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Roles for nucleotide phosphatases in sulfate assimilation and skeletal disease

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Abstract

Sulfur is an essential element to all kingdoms of life and is used in essential cellular processes including the synthesis of sulfur-containing amino acids, maintenance of cellular redox states, and incorporation into various metabolites. Inorganic sulfate, the most abundant source of environmental sulfur, is metabolized into two commonly formed nucleotide precursors: adenosine 5'-phosphosulfate (APS) and 3'-phosphoadenosine 5'-phosphosulfate (PAPS). Donation of activated sulfur occurs through a broad range of enzymatic reactions many of which consume PAPS thereby producing 3'-phosphoadenosine 5'-phosphate (PAP). Two classes of 3'-nucleotide phosphatases then hydrolyze PAP into 5'-AMP: one is evolutionarily conserved from bacteria to man and localizes to the cytoplasmic compartment, while the other is restricted to a subset of eukaryotes and is active within the Golgi lumen. Interestingly, both classes of 3'-nucleotidase are members of a structurally conserved family of lithium-inhibited phosphatases that are targets of the drug in a variety of organisms. In this review we provide an overview of sulfur assimilation and the broad regulatory roles that 3'-nucleotidases play in processes ranging from halotolerance to glycosaminoglycan sulfation. In addition, we discuss recent plant and animal studies that emphasize roles for 3'-nucleotidase function in developmental biology, physiology, and in a rare subset of human patients with skeletal abnormalities.

I. Divergent sulfur assimilation pathways utilize evolutionarily conserved nucleotide intermediates, APS, PAPS, and PAP

Bacteria, fungi, plants and animals all incorporate inorganic sulfate from their environments into either sulfur-containing amino acids or sulfate-containing metabolites (Fig. 1) (Massetot et al., 1975; Klaassen et al., 1997; Takahashi et al., 2011). This pathway known as sulfur assimilation begins by uptake of extracellular sulfate and conversion into the high-energy intermediates adenosine 5'-phosphosulfate (APS) and 3'-phosphoadenosine 5'-phosphosulfate (PAPS) (Fig. 1) via evolutionarily conserved ATP sulfurylase and APS kinase activities (Massetot et al., 1975; Patron et al., 2008). In metazoans, these activities are encoded by bifunctional gene products, PAPSS1/2, harboring both ATP sulfurylase and APS kinase modalities. In contrast, most single cell organisms and plants have maintained these as separate genes, for example *MET3* and *MET14* in budding yeast.

Despite the conserved synthesis of PAPS, its incorporation into downstream metabolites has diverged widely. PAPS, which contains sulfate (SO_4^{2-}) an oxidized form of sulfur, cannot be used directly by bacteria, yeast, or plants for the synthesis of amino acids. Instead, the activated sulfate of PAPS must first be reduced to the bioavailable form of sulfur, sulfide (S^{2-}). In yeast, PAPS reductase, the first step in this process, utilizes the thioredoxin redox system to dissociate the activated sulfate in PAPS (SO_4^{2-}) into inorganic sulfite (SO_3^{2-}) and the byproduct 3'-phosphoadenosine 5'-phosphate (PAP). Sulfite is then further reduced by sulfite reductase to sulfide (S^{2-}) while PAP is metabolized by conserved 3'-nucleotidases to 5'-AMP. Finally, sulfide is combined with O-Acetyl-homoserine by homocysteine synthase

to yield the first sulfur-containing amino acid homocysteine and downstream methionine (Marzluf, 1997). Bacteria, using a nearly identical suite of reactions to those found in fungi, also assimilate sulfate for the purpose of generating sulfur-containing amino acids. In addition, both kingdoms share the commitment step in which the high energy intermediate PAPS is used to generate reduced inorganic sulfite (SO_3^{2-}).

While animals have maintained the proteins necessary for the synthesis of PAPS, they have evolved distinct uses for it including the post-translational modifications of glycosaminoglycans and the sulfation of small endogenous and xenobiotic molecules (Klaassen et al., 1997) (Fig. 1). These reactions are catalyzed by the sulfotransferase superfamily whose members act on a diverse collection of substrates such as bile acids and glycosaminoglycans and will be discussed in greater depth later in this review (Gamage et al., 2006). Interestingly, metazoans no longer possess the enzymes necessary to reduce sulfate (SO_4^{2-}) to sulfite (SO_3^{2-}), perhaps as a result of greater dietary protein consumption (Patron et al., 2008).

