A Role for Cytoplasmic 3'-Nucleotide Hydrolysis in Liver and Intestine Function

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

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ABSTRACT

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Abstract

Bisphosphate 3’-nucleotidase (Bpnt1) is a member of a family of small molecule phosphatases whose activities depend on divalent cations and are inhibited by lithium. While the enzymes share many commonalities, they have distinct and non-overlapping substrate pools. Of the seven mammalian members, two enzymes, gPAPP and Bpnt1, hydrolyze the same small molecule 3’-phosphoadenosine 5’-phosphate (PAP) but act in separate subcellular compartments, the Golgi apparatus and cytoplasm respectively. Hydrolysis of PAP, which is a metabolite of the inorganic sulfate incorporation pathway, is highly conserved throughout evolution from bacteria to yeast to humans. Evidence in multiple species has shown that inhibiting PAP hydrolysis leads to cellular toxicity as a result of its accumulation and also that these effects can be ameliorated by modulating the rate of its production. However, despite the abundant evidence of its importance from studies in lower eukaryotes, the role of the cytoplasmic PAP phosphatase, Bpnt1, in more complicated mammalian physiological remains poorly understood. Here we report for the first time the generation and characterization of mice deficient for Bpnt1. Bpnt1 null mice do not exhibit skeletal defects, but instead develop severe liver pathologies and deficiencies in intestinal iron absorption. Loss of Bpnt1 leads to tissue-specific elevations of the substrate 3’-phosphoadenosine 5’-phosphate (PAP). To test the hypothesis that a toxic cellular accumulation of PAP accounts for the observed
phenotypes, we generated a double mutant mouse that concomitantly down regulates bisphosphorylated nucleotide synthesis in the context of Bpnt1 deficiency. Remarkably, double mutants do not display any detectable physiological defects seen in Bpnt1 null mice. In addition, we have identified and characterized a novel substrate of 3’-nucleotidases, 3’-phosphoadenosine 5’-diphosphate (PAPP) that co-accumulates with PAPS and PAP and might play a role in mediating certain aspects of the physiological defects of Bpnt1 null mice. Overall, our study defines a role for Bpnt1 in mammalian physiology and provides mechanistic insights into the importance of cytoplasmic 3’-nucleotide hydrolysis to normal cellular function.
Dedication

This dissertation is dedicated to the memory of my father Larry Hudson, a beloved educator, mentor, and teacher. Through his actions, he taught me what it means to put family first and to treasure those around you; through his abilities, he instilled in me a love of sharing knowledge with others; and through his perseverance, he showed me how to surmount any and all of life’s challenges.
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1. Introduction

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

Figure 1: Overview of the Sulfate Assimilation Pathway

Bacteria, fungi, plants and animals all incorporate inorganic sulfate from their environments into either sulfur-containing amino acids or sulfate-containing metabolites (Figure 1) (1-3). 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) (Figure 1) via
evolutionarily conserved ATP sulfurylase and APS kinase activities (2, 4). 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 (SO₄²⁻) 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 (S²⁻). In yeast, PAPS reductase, the first step in this process, utilizes the thioredoxin redox system to dissociate the activated sulfate in PAPS (SO₄²⁻) into inorganic sulfite (SO₃²⁻) and the byproduct 3’phosphoadenosine 5’-phosphate (PAP). Sulfite is then further reduced by sulfite reductase to sulfide (S²⁻) 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 (5). 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 (SO₃²⁻).
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 (3) (Figure 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 (6). 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 (4).

In plants, PAPS is also used for a variety of sulfation reactions (Figure 1) (7). 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 (1). 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.
1.2 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 (Figure 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 (2, 8-15).

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 (2, 14, 16). 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, WxxDxDxxT (17, 18). 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, WDXGG, consistent with the notion that they might all be metal-dependent phosphatases (14, 16, 19). 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 αβαβα fold despite having less than 20% overall sequence identity (20-23). 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 (15). 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-Xₙ-EE-Xₙ-DP(i/l)D(s/g/a)T-Xₙ-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 (8, 24).

1.3 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 (2). Epistasis experiments placed MET22 in the first half of methionine biosynthesis in which intracellular inorganic sulfate (SO₄²⁻) is converted into useable
sulfide (S\(^2\)) before being incorporated into homocysteine (2). Confounding though, 

*MET22* was discovered to overlap with five other complementation groups that were 
also necessary for PAPS reductase activity (2). 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 (25, 26). 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 (16).

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 (14, 
27). 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 (14). 
*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 (14). 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” (15, 28). 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 (27). Overexpression of SAL1 alone was sufficient to maintain yeast growth in media supplemented with up to 10 mM Li+ (27). 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 (27).

The first mammalian 3’-nucleotidases were cloned and characterized from rats and mice nearly simultaneously in the late 1990s by two distinct approaches (29, 30). 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 (29). Independently, mouse MET22 (Bpnt1) was discovered in silico on the basis of its family-defining catalytic core motif (30). 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 (24, 29).
Interestingly, a second more divergent mammalian 3′-nucleotidase, gPAPP, was recently identified through a “reverse biochemical” approach (8). 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 (24). 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 (8). Of interest, a study published seven years prior identifying transmembrane and secreted proteins involved in mouse development annotated gPAPP as KST-645 (31); however, it was not realized that the two gene products were allelic until after gPAPP’s localization and function had been determined.

1.4 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, Met22p 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 (15). 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 (27, 32). 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 Met22p 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 Met22p as well as bacterial CysQ and mouse Bpnt1 (30). Although SAL1, SAL2, or AHL were not included in the analyses, Met22p, CysQ, and mouse Bpnt1 were shown to hydrolyze IP$_2$ roughly 1000-fold less efficiently than PAP (30). 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 (24, 30). It was also noted in these studies that IP$_2$ served as a much better competitive inhibitor of PAP 3’-nucleotidase activity than as a
substrate, with $K_i$ and $K_m$ values of 15µM and 113µM 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 PAP is an endogenous substrate, although it does not rule out the possibility that additional as yet unknown substrates exist (8). 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 µM. 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 impossible to assign PAP or PAPS as the kinetically favored substrate. It is worth noting however, that at saturating substrate concentrations Met22p appear to have similar maximal velocities for PAP and PAPS (15, 30). This result confounds interpretations of the presumed roles of 3’-nucleotidases 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.

1.5 3’-Nucleotidases localize to multiple tissues and cellular compartments in plants and animals

1.5.1 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 (10). 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 (33-35). 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 (12, 33-35). 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 (12).

1.5.2 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 (8, 9). 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 (8). 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 (29, 30). In addition, BPNT1 does not appear to localize to any non-cytoplasmic compartments as it has been purified to homogeneity from 22,800 x g supernatants of mouse kidney, lung, liver, and heart and detected by western blotting in 100,000 x g soluble fractions (30).
1.6 *MET22* and its orthologs affect a diverse array of physiological processes in yeast, plants, and animals

1.6.1 *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 (24, 27, 29, 30, 36, 37). Yet despite Met22p’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 (15, 38).

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 (39). 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 *XRN1* and *RAT1* (39). Moreover, it was found that PAP directly inhibited these enzymes *in vitro* (39). 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.
1.6.2 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 XRN inactivating mutants, likely through an accumulation of PAP (11, 39). 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 (11). 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 (11).

Independently, the SAL1 mutant *fry1*, was shown to possess auxin-refractory lateral root formation defects similar to those seen in mutants of XRN4 (40). 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 (40). 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 (41). 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) (41).

Taken together, these results suggest that the regulation of PAP levels by SAL1 and AHL serves to modulate the function of XRNs *in vivo* (11, 40, 41). 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.

### 1.6.3 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 (10, 42). 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 (10). In addition, exposure of the SAL1 mutant *hos2* to cold stress revealed increased steady state levels of cold-specific CBF transcription factor transcripts (42). Given that IP$_2$ and IP$_3$ are crucial components of the inositol phosphate signaling pathway in mammals and that SAL1 possesses IP$_2$ phosphatase activity, it was postulated that SAL1’s regulation of IP$_2$ levels might be involved in mediating the observed stress hypersensitivities. Indeed, it was found that *fry1* mutants contained elevated levels of IP$_3$ in both basal and ABA-stimulated leaf tissue, suggesting a role for
SAL1’s IP$_2$/IP$_3$ phosphatase activity in ABA signaling independent of its 3’-nucleotide hydrolysis (10).

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 Met22p or by downregulating the synthesis of PAPS in a sal1apk1apk2 triple mutant (APK is the Arabidopsis APS Kinase) (35). 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 (13, 43). Clearly, further study is needed to elucidate the precise role of SAL1 in stress response pathways, be it through the inhibition of XRNs or other mechanisms, and to parse out the relative importance of SAL1’s multiple substrates.

1.6.4 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 XRNs 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 (34). Triple sal1apk1apk2 mutants are deficient for PAPS
synthesis, suggesting a role for PAPS or PAP in directly modulating enzymes involved in JA biosynthesis (Figure 1) (34). 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 (44, 45).

