A



Fine-tuning membrane tethering and downstream signalling

B



C

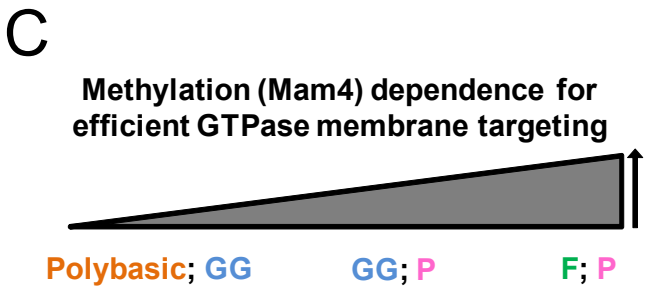


FIGURE 1