In plants, PAPS is also used for a variety of sulfation reactions (Fig. 1) (Kopriva, 2006). In addition plants, like bacteria and fungi, can use inorganic sulfate for the production of sulfur-containing amino acids. However, for this aspect of sulfate assimilation, plants have evolved to use the intermediate APS instead of PAPS for the reduction of activated sulfate to sulfite (Takahashi et al., 2011). Given these widely divergent and specialized uses of PAPS and APS, it is remarkable that the incorporation of inorganic sulfate into these high-energy molecules is so universally conserved throughout evolution. However, it is clear that PAPS and APS serve as key intermediates for multiple varied cellular processes and suggest that their regulation is critical to proper cellular function.

II. Metabolism of sulfur assimilation intermediates occurs through 3'-Nucleotidases: structurally related Li^+ -inhibited, metal-dependent phosphatases

In addition to the reactions involved in the uptake, incorporation, reduction, and utilization of inorganic sulfate, the metabolism of the intermediate PAP by 3'-nucleotidases is now emerging as a key regulatory component of sulfate assimilation (Fig. 1). Early insights into the importance of 3'-nucleotidases came from studies of methionine biosynthesis in yeast while additional roles have been demonstrated by subsequent gain or loss of function reports in yeast, plants and mice (Masselot et al., 1975; Gläser et al., 1993; Murguía et al., 1995; Xiong et al., 2001; Gy et al., 2007; Frederick et al., 2008; Sohaskey et al., 2008; Kim et al., 2009; Wilson et al., 2009).

While phenotypic studies of yeast *MET22* (*HAL2*) and its bacterial ortholog CysQ placed these gene products as potential regulators of sulfur assimilation, their discovery as 3'-nucleotidase enzymes had not yet been made (Masselot et al., 1975; Neuwald et al., 1992; Gläser et al., 1993). In 1990 two metal-dependent Li^+ -inhibited inositol phosphatases, inositol monophosphatase (IMPA) and inositol polyphosphate 1-phosphatase (INPP1) were cloned and reported to share a common sequence motif, WxxDPIDxT (Diehl et al., 1990; York et al., 1990). Comparing yeast *MET22*, bacterial CysQ, and other related bacterial proteins to these phosphatases revealed that they all harbored the original as well as a second shared sequence, WDX₁₁GG, consistent with the notion that they might all be metal-dependent phosphatases (Neuwald et al., 1991; Neuwald et al., 1992; Gläser et al., 1993). X-ray crystal structures of IMPA, INPP1 and a third protein, fructose 1,6-bisphosphatase (FBP) demonstrated that the three proteins shared a common five-layer $\alpha\beta\alpha\beta\alpha$ fold despite having less than 20% overall sequence identity (Ke et al., 1989; Bone et al., 1992; Zhang et

al., 1993; York et al., 1994). Interestingly, the structures revealed that the shared sequence motifs contained the residues necessary for anchoring the catalytic metal and for potentially mediating Li^+ -inhibition. Furthermore, the addition of FBP to the family demonstrated that the substrates extended to other phosphorylated small-molecules.

While these data indicated Hal2 may be a member of this structurally-related family, the seminal discovery was made when, combined with the knowledge of its genetic interactions, it was demonstrated that Hal2 functioned as a PAP and PAPS 3'-nucleotidase (Murguía et al., 1995). These data confirmed that 3'-nucleotidases are members of a larger structurally-related family of phosphatases that share the characteristics of Li^+ -sensitivity, metal-dependency, and small molecule substrate selectivity. Searching metazoan genomes with the expanded pattern, D-X_n-EE-X_n-DP(i/l)D(s/g/a)T-X_n-WDX₁₁GG, has revealed the family is comprised of seven gene products, FBP1/2, IMPA1/2, INPP1, BPNT1, and gPAPP that together hydrolyze four different classes of substrates: fructose 1,6-bisphosphate, inositol monophosphates, inositol polyphosphates, and nucleotide 3',5'-bisphosphates respectively (Spiegelberg et al., 2005; Frederick et al., 2008).

