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Review

Agp2: A master regulator of polyamine uptake that signals gene expression

Genetics

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Abstract

Introduction

Polyamines are essential for diverse biological functions in prokaryotic and eukaryotic cells. Both endogenous synthesis and uptake by means of active transport mechanisms provide the pool of intracellular polyamines. Cancer cells rely on polyamine levels to proliferate, thus interrupting key aspects of polyamine metabolism may serve to impede tumour growth. This overview focuses on the latest findings that relate to the polyamine uptake pathway in the yeast model system, and which may help to refine the development of novel therapeutics to retard cancer growth.

Conclusion

We believe that there are inherent complexities within the polyamine uptake pathway and that a detail understanding of this process would lead to the precise targets.

Introduction

Our interest in polyamines can be considered fortuitous as it originates from a historical effort to understand how tumour cells provide resistance to the anticancer drug bleomycin, a polyamine analogue. Polyamines are positively charged molecules derived from the decarboxylation of ornithine to form the primary polyamine putrescine, which is the precursor for spermidine and spermine synthesis[1]. They form electrostatic bonds with negatively charged macromolecules and control a number of biological processes such as genomic stability, DNA replication, transcription, translation, ribosome biogenesis, modulation of ion channels and receptors, and protein phosphorylation[2–5]. At low concentrations, polyamines cause cell cycle arrest and become cytotoxic at elevated levels[6–8]. Therefore, cells must tightly regulate the intracellular pool of polyamines and this implies exerting controls at multiple levels including (i) *de novo* synthesis, (ii) detoxification via efflux pumps and (iii) uptake via transmembrane transporters[9,10]. A number of rapidly dividing cells have elevated levels of polyamines and which might be required for the development of specific cancers[9–11]. Thus, depleting the pool of polyamines has been proposed to be an attractive therapeutic approach to control cancer growth. Targeting one of the rate limiting enzymes, ornitine decarboxylase, in the polyamine biosynthetic pathway with a specific suicide inhibitor, α-difluoromethylornithine, has been employed as a chemotherapeutic agent, but with limited success. This failure can be attributed to compensatory mechanisms that maintain polyamine levels such as increase uptake from extracellular sources[11]. As a result, efforts have been directed to block the polyamine uptake pathway as an alternative strategy to limit the pool of polyamines and this involves using inhibitors that antagonise polyamine uptake such as D-lysine spermine and N1-spermyl-L-lysinamide[12]. The polyamine uptake pathway has provided additional possibilities to target cancer cells, that is, by facilitating the entry of polyamine conjugated cytotoxic and genotoxic drugs, such as naphthalimide, anthraquinone and bleomycin[13,14]. Thus, a combine approach that uses inhibitors to deplete the endogenous pool of polyamine and polyamine-conjugated anticancer drugs that damage the DNA should greatly improve the treatment of cancer cells. The aim of this review was to discuss Agp2 and its role as a regulator of polyamine uptake that signals gene expression.

Discussion

The author has referenced some of its own studies in this review. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed.

Bleomycin: a polyamine analogue

Nearly a decade ago, we set out to investigate the underlying mechanisms that would cause tumour cells to provide resistance to bleomycin. Bleomycin is used in combination therapy to treat a limited set of cancers[15]. It is most effective against testicular carcinomas, but has no chemotherapeutic benefits towards colon or ovarian cancers[14]. The distinct response by different cancer types towards chemotherapy with bleomycin, suggests that there must be a unique mechanism allowing some tumours, but not others, to respond to the drug. While there is extensive knowledge on the structure of bleomycin and its targets, there is still much more to glean from its mechanism of entry into cells. Bleomycin is a hydrophilic drug that possesses three active regions including a metal and a DNA binding domain (Figure 1)[15]. The latter domain has the chemical composition of polyamines and allows the drug to inter-calate with DNA[15]. Hence, bleomycin is considered a polyamine analogue. In the presence