1.6.5 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 (Pi) 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 (46). SAL1 mutants, but not XRN2/3 single or double mutants, displayed an upregulation of the high affinity Pi transporter, PHT1;4, in their roots which was hypothesized to be due to a localized repression of Pi synthesis (46). While enticing to postulate that an important function of SAL1 might be the localized formation of either 5’-AMP or Pi, little evidence has been provided to demonstrate a direct mechanism leading from SAL1 inactivation to the upregulation of PHT1;4 (46). 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 (8, 9, 31). 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 (31). Subsequent studies have revealed that genetic inactivation of gPAPP results in a number of gross morphological defects including shortened limbs and craniofacial defects (8, 9, 31). 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) (8, 9). 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 (8, 9). Their cartilage was also found to display markedly reduced amounts of full length aggrecan, the predominant chondroitin sulfate proteoglycan (9). Additionally, analyses of lung tissue revealed a similar pattern of impaired sulfation for heparan sulfate glycosaminoglycans (8, 9). Mechanistically, it was hypothesized that the absence of Golgi lumenal PAP phosphatase activity in gPAPP mice might result in an accumulation of lumenal 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 (8). Further study will be necessary to elucidate the inner workings of Golgi lumenal PAP metabolism and its role in glycosaminoglycan production.

1.7 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 (47). Four patients born to three unrelated sets of consanguineous parents presented with short stature, chondrodysplasia, brachydactyly, congenital dislocations, cleft palate, and facial dysmorphism (47). 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 (47). 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 (47). In addition, study of the siblings and their parents confirmed that these mutations were adherent to an autosomal-recessive mode of inheritance (47). 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 (47).
Together, these studies have demonstrated that gPAPP is a crucial component of the osteogenesis program throughout development and that the loss of Golgi-lumenal 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.

1.8 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.
2. Mechanistic insights into a role for cytosolic 3’-nucleotide hydrolysis in mammalian physiology: generation of Bpnt1 global knockout mice

2.1 Introduction

The liver is an important hub for a diverse array of metabolic processes including glucose homeostasis, vitamin biosynthesis, and nitrogen remediation. To meet these demands, hepatocytes, the fundamental units of the liver that account for 70% of its weight, perform hundreds of different biochemical reactions simultaneously. One component of hepatocellular function that is essential for numerous downstream physiological processes is the synthesis and export of more than 100 plasma proteins (48, 49). Albumin, which accounts for greater than 60% of the hepatically-produced plasma protein content, transports hormones, fatty acids, metals, and xenobiotics throughout the body and provides roughly 75% of the vascular osmotic pressure (49). Because of its importance, mammals have developed multiple safeguards to maintain consistent serum albumin concentrations, and as a result, significant reductions in its abundance are generally only seen in chronic liver diseases such as cirrhosis. Indeed, loss of oncotic pressure and concurrent presentation of edema is often indicative of liver failure (48).

In addition to producing a majority of the body’s circulating proteins, the liver also provides the first layer of defense against cytotoxic agents. To this effect, the liver employs two complementary strategies for detoxification, Phase I and II biotransformations (50, 51). Phase I reactions decrease the inherent toxicity of molecules
by modifying a functional group and are mediated predominately by the cytochrome P450 family of enzymes. Conversely, Phase II reactions, which are catalyzed by multiple families of small molecule transferases, involve the appending of a glucoronic acid, glutathione, amino acid, or sulfate moiety to an acceptor molecule in order to increase its solubility and expedite removal from the body via the kidneys. One class of Phase II enzymes, sulfotransferases (SULTs), requires the activated sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) in order to generate a sulfated acceptor molecule and 3′-phosphoadenosine 5′-phosphate (PAP) (3, 6, 52). Following sulfate donation, the byproduct PAP is degraded by 3′-nucleotidases to generate 5′-AMP (53).
Figure 2: A family of lithium-sensitive phosphatases and overview of the sulfate assimilation pathway.

(A) The *Mus Musculus* family of metal-dependent/lithium-sensitive phosphomonoesterases and the consensus core motif that defines the family. (B) Schematic of the sulfate assimilation pathway intermediates. *Bpnt1* and *gPAPP* hydrolyze the 3’ phosphate from the sulfation byproduct 3’-phosphoadenosine 5’-phosphate (PAP) in the cytoplasm and Golgi lumen respectively.
Mammalian genomes encode two 3′-nucleotidases, the recently characterized Golgi-resident PAP phosphatase (gPAPP) and Bisphosphate 3′-nucleotidase 1 (Bpnt1), which localize to the Golgi lumen and cytoplasm respectively (Figure 2) (8, 29, 30, 53). gPAPP and Bpnt1 are members of a family of small molecule phosphatases whose activities are both dependent on divalent cations and inhibited by lithium (53). The family is comprised of seven mammalian gene products: fructose bisphosphatase 1 and 2 (Fbp1/2), inositol monophosphatase 1 and 2 (Impa1/2), inositol polyphosphate 1-phosphatase (Inpp1), gPAPP, and Bpnt1 (Figure 2). Although the members display limited overall sequence similarity, their shared properties are defined by a common structural core and the catalytic motif, D-Xₙ-EE-Xₙ-DP(i/l)D(s/g/a)T-Xₙ-WDXₙ₋₁GG (54).

3′-nucleotideases have been studied in a number of model systems and are important for a wide variety of cellular processes including salt tolerance and methionine biosynthesis (10, 12, 13, 15, 16, 27, 36, 55, 56).

Recently, loss of gPAPP activity in mice was shown to result in pulmonary insufficiency, joint defects, and impaired skeletal development likely as a result of inadequate glycosaminoglycan sulfation (8, 9). We hypothesized that loss of Golgi lumenal 3′-nucleotidase activity resulted in these deficiencies through either reduced stimulation of PAPS transport into the Golgi or the direct inhibition of sulfotransferases (8). The studies in mice also provided important mechanistic insights into how mutations in gPAPP give rise to a subset of human skeletal abnormalities (47).
Here we report the generation and analysis of Bpnt1 global knockout mice. Loss of cytoplasmic 3’-nucleotidase function results in markedly distinct phenotypes as compared to those observed in gPAPP deficient animals. Our data provide an unanticipated genetic basis for liver pathologies that in severe cases leads to liver failure, whole body edema, and death. Additionally, we demonstrate a genetic strategy to completely reverse the observed physiological liver defects in Bpnt1 mutant mice. Our study provides insights into the role of Bpnt1 in mammalian physiology and illuminates the unique contributions of compartment specific 3’-nucleotide hydrolysis.

2.2 Results

2.2.1 Bpnt1 Null Mice Develop Edema as a Result of Liver Dysfunction

In order to further our understanding of Bpnt1’s role in mammals we used homologous recombination to generate global knockout mice (Figure 3).
Figure 3: Generation of Bpnt1 null mice.

(A) Targeted disruption of the Bpnt1 locus. Illustration includes the Bpnt1 locus, the genomic Bpnt1 target, the targeting vector, and the resulting disrupted Bpnt1 allele. Note the indicated exons (numbered vertical lines), the HindIII (H) restriction sites for Southern analysis, the genomic region detected by Southern blot (dashed line), the regions of targeted homology, the screening PCR primers (arrows) f1, f2, and r1, and the diphtheria toxin and neo cassettes. Exons 4 and 5 are replaced with the Neo cassette via homologous recombination resulting in the loss of Bpnt1 function. (B) Southern blot analysis of HindIII digested genomic liver DNA from Bpnt1 wild-type, heterozygote, and homozygous null mice. Predicted fragment sizes are 13.0 and 7.1 kB for the wild-type and targeted alleles respectively. (C) PCR analysis of genomic DNA purified from the tails of Bpnt1 wild-type, heterozygote, and knockout mice. Predicted fragment sizes are 2052-bp and 1546-bp for the wild-type and targeted alleles respectively.
Heterozygous and homozygous null animals were viable and developed according to expected Mendelian distributions. Analysis of tissues from homozygous null animals by western blot revealed a complete absence of detectable Bpnt1 (Figure 4).

**Figure 4:** Absence of detectable Bpnt1 protein in knockout animals.

(A) Western blot of soluble protein extracts from wild-type and Bpnt1 null tissues demonstrating an absence of detectable Bpnt1 protein. (B) Cell fractionation by differential centrifugation of wild-type and knockout liver tissue demonstrating that Bpnt1 remains in the soluble fraction following 100,000 x g centrifugation.

At birth and through early development, homozygous null mice appear grossly identical to their wild-type and heterozygote littermates, and we have seen no evidence
of haploinsufficiency in Bpt1 heterozygote animals throughout adulthood. However, most prominently, by 45 days of age on average, homozygous null mice develop a 45% penetrant lethal full body edema (Figure 5A).
Figure 5: Hypoproteinemia and repressed translation in Bpnt1 null animals.

(A) Photograph of Bpnt1 wild-type (left) and homozygous null (right) littermates illustrating the full body edema. Scale bar: 2 cm. (B) Coomassie stained SDS-PAGE of Bpnt1 wild-type and knockout total serum protein. (C) Quantification of serum albumin in wild-type, heterozygote, and knockout mice. Values represent mean +/- SEM. (D) SDS-PAGE of 2 µL of 3H-leucine-labeled serum, dried and visualized by fluorography (right). Identical gel stained with Coomassie brilliant blue (left). (E) Polysome profiles from wild-type and knockout liver tissue. Note the significantly lower levels of polysomes in Bpnt1 null livers.