III. Genetic and reverse-biochemical identification of 3'-Nucleotidases from a variety of organisms

Although its enzymatic function as a 3'-nucleotidase was unknown at the time, *MET22* was identified in a screen designed to classify genes essential for methionine biosynthesis (Massetot et al., 1975). Epistasis experiments placed *MET22* in the first half of methionine biosynthesis in which intracellular inorganic sulfate (SO_4^{2-}) is converted into useable sulfide (S^{2-}) before being incorporated into homocysteine (Massetot et al., 1975). Confounding though, *MET22* was discovered to overlap with five other complementation groups that were also necessary for PAPS reductase activity (Massetot et al., 1975). Additionally, it was found that the *MET16* gene alone encoded the enzymatic activity for PAPS reductase. These findings suggested that *MET22* and the three other complementation groups were somehow involved indirectly in affecting sulfate's assimilation (Massetot et al., 1977; Thomas et al., 1990). Shortly after, it was recognized that *MET22* contained a putative phosphatase domain similar to that of the bacterial 3'-nucleotidase *cysQ* and suggested that *MET22* and *cysQ* might have evolved specifically to prevent the accumulation of PAPS or a derivative thereof that was responsible for inhibiting the incorporation of sulfate into amino acids (Neuwald et al., 1992).

Following *MET22*'s initial connection to the methionine biosynthesis pathway, it was also unexpectedly found to play a role in mediating the halotolerance of yeast (Gläser et al., 1993; Quintero et al., 1996). A genetic screen identifying positive regulators of halotolerance found that *MET22* (annotated by this study as *HAL2*) overexpressing strains were able to sustain growth in significantly higher Na^+ (1–1.5 M) and Li^+ (0.1–0.4 M) concentrations than wild-type (Gläser et al., 1993). *MET22* therefore provided a previously unsuspected genetic link between salt stress and the sulfate assimilation pathway despite any evidence at the time that it might play a direct enzymatic role in either (Gläser et al., 1993). As discussed earlier, *Met22* was found to be a member of the structurally related family of small molecule phosphatases and the efficient 3'-hydrolysis of PAP was postulated to be necessary for preventing the accumulation of PAP and to help drive the thermodynamically unfavorable synthesis of APS by limiting the product inhibition of "PAPS-utilizing enzymes" (Murguía et al., 1995; Murguía et al., 1996). This provided a basis for why gain or loss of function of *MET22* in yeast altered halotolerance or amino acid synthesis respectively.

Like *MET22*, the *A. Thaliana* 3'-nucleotidase ortholog *SAL1* was also identified in a screen looking for *Arabidopsis* proteins that would improve Li^+ tolerance when expressed in yeast (Quintero et al., 1996). Overexpression of *SAL1* alone was sufficient to maintain yeast growth in media supplemented with up to 10 mM Li^+ (Quintero et al., 1996). Sequence analysis of *SAL1* revealed that it shared significant homology with *MET22*, while biochemical studies conclusively linked *SAL1* to the *MET22* family of phosphatases by showing that it exhibited Li^+ -inhibited nucleotidase activity (Quintero et al., 1996).

The first mammalian 3'-nucleotidases were cloned and characterized from rats and mice nearly simultaneously in the late 1990s by two distinct approaches (López-Coronado et al., 1999; Spiegelberg et al., 1999). The rat *MET22/SAL1* ortholog, RnPIP, was cloned from a rat heart cDNA library screened for its ability to rescue methionine auxotrophy in *MET22* null yeast (López-Coronado et al., 1999). Independently, mouse *MET22* (Bpnt1) was discovered *in silico* on the basis of its family-defining catalytic core motif (Spiegelberg et al., 1999). In addition, studies have noted that the rodent enzymes RnPIP and Bpnt1 share significant sequence homology with human BPNT1 and that based on its ability to complement the loss of *MET22* in yeast, human BPNT1 likely possesses similar activity and substrate specificity (López-Coronado et al., 1999; Spiegelberg et al., 2005).