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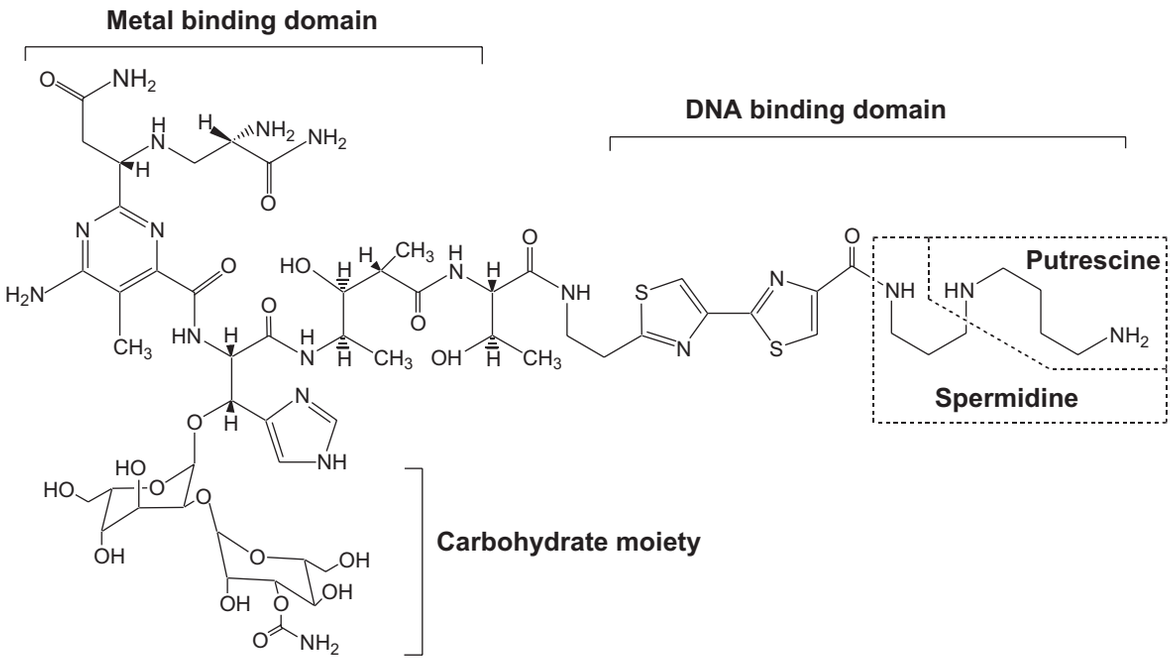

Figure 1: Chemical structure of the polyamine analogue, bleomycin. The three regions are illustrated and the polyamine moiety is required to intercalate with DNA.

of reduced Fe^{2+}, bleomycin undergoes a free radical reaction and becomes an oxidant, which then attacks many macromolecules including DNA and RNA[15]. In the case of DNA, activated bleomycin can produce at least four types of DNA lesions that include both double and single strand breaks, that are terminated with blocked 3′-ends that prevent DNA repair synthesis[15]. Based on the mechanism of action of bleomycin on the DNA, it seems logical at the time to propose that if tumour cells can repair these DNA lesions rapidly then such tumour cells should become resistant to the genotoxic effects of the drug. To test this hypothesis, the yeast *Saccharomyces cerevisiae* has been used as a model system, mainly because of its multitude of facile approaches that can be easily adapted into the laboratory, in order to search for enzymes that will repair bleomycin-induced DNA lesions. As it turns out, two enzymes Apn1 and Apn2 have been identified and biochemically characterised[16]. Both Apn1 and Apn2 can process bleomycin-induced DNA lesions, in particular, removing the blocked group at strand breaks to create a 3′-hydroxyl group for DNA repair synthesis[15]. Deletion of both *APN1* and *APN2* genes resulted in yeast mutants that display hypersensitivity to bleomycin. Although both Apn1 and Apn2 can compete to repair bleomycin-induced DNA lesions, the overproduction of either enzyme did not provide additional resistance to the parent strain. This observation excludes the hypothesis that enhanced DNA repair can account for resistance to the polyamine analogue bleomycin. This tentative conclusion prompted a more in depth search for alternative mechanisms to explain how cells can become resistant to bleomycin. One prediction is that cells defective in the uptake of bleomycin should be resistant to the drug. Likewise, cells with enhanced rate for the efflux of bleomycin might be similarly resistant to the drug. While the latter mechanism has been documented as a mode of resistance towards several anticancer drugs by cancer cells, there is no concrete proof that elevated levels of drug efflux pumps play a role in the extrusion of bleomycin[15]. It is noteworthy that besides the uptake and efflux of bleomycin as possible mechanisms that could lead to resistance, there are other predictions that include sequestration and detoxification of the drug in the vacuoles[17]. It would have been a daunting task to asset each of these processes for a contribution to bleomycin resistance, but this has been greatly simplified with the advent of the yeast haploid mutant collection. This collection consists of ~4,000 mutants each deleted for a single non-essential gene and thus provided a highly resourceful approach to search for those mutants displaying resistance to bleomycin[18]. This has been facilitated by a robotic screen that arrayed each mutant onto solid growth media containing a lethal dose of bleomycin followed by visual examination for those mutants that grew in the presence of the drug[18]. From the nearly 4,000 mutants that have been tested, only 5 showed remarkable resistant to bleomycin and each is deleted for one of the following genes *AGP2*, *SKY1*, *PTK2*, *FES1* or *BRP1*. Below, provides a brief description on the current knowledge of these gene products.