One cause of whole body edema is hypoproteinemia as a result of inadequate hepatic protein export. To investigate this possibility, we analyzed serum from wild-type and Bpnt1 null mice by SDS-PAGE and clinical chemistries. Strikingly, Bpnt1 null mice showed significantly lower levels of many serum proteins compared to wild-types (Figure 5B). One protein we found to be markedly repressed, albumin, normally comprises greater than 60% of the total serum protein content and provides the principle component of the osmotic pressure necessary to maintain fluid inside the vasculature. Because of its importance, the liver maintains tight control over its serum concentration, with normal values in mice ranging from 2.7 to 3.3 g/dL. When compared to wild-type animals, Bpnt1 null mice showed a 36% reduction (1.9 vs. 3.0 g/dL) in serum albumin, while mice presenting with edema had further repressed albumin concentrations of 0.9 g/dL (Figure 5C). In addition, serum clinical chemistries revealed that total cholesterol in Bpnt1 null animals was significantly lower than wild-types (69.2 vs. 188.2 mg/dL) and that levels of the liver enzymes ALT, AST, and ALKP (alanine aminotransferase, aspartate
aminotransferase and alkaline phosphatase) were elevated, indicative of substantial hepatocellular damage (Figure 9A and Table 1).

**Table 1: Serum chemistries of Bpnt1 null mice.**

Serum clinical chemistries demonstrating the repressed albumin and hepatocellular damage in Bpnt1 null animals. Values represent mean +/- SEM.

<table>
<thead>
<tr>
<th></th>
<th>Alb (g/dL)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>ALKP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bpnt1+/+</td>
<td>3.0 ± 0.2</td>
<td>33 ± 2</td>
<td>108 ± 9</td>
<td>327 ± 22</td>
</tr>
<tr>
<td>Bpnt1+/−</td>
<td>3.0 ± 0.1</td>
<td>28 ± 1</td>
<td>133 ± 23</td>
<td>360 ± 20</td>
</tr>
<tr>
<td>Bpnt1−/−</td>
<td>1.9 ± 0.1</td>
<td>382 ± 102</td>
<td>282 ± 56</td>
<td>1083 ± 127</td>
</tr>
<tr>
<td>Bpnt1−/− w/ edema</td>
<td>0.9 ± 0.03</td>
<td>454 ± 94</td>
<td>658 ± 99</td>
<td>865 ± 307</td>
</tr>
</tbody>
</table>

To investigate whether the hypoproteinemia and edema in Bpnt1 null mice might be a direct result of insufficient hepatic protein production, we used *in vivo* radioactive metabolic labeling to measure the rates of serum protein synthesis. Following a pulse of intraperitoneally-injected $^3$H-leucine in rats and mice, radiolabeled albumin begins to appear in the blood within 30 minutes (57). Indeed, we found that after 30 minutes, Bpnt1 null mice incorporated dramatically less radioactivity into newly synthesized albumin (Figure 5D, top band). In addition to decreased incorporation of $^3$H-leucine into albumin, we detected two other bands that showed slower kinetics of production in knockout animals, which we identified by mass-spectrometry as apolipoproteins E and A1, two of the major protein components of *de novo* hepatically synthesized lipoproteins such as LDL (Figure 5D, lower bands).
In order to address whether the slower serum protein production was due to a defect in hepatic translation, we examined polysome profiles of wild-type and Bpnt1−/− livers. In general, mRNAs undergoing more active translation are populated by a greater number of ribosomes than less actively translated messages and therefore will occupy denser sucrose fractions following ultracentrifugation. Thus, comparing the relative quantities of ribosomes in light and dense fractions provides insight into the global translational status of the cell. Relative to wild-type, ribosomes from Bpnt1 null livers were greatly enriched in the light sucrose fractions containing individual subunits and 80S monosomes and less abundant in the more dense polysome-containing fractions (Figure 5E).

### 2.2.2 Bpnt1 Null Mice Have Aberrant Hepatocellular Morphology

Given the striking deficiencies in liver function, we sought to understand what underlying hepatocellular defects were leading to hypoproteinemia, edema, and death. Grossly, Bpnt1 null livers appeared pale and enlarged (Figure 6), while hematoxylin and eosin staining revealed striking alterations to hepatocellular morphology including hypertrophied nuclei and large abnormal subnuclear structures (Figure 7A).
Figure 6: Pale livers in Bpnt1 null mice.

(A) Photograph of peritoneal cavity of wild-type and Bpnt1<sup>−/−</sup> mice. Note the pale appearance and visible lobule outlines.

Importantly, the abnormalities were not localized to a particular liver lobule or zone, but instead were evenly distributed throughout the entire liver. The homogenous pattern of expression was distinct from most models of liver injury, which tend to affect either periportal or perivenous zones disproportionately, and suggested that the primary defect was intrinsic to hepatocytes (58).
Figure 7: Aberrant hepatocellular morphology in Bpnt1 null mice.

(A) 5 µm H&E stained liver sections from wild-type and knockout mice demonstrating the hypertrophied nuclei and condensed nucleoli. Scale bar: 20 µm. (B and C) Transmission electron micrographs of wild-type and knockout hepatocytes. Note the absence of visible inner nuclear-membrane bound DNA and rough endoplasmic reticulum in knockouts. Scale bars: (B) 2 µm, (C) 500 nm.

Because mouse hepatocytes normally contain between three and five visible nucleoli within each nucleus, we wondered whether the structure might be a single
condensed nucleolus. Indeed, indirect immunofluorescence for the nucleolar-resident proteins fibrillarin and B23 confirmed that the observed structures contained both fibrillar centers and dense fibrillar components respectively (Figure 8).

Figure 8: Condensed nucleoli and elevated levels of nucleolar components.

(A) Immunofluorescence staining of nucleolar-resident Fibrillarin (green) and B23 (8), which localize to the fibrillar center and dense fibrillar component respective. The white dashed circle represents the outline of the nuclear membrane. (B) Western blot of Fibrillarin, B23, and Gapdh in wild-type, Bpnt1<sup>−/−</sup>, and double KO liver tissue demonstrating the accumulation of nucleolar proteins specifically in Bpnt1<sup>−/−</sup> mice. Note the rescue in double KO livers.

Ultrastructural examination by TEM revealed that the nuclei contained dramatically less inner membrane-bound DNA and little detectable contiguous rough endoplasmic reticulum (Figure 7B and Figure 7 C). In addition, we observed a number
of changes to the cytoplasmic composition including small irregular mitochondria, reduced glycogen, and abundant lipid droplets. Indeed, Oil Red O staining of frozen liver tissue demonstrated a striking quantity of accumulated neutral lipids distributed throughout the hepatocytes of Bpnt1 null mice that upon further analysis were found to contain cholesteryl esters and triglycerides, the lipids most abundant in hepatically produced lipoproteins such as LDL (Figure 9).

Figure 9: Bpnt1 null mice are hypocholesterolemic and accumulate hepatic lipids.
(A) Total serum cholesterol from wild-type and Bpnt1 null animals. Bar represents the sample mean. (B) Oil Red O staining of 10 µm wild-type and knockout frozen sections showing strong accumulation of neutral lipids in Bpnt1 null tissue. Scale bar: 10 (C) One-dimensional TLC of total lipid extracts from two wild-type and Bpnt1 null livers. Knockout animals show elevated levels of triglycerides and cholesteryl esters, the main components of hepatically produced lipoproteins.

2.2.3 Bpnt1 Null Livers Accumulate Nucleotides and Immature Ribosomal RNA

Because Bpnt1 is a 3’-nucleotidase that hydrolyzes PAP into 5’-AMP (Figure 2B), we were interested in examining whether any alterations to PAP or 5’-AMP pools might be related to the development of the observed hepatic defects. To quantify the amount of PAP within tissues, we developed a simple colorimetric assay suitable for tissue analysis based in part on previously developed methods (59, 60). Briefly, we isolated small molecule extracts from wild-type and knockout livers using boiling glycine. The extracts were then subjected to a PAPS/PAP-dependent enzymatic assay in which the rate of color development is dependent on the combined concentration of PAPS and PAP. Strikingly, we found that Bpnt1 null livers contained roughly 30-50 fold as much PAPS and PAP as wild-type or heterozygote livers (Figure 10A). Because the colorimetric assay is unable to distinguish PAPS from PAP, we isolated small molecule extracts from wild-type and knockout livers by TCA/ethyl-ether and separated the nucleotides by strong anion exchange HPLC-UV. Although the levels of PAPS and PAP in normal liver were below the limit of detection for our assay, in knockout livers we were able to detect adenosine-based peaks corresponding to accumulated PAPS and PAP (Figure 10B). Relative to PAPS, we observed a 10-fold increase in accumulated PAP in Bpnt1 null
livers, suggesting that PAP accounts for the majority of the enzymatic assay’s results (Figure 10B).

Figure 10: Accumulation of PAP and PAPS in Bpnt1 null livers.

(A) Quantification of PAPS and PAP content in wild-type, heterozygote, and knockout livers as measured by enzymatic assay. Values represent mean +/- SEM. (B) Chromatogram of small molecule extracts from wild-type and knockout liver tissue analyzed by SAX-HPLC-UV. Note the significant accumulation of PAP relative to PAPS. (C) Accumulation of aberrant 5’-extended 5.8S rRNA subunits in knockout liver RNA as detected by Northern blot.
In yeast deficient for Bpnt1, PAP has been shown to inhibit the activity of Xrn2 (Rat1p), a conserved 5’-3’ exoribonuclease, and impair the enzyme’s ability to process the pre-5.8S ribosomal RNA subunit (39). Despite the significant evolutionary divergence between yeast and mammals, we wondered whether high levels of intracellular PAP might also inhibit mouse Xrn2, and thus serve as a measure of PAP accumulation in vivo. To examine this possibility, we isolated total RNA from wild-type, heterozygote, and Bpnt1 null livers and used northern blotting to probe for both 5’-extended and total 5.8S rRNA subunits (Figure 10C). Remarkably, only knockout livers had any detectable accumulation of 5’-extended 5.8S rRNA, corroborating our in vitro PAP analyses and demonstrating the potential for evolutionary conservation of PAP-sensitive cellular machinery (Figure 10C).