Interestingly, a second more divergent mammalian 3'-nucleotidase, gPAPP, was recently identified through a "reverse biochemical" approach (Frederick et al., 2008). Initially this gene was annotated as a member of the Li^+ -inhibited, metal-dependent phosphatase family (GenBank accession no. AAK52336), however its putative substrate(s) remained elusive for a number of years (Spiegelberg et al., 2005). While most similar to Inpp1 and Bpnt1, initial attempts to show that recombinant protein produced in bacteria possessed inositol or nucleotide phosphatase activity were unsuccessful. Following the realization that the gene product harbored a single-pass transmembrane domain and an N-linked glycosylation site, features that impaired its proper folding in *E. Coli*, expression through a Golgi-localized secretory pathway in baculovirus-induced insect cells resulted in the determination of its 3'-nucleotidase activity (Frederick et al., 2008). Of interest, a study published seven years prior identifying transmembrane and secreted proteins involved in mouse development annotated gPAPP as KST-645 (Mitchell et al., 2001); however, it was not realized that the two gene products were allelic until after gPAPP's localization and function had been determined.

IV. The substrate specificity of 3'-Nucleotidases

While all of the 3'-nucleotidases discovered thus far have shared the common sulfate assimilation metabolite PAP as a substrate, a number of the orthologs display different degrees of specificity for PAP, PAPS, IP_2 , and IP_3 . In yeast, *Met22* was initially found to hydrolyze both PAP and PAPS at equivalent rates when in the presence of saturating substrate concentrations while no phosphatase activity was detected against IP_2 or various other non-nucleotide substrates (Murguía et al., 1995). In plants, the three known 3'-nucleotidases (*SAL1*, *SAL2*, and *AHL*) were shown to be somewhat less stringent in their substrate selectivity. Multiple reports suggested that *SAL1* and *SAL2* possessed phosphatase activity against PAP, PAPS, IP_2 , and IP_3 , whereas *AHL* hydrolyzed only PAP; however, PAP was preferred over IP_2 by approximately 300-fold in *SAL1* and *SAL2* (Quintero et al., 1996; Gil-Mascarell et al., 1999). The ability of *SAL1* to hydrolyze a broader range of small molecules linked the 3'-nucleotidase and inositol polyphosphate 1-phosphatase branches providing further evidence of their common evolutionary ancestry.

Importantly, a caveat to these early biochemical descriptions of *Met22* and its orthologs was the limited sensitivity inherent to malachite green phosphate detection assays used for their analyses. The development of more sensitive radiolabeled ligands has provided further

insight into the substrate preferences of Met22 as well as bacterial CysQ and mouse Bpnt1 (Spiegelberg et al., 1999). Although SAL1, SAL2, or AHL were not included in the analyses, Met22, CysQ, and mouse Bpnt1 were shown to hydrolyze IP₂ roughly 1000-fold less efficiently than PAP (Spiegelberg et al., 1999). Further supporting the notion that 3',5'-bisphosphorylated nucleotides are the relevant targets of 3'-nucleotidases, loss or inhibition of *MET22* in yeast can be complemented with mouse Bpnt1 or bacterial CysQ but not human INPP1 as well as by the downregulation of PAPS synthesis via genetic ablation or chemical inhibition (Spiegelberg et al., 1999; Spiegelberg et al., 2005). It was also noted in these studies that IP₂ served as a much better competitive inhibitor of PAP 3'-nucleotidase activity than as a substrate, with K_i and K_m values of 15 μ M and 113 μ M respectively. This raised an interesting possibility that inositol phosphate signaling may actually serve to regulate 3'-nucleotidase activity; however this hypothesis awaits further study for validation *in vivo*.

One critical question that remains largely unanswered is whether PAP or PAPS is the physiologically relevant substrate of 3'-nucleotidases. For the Golgi-localized branch of nucleotidases, gPAPP has been shown to hydrolyze only PAP, providing strong evidence that it alone is a physiologically relevant substrate (Frederick et al., 2008). In contrast, the *MET22/BPNT1* branch of the family appears to utilize both nucleotides, thereby confounding interpretation. Kinetic studies demonstrated that PAPS competitively inhibits the hydrolysis of PAP by mouse Bpnt1 with an inhibition constant of 0.7 μ M. This finding suggests that PAP and PAPS possess nearly equivalent binding affinities for mouse Bpnt1 but does not allow for determination of a Michaelis constant (K_m) or catalytic efficiency, making it unreasonable to assign PAP or PAPS as the kinetically favored substrate. It is worth noting however, that at saturating substrate concentrations Met22 appear to have similar maximal velocities for PAP and PAPS (Murguía et al., 1995; Spiegelberg et al., 1999). This result confounds interpretations of the presumed roles of 3'-nucleotidases in the sulfate assimilation pathway, which postulated that the degradation of PAP was specifically thought to drive the energetically unfavorable synthesis of PAPS. Equal rates of hydrolysis for PAPS and PAP would lead significant production of APS, thereby reversing the flow of the pathway and leading to a futile ATP-consuming cycle. Thus, further kinetic analysis and genetic studies are required to resolve this issue. Alternative models propose that the consumption of PAPS by sulfotransferases or reductases is favored over degradation by 3'-nucleotidase activity, or that PAPS, but not PAP, is somehow sequestered from 3'-nucleotidases in order to safeguard it from degradation.