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The five yeast proteins that control bleomycin and polyamine uptake

Agp2 was previously shown to be a plasma membrane transporter, which belongs to the general amino acid family of permeases. It was first characterised as a high affinity transporter of L-carnitine, which serves as a carrier of Acetyl-CoA from the peroxisomes to the mitochondria for further energy production[19]. Both Ptk2 and Sky1 are kinases also shown previously to regulate the high affinity plasma membrane polyamine transporter[18]. Ptk2 positively regulates the major plasma membrane H^+/ATPase, Pma1, which pumps H^+ ions out of the cell and creates an electrochemical proton gradient that drives the import of nutrients across the plasma membrane[18]. The role of Sky1 kinase in the polyamine transport pathway is less clear, and the functions of Fes1 and Brp1 are not known. It should be noted that any further studies involving Fes1 with respect to bleomycin resistance would be complicated by the fact that the original deletion mutant in the collection is wrongly assigned (Claes Andréasson, personal communication 2013).

Since both Ptk2 and Sky1 kinases have been shown to also play a role in the resistance to polyamines, this prompted experiments to check if the additional three deletion mutants *agp2Δ*, *fes1Δ* and *brp1Δ* discovered from the bleomycin screen would be resistant to polyamines. Indeed, four of these deletion mutants *agp2Δ*, *sky1Δ*, *ptk2Δ* and *fes1Δ*, except *brp1Δ*, display striking resistance to polyamines (Figure 2) and thus, set the foundations for a more comprehensive study on the role of these four proteins in protecting cells against polyamine toxicity.

We designed studies to focus primarily on Agp2 functions and established that it is responsible for the high affinity uptake of polyamines operating with a K_m of ~15 μM[20]. *agp2Δ* mutants are completely defective in the uptake of low concentrations of labelled spermidine, underscoring the need for this protein to mediate high affinity polyamine uptake[20]. Thus, it seems that Agp2 might recognise and mediate the transport of a broad range of polycations including L-carnitine, polyamines and bleomycin due to its polyamine moiety. It is anticipated that *agp2Δ* mutants would be highly resistant to other toxic and highly charge polycations. Once these toxic polycations enter into the cell, they are channelled to the vacuoles for detoxification[17].