### 2.2.4 Genetic Suppression of Hepatic Defects in Bpnt1 Null Mice

The mechanistic link between loss of Bpnt1 and the resulting hepatic defects could be explained by multiple possibilities: (i) elevated levels of the substrate PAP, (ii) reduced levels of the end product 5’-AMP, or (iii) a combination thereof. To address this, we took advantage of a mouse strain known as the brachymorphic mouse (Papss2<sup>bm/bm</sup>) that harbors a hypomorphic mutation in PAPS Synthase 2, one of two gene products responsible for the production of PAPS from inorganic sulfate (Figure 2B and Figure 11A) (52, 61, 62).
Figure 11: Rescue of hepatic insufficiencies by repressing bisphosphorylated nucleotide synthesis.

(A) Schematic illustrating the production of PAPS, PAP, and 5’-AMP in wild-type, Bpnt1−/−, and double KO animals. Values represent mean +/- SEM. (B) Quantification of combined PAPS and PAP levels in liver tissue as measured by enzymatic assay. Note the significant reduction in PAPS and PAP in double KOs. (C) Northern blot of 5’-extended 5.8S rRNA subunits. Note that while double KOs still contain detectable levels of unprocessed 5.8S, the quantity compared to Bpnt1−/− is greatly reduced. (D)
Photographs of wild-type, Bpnt1+/−, and double KO liver tissue stained for nucleolar-resident fibrillarin by immunohistochemistry. Scale bar: 10 µm. (E) Quantification of serum albumin levels in wild-type, Bpnt1+/−, and double knockout animals. Values represent mean +/- SEM.

We hypothesized that by expressing the brachymorphic allele, which has slower kinetics of hepatic PAPS synthesis (dashed arrows), in a Bpnt1 null background we would be able to repress PAP accumulation in the absence of 5′-AMP production (Figure 11A) (62, 63). In order to test this, we crossed Bpnt1+/− and Papss2+/−/−/− mice to obtain double heterozygotes, which were subsequently intercrossed to yield Bpnt1+/−Papss2+/−/−/− “double knockouts.” Compared to Bpnt1+/−Papss2+/−/−/− littermates, double knockout livers contained a significant reduction in the levels of accumulated PAP and a corresponding repression of unprocessed 5.8S rRNA (Figure 11B and Figure 11C). Analysis of serum chemistries revealed that double knockout animals contained lower levels of the liver damage markers ALT, AST, and ALKP and histologically double knockout hepatocytes appeared grossly normal with fewer than 10% of nuclei containing visibly condensed nucleoli (Table 2 and Figure 11D). However, most strikingly, double knockouts possess near wild-type levels of serum albumin and we have detected no incidence of edema throughout adulthood (Figure 11E).

Table 2: Serum chemistries of Bpnt1 Papss2 double KO mice.

Serum clinical chemistries from wild-type, Bpnt1+/−, double KO, and Papss2+/−/−/−/− animals illustrating the repression of hepatic defects by downregulation of bisphosphorylated nucleotide synthesis. Value represent mean +/- SEM.
2.3 Discussion

Our data demonstrate an important role for the cytoplasmic 3’-nucleotidase Bpnt1 in normal liver function. Loss of Bpnt1 results in severe hepatic deficiencies and frequently leads to liver failure and death. These phenotypes are markedly distinct from the skeletal abnormalities we reported for Golgi 3’-nucleotidase deficient mice and suggest that compartmentalization, or possibly differential tissue distribution, may account for the evolution of fundamentally unique roles for 3’-nucleotidases in cellular and organismal physiology. Overall, Bpnt1 null mice provide a unique model for studying cytoplasmic PAP metabolism, nucleolar dynamics, and liver disease and give insight into the biological roles of 3’-nucleotidases in mammals.

Mechanistically, our ability to rescue the defects of Bpnt1 null mice by concomitantly repressing bisphosphorylated nucleotide synthesis provides an important molecular basis for how loss of cytoplasmic 3’-nucleotidase activity might lead to impaired hepatic function. Our results are most consistent with a model in which the accumulation of PAP or another substrate of Bpnt1, as opposed to inadequate 5’-AMP synthesis, leads directly to impaired hepatic translation and hypoproteinemia. This

<table>
<thead>
<tr>
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<th>Alb (g/dL)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>ALKP (U/L)</th>
<th>Incidence of Edema</th>
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<tr>
<td>wild-type</td>
<td>3.1 ± 0.12</td>
<td>40 ± 3</td>
<td>116 ± 26</td>
<td>207 ± 14</td>
<td>0/122</td>
</tr>
<tr>
<td>Bpnt1⁻/⁻</td>
<td>0.9 ± 0.08</td>
<td>333 ± 75</td>
<td>580 ± 75</td>
<td>1628 ± 452</td>
<td>17/38</td>
</tr>
<tr>
<td>double KO</td>
<td>2.7 ± 0.29</td>
<td>239 ± 131</td>
<td>270 ± 83</td>
<td>484 ± 137</td>
<td>0/28</td>
</tr>
<tr>
<td>Papss²⁰⁴⁶⁷/⁴⁶⁷</td>
<td>3.4 ± 0.10</td>
<td>39 ± 7</td>
<td>121 ± 13</td>
<td>230 ± 55</td>
<td>n.d.</td>
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model appears to be evolutionarily conserved as it consistent with observations reported in yeast. In contrast, the skeletal abnormalities and neonatal lethality that arise from loss of the Golgi-localized 3’-nucleotidase were not rescued by concomitant repression of bisphosphorylated nucleotide synthesis, in fact gPAPP<sup>gt/gt</sup>Papss<sub>2<sup>bm/bm</sup></sub> double mutant embryos appear more affected than inactivation of gPAPP alone (Table 3).

**Table 3: Outcomes of gPAPP Papss2 double heterozygote matings.**

Genotypes of pups resulting from gPAPP Papss2 double heterozygote crosses that survived to 13 days postnatal. Note the complete absence of gPAPP<sup>gt/gt</sup> pups in Papss<sub>2<sup>++/+</sup></sub>, Papss<sub>2<sup>+/bm</sup></sub>, or Papss<sub>2<sup>bm/bm</sup></sub> backgrounds.

<table>
<thead>
<tr>
<th>Genotype</th>
<th># of mice</th>
<th>Expected frequency</th>
<th>Actual frequency</th>
</tr>
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<tbody>
<tr>
<td>gPAPP&lt;sup&gt;+/+&lt;/sup&gt; Papss&lt;sub&gt;2&lt;sup&gt;+/+&lt;/sup&gt;&lt;/sub&gt;</td>
<td>11</td>
<td>6.25%</td>
<td>11.34%</td>
</tr>
<tr>
<td>gPAPP&lt;sup&gt;+/+&lt;/sup&gt; Papss&lt;sub&gt;2&lt;sup&gt;+/bm&lt;/sup&gt;&lt;/sub&gt;</td>
<td>12</td>
<td>12.50%</td>
<td>12.37%</td>
</tr>
<tr>
<td>gPAPP&lt;sup&gt;+/+&lt;/sup&gt; Papss&lt;sub&gt;2&lt;sup&gt;bm/bm&lt;/sup&gt;&lt;/sub&gt;</td>
<td>6</td>
<td>6.25%</td>
<td>6.19%</td>
</tr>
<tr>
<td>gPAPP&lt;sup&gt;+/gt&lt;/sup&gt; Papss&lt;sub&gt;2&lt;sup&gt;+/+&lt;/sup&gt;&lt;/sub&gt;</td>
<td>19</td>
<td>12.50%</td>
<td>19.59%</td>
</tr>
<tr>
<td>gPAPP&lt;sup&gt;+/gt&lt;/sup&gt; Papss&lt;sub&gt;2&lt;sup&gt;+/bm&lt;/sup&gt;&lt;/sub&gt;</td>
<td>31</td>
<td>25.00%</td>
<td>31.96%</td>
</tr>
<tr>
<td>gPAPP&lt;sup&gt;+/gt&lt;/sup&gt; Papss&lt;sub&gt;2&lt;sup&gt;bm/bm&lt;/sup&gt;&lt;/sub&gt;</td>
<td>18</td>
<td>12.50%</td>
<td>18.56%</td>
</tr>
<tr>
<td>gPAPP&lt;sup&gt;gt/gt&lt;/sup&gt; Papss&lt;sub&gt;2&lt;sup&gt;+/+&lt;/sup&gt;&lt;/sub&gt;</td>
<td>0</td>
<td>6.25%</td>
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<tr>
<td>gPAPP&lt;sup&gt;gt/gt&lt;/sup&gt; Papss&lt;sub&gt;2&lt;sup&gt;+/bm&lt;/sup&gt;&lt;/sub&gt;</td>
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<td>12.50%</td>
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<tr>
<td>gPAPP&lt;sup&gt;gt/gt&lt;/sup&gt; Papss&lt;sub&gt;2&lt;sup&gt;bm/bm&lt;/sup&gt;&lt;/sub&gt;</td>
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<td>6.25%</td>
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<tr>
<td><strong>total</strong></td>
<td><strong>97</strong></td>
<td><strong>100.00%</strong></td>
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This provides a mechanistic and physiological distinction between the two mammalian 3’-nucleotidases and substantiates our conclusion that loss of Bpnt1 results in impaired cellular function through toxic accumulation of PAP and/or potentially additional substrates.
In addition to PAP, Bpnt1 has been shown to hydrolyze its sulfated precursor PAPS and a number of other 3’-phosphorylated nucleotides including PCP and PGP (30). Although Michaelis-Menten constants for PAPS hydrolysis have not been reported, competitive inhibition experiments have suggested that murine Bpnt1 can degrade PAP and PAPS with roughly equivalent catalytic efficiencies (30). Relative to wild-type, Bpnt1 null livers contain elevated levels of PAP and PAPS, suggesting that both are substrates in vivo. However, we observed 10-fold higher levels of PAP than PAPS and did not detect the accumulation of any other previously reported substrates. Therefore, while we cannot rule out that PAPS or another 3’-phosphorylated nucleotide contributes, we postulate that the accumulation of PAP is primarily responsible for the development of liver failure in Bpnt1 null mice.