V. 3'-Nucleotidases localize to multiple tissues and cellular compartments in plants and animals

SAL1 is expressed in all plant tissues and localizes to plastids

Unlike in yeast, the expression of 3'-nucleotidases in plants and animals must be coordinated throughout development and adulthood and in a variety of tissues and subcellular environments. In *Arabidopsis*, northern blotting and SAL1 promoter-GUS reporter analyses have shown that SAL1 is detectable in all plant organs (Xiong et al., 2001). Unfortunately, no tissue-wide expression studies of SAL2 and AHL have been performed so it remains unclear whether the three homologues spatially overlap within plant tissues. At the cellular level, AHL and SAL2 have been demonstrated by fluorescent fusion protein analysis to localize to the cytoplasm whereas SAL1 contains a chloroplast transit peptide sequence and appears to be localized to plastids (Peltier et al., 2006; Rodríguez et al., 2010; Chen et al., 2011). However, it has also been detected in the cytoplasm and nucleus by fluorescent fusion protein studies, suggesting that further experiments are necessary to delineate its precise distribution within the cell (Peltier et al., 2006; Kim et al.,

2009; Rodríguez et al., 2010; Chen et al., 2011). In addition to varying spatially, the expression of SAL1 is also regulated diurnally, with mRNA levels peaking during light exposure and strongly repressed within one hour of darkness (Kim et al., 2009).

Bpnt1 and gPAPP are expressed in distinct tissue sets and localize to unique subcellular locations in mammals

Examinations of gPAPP's tissue distribution through the use of a β -galactosidase gene-trap insertion mouse mutant revealed that it is most strongly expressed in cartilage, lungs, spinal cord, brain, and the developing growth plates of bones (Frederick et al., 2008; Sohaskey et al., 2008). Further, the single-pass transmembrane domain and N-linked glycosylation site indicated that gPAPP might transit through the ER/Golgi. Indeed, it was found that gPAPP localizes in a Type II orientation with the active site present in the interior of the Golgi lumen (Frederick et al., 2008). The function of gPAPP in modulating Golgi-lumen sulfur assimilation pathways will be discussed in greater detail below.

While gPAPP and BPNT1 share the common substrate PAP, BPNT1 appears to perform its 3'-nucleotidase activity in a distinct but broader set of tissues. Northern blotting of normal human and rat tissues has shown that it is expressed in the liver, kidneys, pancreas, brain, spleen, testis, skeletal muscle, and heart (López-Coronado et al., 1999; Spiegelberg et al., 1999). In addition, BPNT1 does not appear to localize to any non-cytoplasmic compartments as it has been purified to homogeneity from $22,800 \times g$ supernatants of mouse kidney, lung, liver, and heart and detected by western blotting in $100,000 \times g$ soluble fractions (Spiegelberg et al., 1999).

VI. MET22 and its orthologs affect a diverse array of physiological processes in yeast, plants, and animals

MET22 regulates 5'-3' exoribonuclease activity through the accumulation of PAP

In the years following the initial discovery and characterization of *MET22*, orthologs have been described in multiple species including rice, thale cress, mice, rats, guinea pigs, and humans (Ramaswamy et al., 1987; Peng et al., 1995; Quintero et al., 1996; López-Coronado et al., 1999; Spiegelberg et al., 1999; Spiegelberg et al., 2005). Yet despite *Met22*'s principle substrate PAP having been implicated *in vitro* as early as 1958 in the inhibition of sulfotransferases, direct targets of PAP *in vivo* remained unclear (Brunngraber, 1958; Murguía et al., 1995).