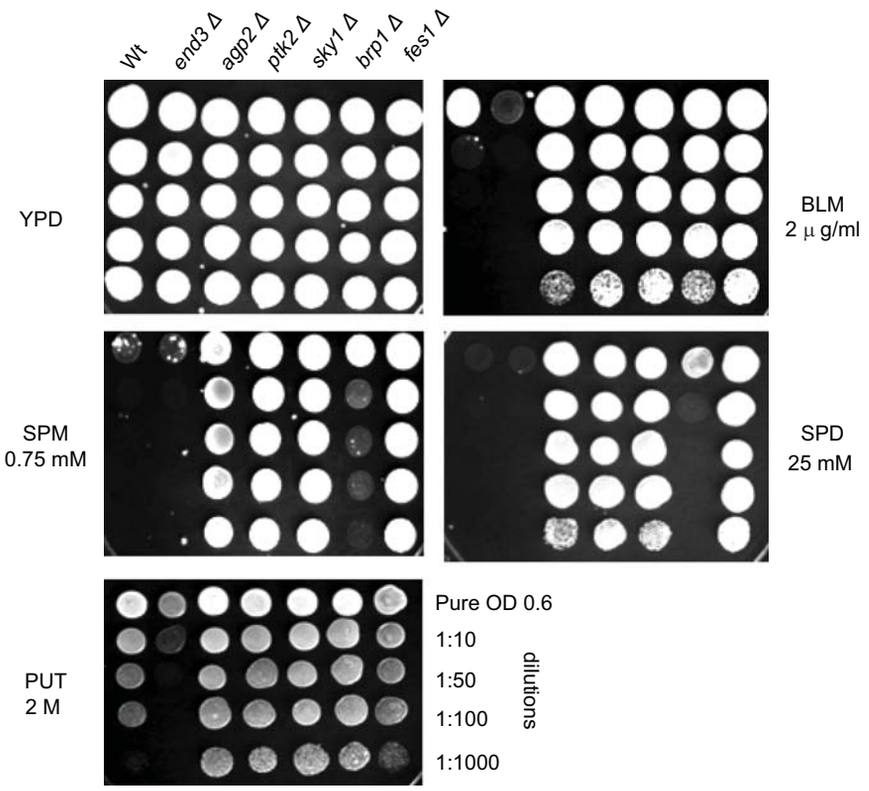

Figure 2: Spot test assay showing yeast mutant strains that are resistant or sensitive to both bleomycin and polyamines. Exponentially growing cultures have been diluted, spotted onto solid media containing the indicated agents and photographed after 48 hours of growth at 30°C. Wild-type (WT) is the parent strain from which the isogenic mutants have been derived. The *end3*Δ mutant is defective in endocytosis to the vacuoles. BLM, bleomycin; PUT, putrecine; SPM, spermine; SPD, spermidine.

Although Agp2 has been characterised as a high affinity transporter for L-carnitine and polyamines, subsequent observations cast doubt whether Agp2 is an actual transporter. This emanated from two tantalising findings (i) a 200-fold excess of L-carnitine did not block polyamine uptake as would be expected if Agp2 is indeed a high affinity transporter for both L-carnitine and polyamines, and (ii) the addition of a 100-fold excess of L-carnitine in the media did not protect the parent strain from spermine toxicity[21]. As such, it seems imperative to reconsider the role of Agp2 as a transporter, and instead we postulate that Agp2 might be a regulator that controls the high affinity transport of polyamine. In support of this latest model (Figure 3), the Igarashi group (Japan) reported the presence of two high affinity polyamine transporters Dur3 and Sam3, that are on the plasma membrane of yeast cells[22]. These combined data prompted us to examine if there is a

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connection between Agp2 and the latter two transporters. One possibility is that Agp2 exerts regulatory control on the expression of *DUR3* and *SAM3* genes. In such a scenario, Agp2 would act as a receptor that transmits a signal to activate gene expression.

Deletion of either the *DUR3* or *SAM3* gene resulted in single mutants that showed the same level of sensitivity to polyamines as the parent[21]. However, mutants lacking both genes are completely blocked for the uptake of labelled spermidine and display sharp resistance to polyamines when compared to the parent[21]. The phenotypic similarities that exist between the *agp2Δ* mutant and the *dur3Δ sam3Δ* double mutant, suggest that Agp2 belongs to the same uptake pathway as Dur3 and Sam3. To date, there are many examples whereby plasma membrane proteins such as Ssy1, which possesses the structural features of an amino acid transporter and belongs to the yeast amino acid permease family, as in the case of Agp2, but lacks the ability to function as a transporter[23]. Instead, these proteins serve the role of a sensor to activate gene expression[23]. Ssy1 senses amino acid availability by direct interaction with extracellular amino acids and triggers the expression of several downstream target genes, that encode amino acid permeases such as *AGP1*, *BAP2, BAP3, DIP5,* and *TAT1* via the formation of an intermediary complex, the SPS sensor (Ssy1-Ptr3-Ssy5), with the plasma membrane proteins Ptr3 and Ssy5[23,24]. In this sensory system, Ptr3 becomes hyper-phosphorylated allowing the activation of the protease Ssy5, which cleaves the latent form of the two homologous zinc-finger transcription factors, Stp1 and Stp2, present in the cytosol, that