There are a number of potential cellular targets that may be influenced by the dramatic accumulation of PAP observed in hepatocytes. It is well established that PAP competes strongly with PAPS in vitro for binding to SULTs, leading to a nonproductive SULT/substrate/PAP complex (64, 65). Yet, because we are able to rescue the defects of Bpnt1 null mice by repressing PAPS synthesis, and thereby depleting the substrate of SULTs, it is unlikely that impaired sulfation is responsible for the liver failure. As mentioned previously, PAP has also been shown to directly inhibit the nuclear-resident 5’-3’ exoribonuclease Xrn2. In yeast, high levels of PAP inhibit Xrn2 and lead to the accumulation of aberrant unprocessed RNA substrates, including the 5.8S rRNA
subunit. Indeed, we were able to detect immature partially unprocessed 5.8S rRNA species exclusively in Bpnt1 null livers (Figure 10C). In addition, its accumulation is repressed in double KOs and correlates with the reduction in PAP (Figure 11B). However, it is important to note that the relative levels of mature 5.8S rRNA appear normal in Bpnt1 null liver tissue. These data indicate that the rate of rRNA processing may be altered or that a specialized minor pool of substrates is failing to mature. Thus, while we suspect that impaired Xrn2 activity may contribute to the development of liver failure in Bpnt1 null mice, further study will be necessary to delineate its precise role.

The observed globally repressed translation, reduced levels of rough endoplasmic reticulum, aberrant nucleolar structure, and accumulation of immature ribosomal RNA in Bpnt1 null liver are consistent with a model in which PAP accumulation may alter ribosome biogenesis. Mammalian ribosomes, which contain nearly 7kb of RNA and more than 100 core and accessory ribosomal proteins, represent an immensely complex assembly process and comprise a significant fraction of total energy expenditure, particularly in professional metabolic cells such as hepatocytes (66, 67). Of interest, in our mutant animals, we did not observe other reported markers of impaired ribosome biogenesis including the stabilization of p53 and cell cycle arrest (data not shown) (68). Our results indicate that certain aspects of hepatic ribosome biogenesis or functionality are disrupted in the absence of Bpnt1 and provide a basis for directing future studies aimed at determining precisely how this process is affected.
Our data describe an unexpected role for the 3′-nucleotidase Bpnt1 in hepatic function. As a result of global translation repression in the liver, Bpnt1 null mice are unable to maintain sufficient serum protein levels, leading to whole body edema and lethality. Through the use of biochemical analysis and forward genetics we were able to determine that the accumulation of Bpnt1’s substrate PAP is necessary for the development of liver failure. We also discovered a significant morphological alteration to Bpnt1 null hepatocytes that strongly correlated with the accumulation of PAP and suggested that condensed nucleoli might be useful as a histological biomarker for impaired cytoplasmic 3′-nucleotidase activity in future studies. Because of the unique presentation, our findings help to define a novel murine model of intrinsic hepatocellular disease, one that is due to a previously undescribed molecular defect and not the result of a dietary change or pharmacological insult. In addition, because the key components of the sulfate assimilation pathway are strongly conserved among mammals, we suspect that loss of function mutations in human Bpnt1 might recapitulate the hepatic defects seen in null mice and provide a genetic basis for a subset of idiopathic liver pathologies.

2.4 Materials and Methods

2.4.1 Animals

Bpnt1 null mice were generated using a standard homologous recombination approach (Figure 3). Briefly, we replaced the 4th and 5th exons of the Bpnt1 locus in
129/SvEv ES cells with a neomycin resistance cassette. Cells resistant to neomycin were confirmed by PCR and Southern blotting, injected into blastocysts and implanted into pseudopregnant females by the University of North Carolina Animal Models Core. Two chimeric founders were identified by Southern blotting and bred into the C57BL/6J background. Bpnt1 mice were genotyped by multiplex PCR using the following primers: (a) 5’-ctatatgtcctagcacttgagagg-3’; (b) 5’-accaagaacggagccggttggcg-3’; and (c) 5’-aggtcggacaccttgtctctagtc-3’. Papss2 brachymorphic mice (B6C3Fe a/a-Papss2\textsuperscript{bm}/J from The Jackson Laboratory), were crossed with Bpnt1 heterozygotes to obtain F1 Bpnt1\textsuperscript{+/-} Papss2\textsuperscript{+/bm} progeny, which were subsequently intercrossed to generate double knockouts. Papss2 mice were genotyped by amplifying the brachymorphic locus using the following primers: (a) 5’-accaccattcctgttgcggttc-3’ and (b) 5’-cgcctggtgcccacatcacc-3’. Following digestion with FokI, which specifically cuts the wild-type allele, the 180 bp (wild-type) and 210 bp fragments (Papss2\textsuperscript{bm/bm}) were separated on 3% agarose gels.

Animal care and experiments were performed in accordance with the National Institutes of Health guidelines and approved by the Duke University Institutional Animal Care and Use Committee.

2.4.2 Serum Analysis and In Vivo 3H-Leucine Protein Labeling

Following sacrifice by CO\textsubscript{2} exposure, whole blood was collected from the right ventricle. Serum was purified using serum separator tubes (BD), snap frozen in liquid nitrogen, and stored at -80°C. Serum chemistries including albumin levels and
liver/kidney panels were analyzed by the University of North Carolina Animal Clinical Chemistry and Gene Expression Laboratory. For in vivo serum protein labeling, two heterozygote and two homozygous null animals were each injected intraperitoneally with 150µCi of 3H-Leucine (Perkin-Elmer). 30 minutes following injection, animals were sacrificed and serum was isolated as above. 2µL of each serum sample were run on duplicate 10% SDS polyacrylamide gels. One gel was treated for fluorography using EN3HANCE (Perkin-Elmer) according to the manufacturer’s instructions and exposed to BioMax MS film (Kodak) for 30 days at -80°C. The other gel was stained with Coomassie Blue for reference, band isolation, and mass spectrometry. Mass spectrometry was performed in collaboration with T. Haystead’s lab at Duke University.

2.4.3 Histology, Staining, and Electron Microscopy

For tissue analysis, animals were sacrificed by CO₂ exposure, blood was collected by cardiac stick, and the body was perfused transcardially with 30mL of phosphate-buffered saline pH 7.4. Tissues for histology were fixed in 10% formalin (VWR) for 2 days then embedded in paraffin by the Duke University Medical Center Immunohistology Research Laboratory. 5µm sections were stained for H&E by standard protocols. For immunohistochemistry, sections were blocked, stained, and visualized with DAB according to standard procedures. Primary antibodies recognizing fibrillarin (Abcam) and B23 (Santa Cruz Biotechnology) were incubated at 4°C overnight. Tissues for Oil Red O staining were fixed in 4% paraformaldehyde/1X PBS overnight,
cryoprotected overnight in 30% sucrose 0.1M phosphate buffer at 4°C, and sectioned at 10μm using a Leica CM3050 S cryostat. Oil Red O staining was performed according to standard procedures. Slides were imaged on a Nikon TE2000 inverted fluorescent microscope. Tissues for electron microscopy were fixed, processed, and stained by the Duke Electron Microscopy Service in the Department of Pathology. Hepatic lipids were extracted by the Folch method, separated on silica gel TLC plates (GE Healthcare), and visualized by charring.

2.4.4 Liver PAPS and PAP analysis

Hepatic PAPS and PAP levels were measured using a combination of two previously published protocols (59, 60). Briefly, frozen liver slices (~150 mg) were boiled for 3 minutes in 5 μL of PAP isolation buffer [50 mM glycine (pH 9.2)] per mg of tissue and disrupted using a PowerGen 700 homogenizer (Fisher Scientific – power “4”) and disposable hard tissue generators (Omni International). This process was repeated once more before transferring the samples to ice. Homogenates were clarified by at 16,100 x g, 4°C for 20 minutes. Following addition of 0.2 volumes of CHCl₃, mixtures were shaken vigorously and then centrifuged at 16,100 x g, 4°C for 20 minutes. Finally, the upper aqueous phases were collected. The final extract was stable at -80°C for at least 3 months. To quantify PAP levels, we developed a simple colorimetric microplate absorbance assay in which recombinant mouse Sult1a1-GST is used to transfer a sulfate group from p-nitrophenyl sulfate to 2-naphthol, using PAPS or PAP as a catalytic cofactor. Briefly,
10 µL of the tissue lysate or PAP standard was incubated with 190 µL of PAP reaction mixture [100 mM bis-tris propane (pH 7.0), 2.5 mM β-mercaptoethanol, 2.5 mM p-nitrophenyl sulfate, 1 mM β-naphthol, and 1 µg of PAP-free recombinant mouse GST-Sult1a1]. Reactions velocities were determined by monitoring the production of 4-nitrophenol at 400 nm. Unknown concentrations of PAP in lysates were interpolated from Michaelis-Menten one-site binding curves of initial velocity vs. PAP concentration via Prism 5 software (GraphPad).