Unexpectedly, the first mechanistic off-axis target for PAP was discovered in a screen for yeast genes involved in ribosomal RNA (rRNA) processing, in which *MET22* was found to be synthetically lethal (sl) with a temperature sensitive mutant of the rRNA endoribonuclease RNase MRP (Dichtl et al., 1997). Notably, the observed inhibition of ribosomal RNA processing in *MET22* null strains, or in wild-type strains grown in the presence of $0.2M Li^+$, closely resembled mutants of the 5'-3' exoribonucleases *XRNI* and *RAT1* (Dichtl et al., 1997). Moreover, it was found that PAP directly inhibited these enzymes *in vitro* (Dichtl et al., 1997). PAP's unforeseen involvement in the 5'-3' exoribonuclease-dependent degradation of various classes of RNAs presented a potential mechanism for mediating the downstream effects of 3'-nucleotidase inhibition and has prompted further investigations into the links between 3'-nucleotidase and 5'-3' exoribonuclease function in more complex multicellular organisms.

Loss of SAL1 phenocopies inactivating mutations in 5'-3' exoribonucleases

As was initially described in yeast, a number of recent studies have shown that loss of 3'-nucleotidase activity in plants also appears to phenocopy RNA misprocessing seen in *XRNI*

inactivating mutants, likely through an accumulation of PAP (Dichtl et al., 1997; Gy et al., 2007). Screening for genes involved in posttranscriptional gene silencing (PTGS), a defense mechanism for foreign RNA, it was found that loss of either SAL1, XRN2, or XRN3, the nuclear-resident *Arabidopsis* XRNs, was sufficient to restore PTGS in an argonaute-compromised background (Gy et al., 2007). To explain these findings, it was hypothesized that the loss of SAL1's 3'-nucleotidase activity resulted in an accumulation of PAP, which inhibited XRN2/3/4, prevented the degradation of aberrant RNAs, and activated the PTGS pathway (Gy et al., 2007).

Independently, the SAL1 mutant *fry1*, was shown to possess auxin-refractory lateral root formation defects similar to those seen in mutants of XRN4 (Chen et al., 2010). Corroborating the hypothesis that accumulated PAP was responsible for the defects, overexpression of yeast *MET22* was sufficient to restore proper auxin-induced lateral root formation (Chen et al., 2010). Further supporting this model, mutants of both SAL1 and the PAP-specific homologue AHL were found to be less capable of inhibiting viral RNA recombination (Jaag et al., 2010). Building on work that established a role for yeast *XRN1* (XRN4 in *Arabidopsis*) in the degradation of viral RNA recombination intermediates, it was shown that *Arabidopsis* mutants deficient for SAL1, AHL, or XRN4 were defective in repressing the recombination and replication of *Tomato bushy stunt virus* (TBSV) (Jaag et al., 2010).

Taken together, these results suggest that the regulation of PAP levels by SAL1 and AHL serves to modulate the function of XRNs *in vivo* (Gy et al., 2007; Chen et al., 2010; Jaag et al., 2010). Unfortunately, to date, only genetic evidence has linked impaired XRN function in *Arabidopsis* to a loss of SAL1 phosphatase activity. Therefore, detailed mechanistic studies are still needed to fully characterize the role of PAP in modulating 5'-3' exoribonuclease activity.

Loss of SAL1 leads to stress hypersensitivity that can be rescued by reducing PAPS synthesis

Like *MET22*, which is closely tied to the modulation of salt stress in yeast, two mutant alleles of SAL1, *fry1* and *hos2*, were discovered through genetic screens designed to identify *Arabidopsis* mutants that exhibited aberrant stress responses (Xiong et al., 2001; Xiong et al., 2004). Following exposure to freezing, high osmolarity, high salt, or abscisic acid (ABA) *fry1* mutants display a stronger induction of RD29A stress-related genes than wild-type plants (Xiong et al., 2001). In addition, exposure of the SAL1 mutant *hos2* to cold stress revealed increased steady state levels of cold-specific CBF transcription factor transcripts (Xiong et al., 2004). Given that IP₂ and IP₃ are crucial components of the inositol phosphate signaling pathway in mammals and that SAL1 possesses IP₂ phosphatase activity, it was postulated that SAL1's regulation of IP₂ levels might be involved in mediating the observed stress hypersensitivities. Indeed, it was found that *fry1* mutants contained elevated levels of IP₃ in both basal and ABA-stimulated leaf tissue, suggesting a role for SAL1's IP₂/IP₃ phosphatase activity in ABA signaling independent of its 3'-nucleotide hydrolysis (Xiong et al., 2001).