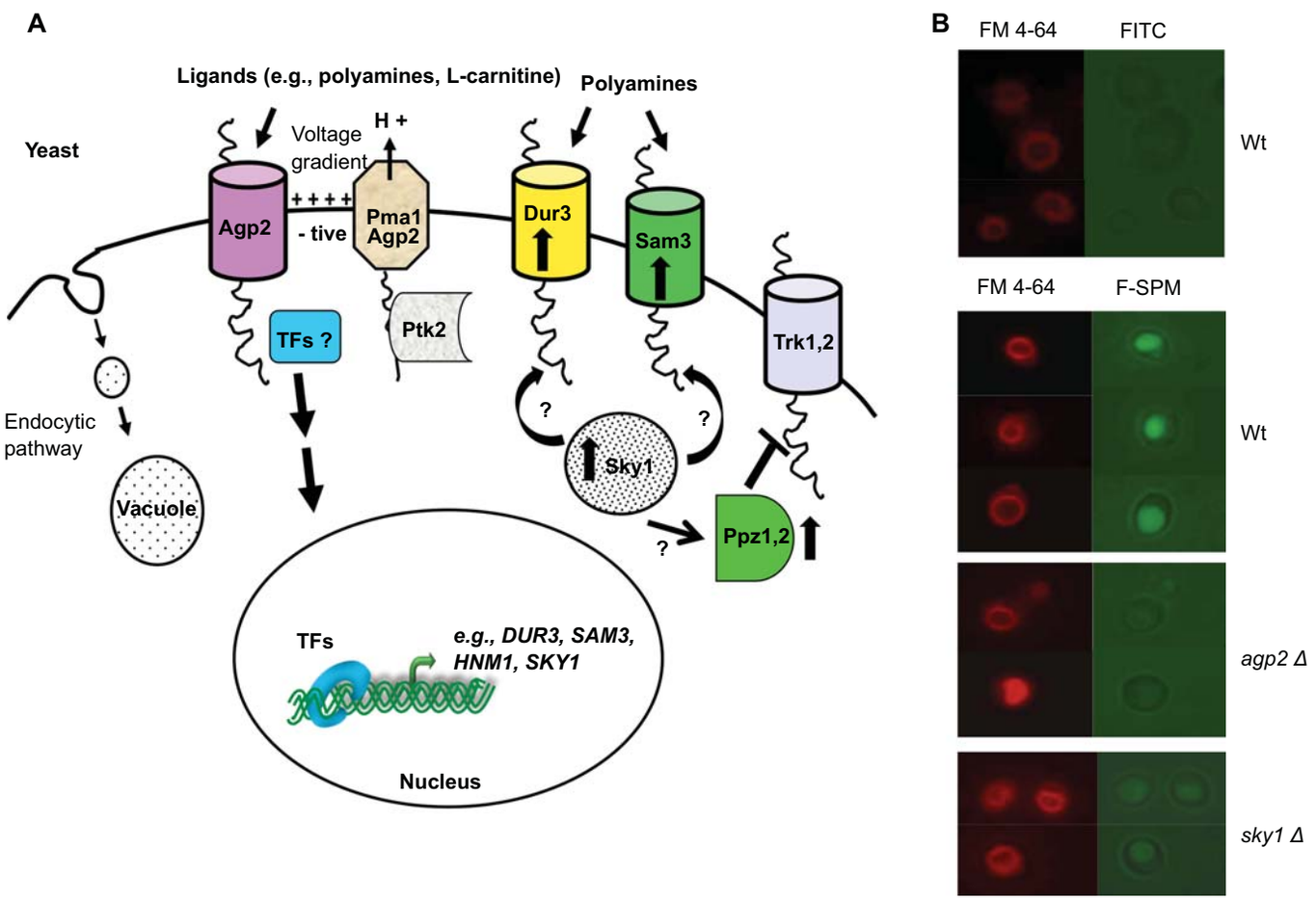

Figure 3: A model depicting factors that are involved in controlling polyamine uptake. (A) Agp2 is believed to be a non-transporting receptor that senses several nutrients including polyamines and transmits a signal to a transcriptional activator that maintains the expression of many genes, e.g., the regulatory kinase Sky1 and the two high-affinity polyamine transporters Dur3 and Sam3. Since Sky1 does not affect *DUR3* or *SAM3* expression, it may therefore activate these transporters by post-translational modification, e.g., by phosphorylation. The transcription factors (TFs) responsible for conveying Agp2 sensory function and culminating in gene activation remain to be identified. (B) Accumulation of fluorescein-labelled spermine (F-SPM) into the vacuoles of the parent (WT), but not in the *agp2*Δ or *sky1*Δ mutant. FM4-64 is a dye that stains the vacuolar membrane and used for localisation of this organelle.