For HPLC analysis, extracts were isolated with 0.6 M TCA by homogenization. Following TCA extraction and neutralization with ethyl-ether, lysates were clarified by centrifugation. Nucleotides were then separated by HPLC (Waters) using a 5 µm partisphere SAX column (Whatman) on a 60 minute linear gradient from 10 mM NH₄H₂PO₄ (pH 3.7) to 500 mM KCl, 250 mM NH₄H₂PO₄ (pH 4.5). Eluted nucleotides were detected by inline UV absorption at 259 nm (Waters).

### 2.4.5 Northern Blotting

Total tissue RNA was isolated using a standard Trizol (Invitrogen) extraction protocol. 5 µg of total liver RNA was separated on 5% denaturing TBE-urea polyacrylamide gels and transferred to Hybond-N+ membranes (Amersham). Blots were UV crosslinked then dried at 80°C for two hours. Blots were hybridized in ExpressHyb buffer overnight at 55°C using a rotating micro hybridization oven (Bellco). ³²P-radiolabeled probes were generated from the following primers: 5.8S long antisense (5’-
CGTATCGGTATTTCGGGTGTGAGCG (5’-3‘) and 5.8S total antisense (5’-CGAAGTGTCGATGATCAATGTGTCC-3‘) using 5’ end labeling with T4 polynucleotide kinase (New England Biolabs) and purified by G-25 sephadex spin columns (GE Healthcare). All hybridizations were performed using 5x10^6 DPM and visualized by phosphorimagery (GE Healthcare).

2.4.6 Cell Fractionation and Western Blotting

For tissue fractionation, animals were sacrificed by CO₂ exposure and perfused with 30mL of 1x PBS (pH 7.4) transcardially. Fresh liver tissue (~100mg) was isolated, weighed, and homogenized in 3 volumes of ice-cold isotonic lysis buffer [250 mM sucrose, 50 mM Tris-Cl (pH 8.0), 25 mM KCl, 5 mM MgCl₂, and 1x complete protease inhibitors (SigmaFast tablets)] using a 15-mL glass-glass dounce homogenizer. The samples were then filtered through a 70 µm cell strainer and centrifuged sequentially at 800 x g, 16,100 x g, and 100,000 x g, to separate the various soluble and pellet fractions. Protein concentrations were determined and normalized using the BioRad DC Protein Assay. Samples were then separated by SDS-PAGE and transferred to nitrocellulose for western blotting using standard procedures. Polyclonal rabbit antibodies recognizing mouse Bpnt1 were raised against full-length recombinant protein in rabbits, affinity purified, and incubated 1/1000 overnight at 4°C. Primary antibodies recognizing PDI and Ribosomal Protein S6 were purchased from Cell Signaling Technology. Blots were visualized using the LI-COR infrared detection system. For Bpnt1 multi-tissue expression analysis, tissues were isolated and rapidly frozen in liquid nitrogen. Frozen tissues were then weighed and
homogenized as above in 20 volumes of soluble lysis buffer [50 mM Na-HEPES (pH 7.5),
150 mM NaCl, 1mM DTT, and 1x complete protease inhibitors (SigmaFast tablets)] on
ice. Lysates were clarified at 16,100 x g and normalized using the BioRad Protein Assay.
Samples were then blotted for Bpnt1 using the procedure described above.
3. Loss of Bpnt1 in the small intestine leads to defects in iron absorption and anemia

3.1 Introduction

Iron is an essential dietary input that serves as a cofactor for numerous metabolic reactions and is necessary for the transport of oxygen throughout the body (69, 70). In mammals, iron is absorbed exclusively by the small intestine (69). Numerous disease states result from an imbalance in systemic iron stores including hereditary hemochromatosis (excess iron) and anemia (insufficient iron) (69). Hereditary hemochromatosis, the most common genetic iron imbalance in humans, results in tissue iron overload and eventual organ failure (71). Likewise, anemia due to chronic dietary iron deficiency is a pervasive world-wide problem, affecting as many as 2 billion people (72).

Because mammals are unable to modulate the excretion of iron, bodily stores are regulated by the rate of absorption in the small intestine (70). Iron is ingested either in complex as heme or as iron salts. Following solubilization in the acidic stomach environment, free iron is absorbed by enterocytes in the duodenum, a short segment of the small intestine immediately downstream of the stomach. Before absorption, iron is first reduced from Fe$^{3+}$ (ferric) to Fe$^{2+}$ (ferrous) by duodenal cytochrome $b$ (Dcytb) (73). Although it has been postulated to be important for iron uptake, Dcytb knockout mice display normal iron absorption, suggesting that mice have evolved additional mechanisms, perhaps via other reductases (74) or the endogenous biogenesis of the
reducing agent ascorbic acid (75). Following reduction to Fe\(^{2+}\), iron is transported across the apical cell membrane by divalent metal transporter 1 (Dmt1) (76, 77). Once intracellular, iron can be stored in large ferritin complexes or exported across the basolateral membrane to the blood stream by ferroportin (Fpn) (78-80), where it is subsequently reoxidized to Fe\(^{3+}\) by hephaestin (Hfe) (81), bound to the plasma iron transporter transferrin (Tf), and delivered to tissues expressing the transferrin receptor (Tfr1) throughout the body.

In order to regulate iron absorption, both the duodenum and liver are able to monitor and adapt to changes in bodily iron stores. In iron replete situations, the liver produces the excreted peptide hepcidin (82) (82, 83), which binds to Fpn and promotes its internalization and degradation (84). This leads to an accumulation of iron in enterocyte ferritin granules (85), which are eventually excreted following the rapid turnover of enterocytes as they are sloughed off into the lumen. In addition to the production of Hamp, iron availability is also monitored by the RNA binding proteins iron-regulatory proteins 1 and 2 (Irp1/2) in the duodenum. In response to low intracellular iron concentrations, Irp1/2 bind to and stabilize transcripts encoding iron response elements, resulting in an increase in iron absorption (86).

Here we report the unexpected finding that mice deficient for the cytosolic 3’-nucleotidase phosphatase bisphosphate 3’-nucleotidase 1 (Bpnt1) in the small intestine develop iron deficiency anemia as a result of impaired iron uptake by the duodenum.
Loss of Bpnt1, which is enriched in the duodenum and proximal jejunum, results in an accumulation of its substrate PAP and defects in dudodenal morphology and functionality. Our results suggest that Bpnt1 plays an important role in maintaining the viability of the proximal small intestine and is essential for normal iron metabolism.

3.2 Results

3.2.1 Global and intestine specific Bpnt1 null mice are iron deficient anemic

Bpnt1 is expressed in a number of tissues but is particularly enriched in the small intestines and kidney (Figure 12).
Figure 12: Expression profile of Bpnt1 in mice.

(A) Western blot of Bpnt1 protein and B-tubulin loading control from various tissues of wild-type and Bpnt1 null mice. (B) Relative specific activity of Bpnt1 in the same tissues normalized to total soluble protein content.

In the small intestine Bpnt1 is most strongly expressed in the early intestine and decreases in abundance towards the ileum and colon (Figure 13).
Expression of Bpnt1 in wild-type mice along the small intestine measured by Western blot and normalized to liver. Note that Bpnt1 is most strongly expressed in the early small intestine.

To study the role of Bpnt1 specifically in the intestines, we used homologous recombination to generate mice harboring LoxP recombination sites flanking the 4th and 5th exons of the Bpnt1 locus (Figure 14A). The floxed mice were crossed with mice harboring a Villin-Cre transgene that drives the expression of Cre recombinase in the enterocytes of the small intestine and to a lesser extent in the colon (87). Offspring of these crosses yielded mice specifically lacking Bpnt1 in the small intestine (Figure 14B and Figure 14C). Importantly, these mice accumulate PAP and PAPS only in the small intestine with no detectable elevation in the liver or kidneys (Figure 15).
Figure 14: Generation of intestine specific Bpnt1 null mice.

(A) Bpnt1 genomic locus and floxed allele targeting scheme. Note the flanking of the 4th and 5th exons with LoxP recombination sites. (B) Multiple tissue western blot of
Bpnt1<sup>−/−</sup> mice with (right) or without (left) the expression of Villin-Cre to induce intestine-specific recombination. (C) Immunohistochemistry for Bpnt1 in the small intestines of wild-type (left) and intestine-specific Bpnt1 null mice (right). Note the lack of detectable protein in the enterocytes lining the lumenal side face of the intestinal villi.

![Graph showing accumulation of PAPS and PAP specifically in the intestines of Bpnt1<sup>−/−</sup> mice.](image)

Figure 15: Accumulation of PAPS and PAP specifically in the intestines of Bpnt1<sup>−/−</sup> mice.
Intestine specific Bpnt1 null mice (Bpnt1^{int}) gain weight normally and are indistinguishable from their wild-type and heterozygote littermates from birth through adolescence. However, by 8 weeks of age the fur of Bpnt1^{int} mice appears dull and they develop moderate alopecia (Figure 16B). Because alopecia is frequently associated with anemia, we investigated whether Bpnt1^{int} mice had defects in their hematopoiesis. First, we isolated whole blood from 8-week-old mice and measured complete blood counts (CBCs). We found that Bpnt1^{int} mice displayed symptoms of anemia and presented with repressed levels of hemoglobin (Hb) and reduced mean corpuscular volumes (MCV) (Figure 16B). Examination of whole blood stained with Wright-Giemsa revealed the presence of hollow, hypochromic red blood cells, confirming that Bpnt1^{int} mice have microcytic anemia (Figure 16C).
Figure 16: Alopecia and microcytic anemia in Bpnt1 null mice.