However, subsequent studies have provided conflicting evidence, reporting that loss of PAP phosphatase activity in SAL1 mutants is responsible for the hypersensitivity and that the hyperinduction of RD29A genes can be repressed by overexpression of the PAP-specific *Met22* or by downregulating the synthesis of PAPS in a *sallapk1apk2* triple mutant (APK is the *Arabidopsis* APS Kinase) (Chen et al., 2011). In addition, multiple other groups have recently disputed the role of SAL1 in the stress response pathway, detecting either no hypersensitivity or an increase in stress tolerance, including to drought and solute-induced hyperosmolarity, in various SAL1 mutants (Rossel et al., 2006; Wilson et al., 2009). Clearly,

further study is needed to elucidate the precise role of SAL1 in stress response pathways, be it through the inhibition of XRN's or other mechanisms, and to parse out the relative importance of SAL1's multiple substrates.

Loss of SAL1 alters the levels of metabolites in the jasmonic acid biosynthesis pathway and can be rescued by repressing PAPS synthesis

In addition to the inhibition of XRN's by PAP, recent genetic studies have suggested that PAPS or PAP might directly regulate components of the jasmonic acid (JA) biosynthesis pathway. The SAL1 mutant allele *fou8*, which displays roughly 2-fold higher levels of JA in resting leaf tissue, can be rescued through concomitant suppression of APK1 and APK2 (Rodríguez et al., 2010). Triple *sallapk1apk2* mutants are deficient for PAPS synthesis, suggesting a role for PAPS or PAP in directly modulating enzymes involved in JA biosynthesis (Fig. 1) (Rodríguez et al., 2010). While these findings were consistent with other studies that had shed light on a connection between impaired chloroplastic PAPS synthesis and alterations to the JA biosynthesis pathway, it remains unclear whether this is a direct or indirect mechanism and how the impaired hydrolysis of PAPS and PAP might play a role (Mugford et al., 2009; Yatusovich et al., 2010).

Loss of SAL1 locally upregulates phosphate starvation genes

Given the wealth of studies validating SAL1's substrates as the key intermediates in moderating the phenotypes of SAL1 null mutants, it has been easy to overlook the possibility that SAL1's products, 5'-AMP and inorganic phosphate (P_i) may also be relevant to SAL1's physiological role. Indeed, a recent report suggests that not all phenotypes downstream of SAL1 inactivation can be attributed to an accumulation of its substrates (Hirsch et al., 2011). SAL1 mutants, but not XRN2/3 single or double mutants, displayed an upregulation of the high affinity P_i transporter, PHT1;4, in their roots which was hypothesized to be due to a localized repression of P_i synthesis (Hirsch et al., 2011). While enticing to postulate that an important function of SAL1 might be the localized formation of either 5'-AMP or P_i, little evidence has been provided to demonstrate a direct mechanism leading from SAL1 inactivation to the upregulation of PHT1;4 (Hirsch et al., 2011). Hopefully, future studies will follow up on this exciting new paradigm.

gPAPP regulates glycosaminoglycan sulfation, chondrocyte proliferation, and osteogenesis in mice

The role of gPAPP in mice has been investigated through the use of two unique gene-trap insertions that abrogate the expression of the wild-type transcript (Mitchell et al., 2001; Frederick et al., 2008; Sohaskey et al., 2008). Initially identified as a murine transmembrane or secreted gene product resulting in homozygous neonatal lethality, gPAPP was first shown to play an important but unknown role in osteogenesis (Mitchell et al., 2001). Subsequent studies have revealed that genetic inactivation of gPAPP results in a number of gross morphological defects including shortened limbs and craniofacial defects (Mitchell et al., 2001; Frederick et al., 2008; Sohaskey et al., 2008). In addition to the growth plate, cartilage, and periosteum of long bones, gPAPP is widely expressed in a variety of other tissues such as costal cartilage, lungs, kidneys (medulla and cortex), spinal cord, and brain (cerebellum, pons, medulla oblongata, and neopallial cortex) (Frederick et al., 2008; Sohaskey et al., 2008). Cartilage of gPAPP mutant mice was shown to be deficient in chondroitin 4-sulfate that was concomitant with an increase in the levels of nonsulfated chondroitin (Frederick et al., 2008; Sohaskey et al., 2008). Their cartilage was also found to display markedly reduced amounts of full length aggrecan, the predominant chondroitin sulfate proteoglycan (Sohaskey et al., 2008). Additionally, analyses of lung tissue revealed a similar pattern of impaired sulfation for heparan sulfate glycosaminoglycans (Frederick et