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then enter the nucleus and induce the Ssy1-targets[25]. Other examples include Gap1 and Mep2 that have dual functions, not only acting as a general amino acid transporter and ammonium permease, respectively, but also as a sensor that signals through the rapid activation of protein kinase A (PKA) targets[26,27]. Thus, in view of the increasing number of sensors that exist, it would appear that Agp2 might also belong to this category of regulatory functions.

Agp2 maintains the expression of genes involved in polyamine uptake

Based on the foregoing knowledge, it seems logical then to perform a microarray analysis and look for differences in gene expression between the parent and the *agp2Δ* mutant under normal growth conditions. The analysis revealed that several transporter genes including *DUR3* and *SAM3*, as well as *SKY1* are downregulated in the *agp2Δ* mutant, while several others are unaffected such as *GAP1*[21]. Re-introduction of the *AGP2* gene into the *agp2Δ* mutant restored expression of all the affected genes. Since the Sky1 kinase is downregulated in the *agp2Δ* mutant, raises the question whether Agp2 might regulate the transporters via Sky1. In fact, deletion of the *SKY1* gene did not affect the expression of either *DUR3* or *SAM3*, thus excluding the possibility that Sky1 is a mediator that influences expression of Agp2 target genes. Sky1, like Agp2, can also regulate low-affinity polyamine uptake. Sky1 has been shown to negatively regulate the Trk1 and Trk2 potassium transporters, which is believed to occur through activation of the Ppz1 and Ppz2 phosphatases, leading to the hyperpolarisation of the cells and the stimulation of polyamine uptake[28–30]. In the absence of Sky1, the uninhibited Trk1, 2 K^+ permeases cause depolarisation of the cells, by opposing the proton motive potential built up by the Pma1 H^+-pump, blocking uptake and consequently resistance to polyamine. Therefore, the positive action of Agp2 on Sky1 may ultimately increase low affinity polyamine uptake activity via the general increase in membrane potential that results from the inhibition of Trk1 and Trk2 K^+ permeases as illustrated by the model (Figure 3). Consistent with this model, *ppz1Δ ppz2Δ* double mutants have been reported to be highly resistant to spermine in the presence of functional Trk1 and Trk2[28,29]. A logical extension of this model would be to delete the *TRK1* and *TRK2* gene in the *ppz1Δ ppz2Δ* double mutant and check if the quadruple mutant restores polyamine uptake.

Conclusion

In short, we believe that Agp2 senses various polycationic ligands, for example, polyamines, and transmits a signal to activate one or more transcription factors, which then turn on the transporter genes to maintain polyamine homeostasis (Figure 3). In such a scenario, Agp2 would behave as a plasma membrane sensor or non transporting receptor for polyamines in a manner similar to the Ssy1 transceptor that senses amino acids. The role of Sky1 kinase in this model remains elusive. Since Sky1 kinase does not affect the expression of the polyamine transporter genes *DUR3* or *SAM3*, there is a distinct possibility that it could act to maintain the activity of these transporters. Precedent for this exists, as the Ptk2 kinase has been documented to phosphorylate at least Dur3. Nonetheless, the unrelenting issue regarding this model is to find the transcription factor that communicates with Agp2 and turns on *DUR3* and *SAM3* expression levels. Exploring the YEASTRACT database, which uses an algorithm that can assign a potential transcriptional activator to a subset of genes based on a common promoter element, failed to reveal the candidate transcription factor. Thus, any advancement of this model will depend on additional approaches that can reliably identify the transcription factor. It is noteworthy that many compounds have been developed to target the polyamine pathway by blocking either endogenous synthesis or polyamine uptake. However, these compounds have limited effects when tested in clinical trials for treating cancers, raising the possibility that there are auxiliary mechanisms to bypass both inhibition of polyamine synthesis and uptake. We believe that there are inherent complexities within the polyamine uptake pathway and that a detail understanding of this process would lead to the precise targets.

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