(A) Schematic of a portion of the Bpnt1 genomic locus depicting the various genotypes of the mice used for subsequent experiments. (B) Photograph of Bpnt1^fl/fl and Bpnt1^int/int mice at 8 weeks postnatal demonstrating the gray and thinning hair that develops progressively with age in intestine-specific Bpnt1 null mice. (C) Hematological parameters of Bpnt1^fl/fl and Bpnt1^int/int mice. (D) Wright-Giemsa stain of Bpnt1^fl/fl (left) and Bpnt1^int/int (right) blood smears. Note the reduced volume and hemoglobin content of Bpnt1^int/int RBCs, indicative of microcytic anemia.

One cause of hypochromic, microcytic anemia is systemic iron deficiency. To investigate whether Bpnt1 null mice were iron deficient, we measured the total iron burden in liver and splenic tissue. Livers and spleens of Bpnt1^int/int mice contained approximately 50% and 33% as much iron respectively as wild-type tissues, indicating that the anemia is likely the result of a systemic iron deficiency (Figure 17A). To confirm this, we isolated bone marrow from the femurs of Bpnt1^fl/fl and Bpnt1^int/int mice and stained for the presence of iron-containing macrophages. Compared to the readily detectable iron-positive macrophages in Bpnt1^fl/fl mice, bone marrow from Bpnt1^int/int mice contained no detectable iron-positive cells, demonstrating that the hematopoietic insufficiency is likely a direct result of depleted iron stores (Figure 17B).

In mammals, iron stores are regulated by the rate of iron absorption in the intestine. In situations of iron overload, the liver secretes hepcidin, a small peptide that binds to the iron transporter ferroportin (Fpn) at the basolateral membrane of enterocytes and stimulates its internalization and degradation. The sequestered intracellular iron is then released into the feces through the normal sloughing of enterocytes from the tips of the villi. In order to determine whether the livers of Bpnt1^int/
mice were responding appropriately to iron deficiency, we isolated total liver RNA and assessed the levels of Hamp mRNA by quantitative reverse transcriptase PCR (qRT-PCR). Relative to Bpnt1⁻/⁻ mice, intestine-specific knockouts contained approximately 1000-fold less liver Hamp mRNA (Figure 17C). This demonstrated that the livers of Bpnt1⁻/⁻ mice were compensating for the iron deficiency by repressing Hamp expression, but that the duodenum was unable to integrate the signal or stimulate additional iron absorption.

![Graph showing iron levels in spleen and liver tissue](image)

![Perls' iron stain of femur-derived bone marrow](image)

![qRT-PCR for Hepcidin](image)

**Figure 17:** Bpnt1 null mice are iron deficient.

(A) Total iron concentrations of spleen and liver tissue from intestine-specific Bpnt1 null mice. Note that only Bpnt1⁻/⁻ mice are significantly anemic. (B) Perls’ iron stain of femur-derived bone marrow where blue represents intracellular iron deposits demonstrating the absence of iron containing macrophages in Bpnt1⁻/⁻ mice (right). (C) qRT-PCR for Hepcidin in Bpnt1⁻/⁻ and Bpnt1⁻/⁻ livers.
To confirm that the observed anemia was due to a defect solely in iron absorption, we supplemented severely anemic 1-year-old global Bpnt1 null mice with subcutaneous iron-dextran. Mice were injected with 5 mg of iron-dextran or saline weekly for a total of 3 weeks and then assessed for the continued presence of iron deficiency and anemia. Compared with saline injected controls, Bpnt1 null mice that received exogenous iron had replete iron stores and presented with normalized hematological values (Figure 18). This suggested that Bpnt1 null mice are able to transport, store, and use available iron for normal hematopoiesis but are unable to obtain sufficient quantities due to a defect in dietary absorption.
Figure 18: Exogenous subcutaneous iron is able to rescue the anemia in Bpnt1 null mice.

(A) Total iron content of liver and splenic tissue following iron supplementation. Note that both wild-type and Bpnt1 null animals are able to store excess iron. (B) Hemoglobin concentrations following 3 weeks of subcutaneous iron injections.

3.2.2 Loss of Bpnt1 in the intestines results in aberrant duodenal and intestine morphology

The duodenum, which is responsible for iron absorption, is a short segment of the small intestine immediately following the stomach. Gross observation revealed that
Bpnt1 null duodenums are enlarged and have a dilated appearance, suggesting that the iron deficiency anemia might be due to a defect in duodenal function (Figure 19).

Figure 19: Enlarged and dilated proximal small intestines in Bpnt1 null mice.

(A) Photograph of the stomach and first 4 cm of fixed small intestines from 1 year old wild-type and Bpnt1 null animals. The dashed line delineates the pyloric junction between the stomach and duodenum. (B) Average weights of 3 cm small intestine segments from 1 year old mice. Note the significant enlargement of the proximal duodenum as compared to the more distal jejunum and ileum.

Histologically, hematoxylin and eosin staining revealed that Bpnt1\textsuperscript{hi} duodenal enterocytes were abnormally basophilic and displayed aberrant elongated nuclei (Figure
20A and B). Further, the nuclei of enterocytes contained large condensed nucleoli, strikingly similar to those observed in Bpnt1 null hepatocytes (Figure 20C). Immunostaining for the nucleolar-resident protein fibrillarin along the small intestine revealed that the condensed nucleoli were detectable in both the duodenum and early jejunum but not in the late jejunum or ileum.
Figure 20: Aberrant cellular and nuclear morphology in Bpnt1 null intestines.

(A) H&E stain of the distal duodenums of Bpnt1^{−/−} and Bpnt1^{−/int} mice. Note the decrease in eosinophilic (protein) and relative increase in basophilic (nucleic acid) staining intensity in Bpnt1 null enterocytes. (B) Higher magnification of duodenal
villi. Nuclei of Bpnt1 null enterocytes have an elongated and hypertrophied appearance as compared to the more circular nuclei seen in Bpnt1−/− enterocytes. (C) Immunohistochemistry for fibrillarin, a marker of the dense fibrillar layer of the nucleolus. Bpnt1 null enterocytes display a single condensed enlarged nucleolus.

We wondered whether the appearance of condensed nucleoli was related to the accumulation of PAP in Bpnt1 deficient enterocytes as was originally seen in Bpnt1 null livers. Indeed, we found a sharp decline in PAP levels between the early and late jejunum, coinciding closely with the disappearance of the condensed nucleoli (Figure 21).
3.2.3 Loss of Bpnt1 results in impaired apical iron transport in enterocytes

Following requisite solubilization in the acidic environment of the stomach, iron is taken up by differentiated enterocytes lining the upper villi of the duodenum. Because iron must be absorbed across both the apical and basolateral membranes of enterocytes en route from the intestinal lumen to the bloodstream, we first wondered whether the systemic iron deficiency might be due to a defect in iron transport across the basolateral membrane. We hypothesized that if basolateral transport was defective, iron would...
accumulate in enterocytes. To address this, we isolated duodenums from Bpnt1^{-/l} and Bpnt1^{-/int} mice and analyzed their iron content through biochemical and histological techniques. We found that Bpnt1^{-/int} mice had no detectable increase in iron content either by staining and a decrease in iron content by biochemical analysis, suggesting that iron was not accumulating in duodenal enterocytes and that a defect in basolateral transport was not responsible for the iron deficiency (Figure 22).
Figure 22: Bpnt1 null enterocytes do not accumulate any detectable excess iron.

Perls’ iron stain of wild-type and Bpnt1 deficient duodenum reveals cellular proteins in pink and iron deposits in blue. A section of iron-loaded human liver serves as a positive control for the staining quality.

Iron is transported across the apical membrane of enterocytes by a single 12-pass transmembrane channel, divalent metal transporter 1 (Dmt1) (77). Normally, in response to iron deficiency, Dmt1 transcript and protein levels are upregulated to promote the
uptake of dietary iron through the stabilization of Dmt1 transcript by Irp1/2. We hypothesized that if Bpnt1\textsuperscript{-/-} mice were unable to upregulate Dmt1 in response to depleted iron stores, then they would be further sensitized to diet-induced anemia. To determine whether the iron deficiency might be due to a defect in the transport of iron by Dmt1, we fed wild-type and intestine-specific Bpnt1 null mice an iron-deficient diet for 5 weeks starting at weaning (Figure 23A). Indeed, while iron-restricted wild-type and heterozygote animals displayed only moderately repressed levels of hemoglobin, intestine-specific knockouts presented with drastically reductions in hemoglobin concentrations, suggesting that these mice are unable to compensate for the change in dietary iron and that insufficient iron transport across the apical membrane is responsible for the iron deficiency anemia (Figure 23B).
Figure 23: Increased sensitivity to iron deficient diets in intestine-specific Bpnt1 null mice.