al., 2008; Sohaskey et al., 2008). Mechanistically, it was hypothesized that the absence of Golgi luminal PAP phosphatase activity in gPAPP mice might result in an accumulation of luminal PAP and the subsequent inhibition of golgi-resident sulfotransferases. Alternatively, the impairment of glycosaminoglycan sulfation might be due to the repressed synthesis of a sulfation stimulatory factor such as gPAPP's product 5'-AMP (Frederick et al., 2008). Further study will be necessary to elucidate the inner workings of Golgi luminal PAP metabolism and its role in glycosaminoglycan production.

VII. Mutations in human gPAPP phenocopy mice and define a novel type of skeletal dysplasia

Recently, genetic linkage studies have demonstrated that human gPAPP also plays an important role in skeletal development (Vissers et al., 2011). Four patients born to three unrelated sets of consanguineous parents presented with short stature, chondrodysplasia, brachydactyly, congenital dislocations, cleft palate, and facial dysmorphism (Vissers et al., 2011). These characteristics were broadly similar to other well-described skeletal defects such as those seen in Larsen syndrome, Desbuquois dysplasia, diastrophic dysplasia, or pseudodiastrophic dysplasia (Vissers et al., 2011). However, prior analyses had excluded the more common mutations responsible for the above syndromes in DTDST, CHST3, and CANT1 and thus prompted a genome-wide search to identify the potentially novel mutations. Whole exome sequencing of three of the patients, two of whom were siblings, revealed that only gPAPP was consistently mutated and not present in their 177 patient in-house exome variant database (Vissers et al., 2011). In addition, study of the siblings and their parents confirmed that these mutations were adherent to an autosomal-recessive mode of inheritance (Vissers et al., 2011). Traditional Sanger sequencing was used for the fourth patient who was also found to harbor a mutation in gPAPP, confirming the hypothesis that mutations in gPAPP led to the novel skeletal dysplasias (Vissers et al., 2011).

Together, these studies have demonstrated that gPAPP is a crucial component of the osteogenesis program throughout development and that the loss of Golgi-luminal PAP phosphatase activity is strongly deleterious. And yet, while the importance of gPAPP is evident, there is still much to understand regarding the connection between the loss of PAP phosphatase activity in the Golgi and the resulting skeletal dysplasia. In addition, further study is needed to determine the physiological role of gPAPP's cytoplasmic homologue BPNT1 and why the two 3'-nucleotidases localize to unique subcellular compartments.

VIII. Summary

In this review we have described the discovery, characterization, and biological roles of 3'-nucleotide phosphatases. 3'-nucleotidases are intrinsically linked to the process of sulfate assimilation, which possesses a remarkably conserved core machinery despite its different purposes in plants, animals, and fungi. In yeast, the nucleotide phosphatase *MET22* is essential for the incorporation of inorganic sulfate into amino acids and also for growth in environments with high concentrations of Na⁺ and Li⁺. In plants, *SAL1* and its related homologues play roles not only in halotolerance, but also in hormone biosynthesis, abiotic stress response, and posttranscriptional gene silencing. Finally, while metazoan nucleotide phosphatases are just beginning to be understood, they have already proven to be indispensable for proper osteogenesis in mice and to be clinically relevant to human disease. Hopefully, future experimentation will continue to reveal novel and interesting cellular functions for these conserved nucleotide phosphatases.

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Figure 1.

Overview of the sulfate assimilation pathway in metazoans, yeast, and plants. The boxed numbers represent the various enzyme-catalyzed reactions and the genes that encode for the activities from the representative species *M. Musculus*, *S. Cerevisiae*, and *A. Thaliana*. Of note, yeast are capable of incorporating inorganic sulfate into sulfur-containing amino acids while metazoans instead utilize sulfate for the modification of small molecules, lipids, and proteoglycans. Plants contain the enzymes necessary for both aspects of the pathway.