(A) Wild-type or Bpnt1<sup>d<sub>int</sub></sup> (KO) mice were transitioned from standard chow to purified diets with or without iron and maintained on these diets for 5 weeks before being sacrificed. (B) Hemoglobin concentration of mice challenged with iron deficient or control diets for five weeks. Note the decrease in hemoglobin concentrations of Bpnt1<sup>d<sub>int</sub></sup> mice on iron-deficient chow relative to wild-types.

3.3 Discussion

Our data demonstrate an important role for the cytoplasmic 3′-nucleotidase Bpnt1 in normal intestine function. Loss of Bpnt1 either globally or specifically in the
small intestine results in iron-deficiency anemia as a result of impaired iron absorption by duodenal enterocytes. Our study has helped to uncover an unexpected link between cytoplasmic 3′-nucleotide hydrolysis and intestinal function and provides further insight into the roles of Bpnt1 in mammals.

How does loss of Bpnt1 result in impaired iron absorption? As we have previously demonstrated in the liver, inactivation of Bpnt1 in the small intestine results in an accumulation of the substrates PAPS and PAP. In liver, high levels of PAPS and PAP are cytotoxic, resulting in numerous cell defects, hepatocyte dysfunction, and eventual organ failure. Given the compelling similarities observed between the alterations to nuclear morphology in hepatocytes and enterocytes, we suspect that like hepatocytes, the impairment of nuclear-resident 5′-3′ exoribonuclease activity might be mediating at least some of the defects in Bpnt1 null intestines including the nucleolar condensation. These observations provide a set of tools for future studies aimed at determining how PAP accumulation affects nuclear architecture and nucleolar function.

It is interesting that the duodenum and proximal small intestine appear to be more affected both functionally and morphologically by the loss of Bpnt1 than the distal jejunum and ileum. Combined with the roughly 4-fold enrichment in the expression of wild-type Bpnt1 in the duodenum relative to the ileum, our results suggest that Bpnt1 might be essential for normal cellular processes that take place exclusively in the early small intestine. One interesting possibility is that the duodenum and early jejunum are
important for mediating certain aspects of either cytoplasmic or Golgi-resident sulfation and thus require a greater amount of PAPS production to meet this demand. These cells would be more susceptible to PAP accumulation following loss of Bpnt1 relative to cells with slower PAPS flux. A second possibility to account for the uneven distribution of accumulated PAP throughout the small intestine is that Bpnt1 may be necessary for the catabolism of ingested 2’- or 3’-phosphorylated molecules including PAPS, PAP, Coenzyme A, or NADPH and that the absorption of these molecules occurs predominately in the early intestine. Further investigation will be necessary to determine why bisphosphorylated nucleotides accumulate specifically in the proximal small intestine and what functional role Bpnt1 plays in this organ.
3.4 Materials and Methods

3.4.1 Animals

Bpnt1 floxed mice were generated using a standard homologous recombination approach (Figure 14). Briefly, we recombined the 4th and 5th exons of the Bpnt1 locus in 129/SvEv ES cells with a construct containing flanking LoxP sites and a neomycin resistance cassette. Cells resistant to neomycin were confirmed by PCR and Southern blotting, injected into blastocysts and implanted into pseudopregnant females by the University of North Carolina Animal Models Core. Chimeric founders were identified by Southern blotting and PCR and crossed with B6.Cg-Tg(ACTFLPe)9205Dym/J mice (The Jackson Laboratory) expressing FLP recombinase in order to remove the neomycin cassette. Bpnt1^+/fl mice were then backcrossed four generations into the C57BL/6J background, intercrossed and maintained as Bpnt1^fl/fl animals. To obtain intestine-specific knockouts, we first crossed B6.SJL-Tg(Vil-cre)997Gum/J mice (The Jackson Laboratory) expressing Cre recombinase under the control of the villin promoter with Bpnt1^+/ animals to obtain Bpnt1^+/Vil-Cre^+ double heterozygotes. These animals were then crossed to Bpnt1^fl/fl mice to generate Bpnt1^+/fl, Bpnt1^fl/fl, Bpnt1^+/int, and Bpnt1^fl/int mice. Wild-type and conventional knockout Bpnt1 alleles were genotyped by multiplex PCR using the following primers: (a) 5'-cttatagtctctagcctgagg-3'; (b) 5'-accaagaacggcgcggtgtg-3'; and (c) 5'-aggtcggaacctgttctcagtc-3'. Floxed Bpnt1 alleles were genotyped by PCR using the following primers: (a) 5'-cttgggttgtggtgacccttg-3'
and (b) 5’-ctctagccccagtcacagtctcag-3’. Villin-Cre expression was determined by PCR using the following primers: (a) 5’-gcggtctggcagtaaaaactatc-3’ and (b) 5’-gtgaacacgctgtgctcactt-3’. All animals unless otherwise noted were maintained on Purina 5058 natural products chow. Animals receiving supplemental iron were injected into the scruff of the neck weekly for a total of 3 weeks with 5 mg of sterile iron-dextran (Sigma). After 3 weeks, mice were sacrificed and analyzed as described above. Animals challenged with iron-deficient diets were maintained on normal Purina 5058 chow until the time of weaning (P23) at which point they were given either iron-deficient AIN-93G with 2-6 ppm total iron or an identical AIN-93G supplemented with 200 ppm iron(II) sulfate (Harlan-Teklad TD.120105 and TD.120106 respectively). Mice were sacrificed after 5 weeks of dietary treatment and analyzed for hematological parameters as described above. Animal care and experiments were performed in accordance with the National Institutes of Health guidelines and approved by the Duke University Institutional Animal Care and Use Committee.

### 3.4.2 Tissue PAPS and PAP analysis

Tissue PAPS and PAP levels were measured using a combination of two previously published protocols (59, 60). Briefly, frozen liver slices (~150 mg) were boiled for 3 minutes in 5 μL of PAP isolation buffer [50 mM glycine (pH 9.2)] per mg of tissue and disrupted using a PowerGen 700 homogenizer (Fisher Scientific – power “4”) and disposable hard tissue generators (Omni International). This process was repeated once
more before transferring the samples to ice. Homogenates were clarified by at 16,100 x g, 4°C for 20 minutes. Following addition of 0.2 volumes of CHCl₃, mixtures were shaken vigorously and then centrifuged at 16,100 x g, 4°C for 20 minutes. Finally, the upper aqueous phases were collected. The final extract was stable at -80°C for at least 3 months.

To quantify PAP levels, we developed a simple colorimetric microplate absorbance assay in which recombinant mouse Sult1a1-GST is used to transfer a sulfate group from p-nitrophenyl sulfate to 2-naphthol, using PAPS or PAP as a catalytic cofactor. Briefly, 10 µL of the tissue lysate or PAP standard was incubated with 190 µL of PAP reaction mixture [100 mM bis-tris propane (pH 7.0), 2.5 mM β-mercaptoethanol, 2.5 mM p-nitrophenyl sulfate, 1 mM β-naphthol, and 1 ug of PAP-free recombinant mouse GST-Sult1a1]. Reactions velocities were determined by monitoring the production of 4-nitrophenol at 400 nm. Unknown concentrations of PAP in lysates were interpolated from Michaelis-Menten one-site binding curves of initial velocity vs. PAP concentration via Prism 5 software (GraphPad).

### 3.4.3 Hematological analysis and histology

For hematological analysis, mice were sacrificed by CO₂ exposure and blood collected by cardiac stick from the right ventricle. Blood was collected into K₂EDTA tubes (Becton Dickinson) and mixed gently to prevent clotting. Complete blood counts were performed by the Duke Veterinary Diagnostic Laboratory using an Abbot Cell Dyn 3700. For blood smears, whole blood was collected via cardiac stick, spread onto glass
slides (Fisher Scientific), allowed to air-dry, and stained with Wright-Giemsa (Electron Microscopy Services). For histological analysis, mice were sacrificed using the above method and then perfused transcardially with 30 mL of phosphate-buffered saline pH 7.4. Tissues for histology were fixed in 10% formalin (VWR) for 2 days then embedded in paraffin by the Duke University Medical Center Immunohistology Research Laboratory. 5µm sections were stained for H&E and with Perls’ iron stain by the Duke University Medical Center Immunohistology Research Laboratory. For immunohistochemistry, sections were blocked, stained, and visualized with DAB according to standard procedures. Primary antibodies recognizing Bpnt1 (York lab - 2296) or fibrillarin (Abcam) were incubated at 4°C overnight. For bone marrow smears, marrow was isolated from the femur, spread onto glass slides, and stained with Perls’ iron stain as described above. Slides were imaged on a Nikon TE2000 inverted microscope.

3.4.4 Quantification of iron stores

Iron analysis was performed according to standard methodologies with minor modifications. Briefly, livers, spleens, and duodenums for iron analysis were isolated following PBS perfusion, blotted dry, and snap frozen in liquid nitrogen. The tissues were weighed while still frozen and added to 9 volumes of acid lysis buffer (3 M HCl, 0.61 M trichloroacetic acid). The samples were shaken vigorously and incubated at 95°C overnight until completely dissociated. Samples were then centrifuged at 5,000 x g for 10
min. To quantify iron content, 5 volumes of saturated sodium acetate, 5 volumes of milliQ water, and 1 volume of chromogen stock solution (1.86 mM bathophenanthroline, 143 mM thioglycolic acid in milliQ water) were combined to generate the chromogen working solution. 100 µL of supernatant was then added to 1 mL of working chromogen buffer and incubated for 10 min at room temperature to allow for color development. Absorbances was measured on a Beckman Coulter DU730 spectrophotometer and compared to a standard curve of iron(II) sulfate ranging from 0 to 4000 µg/dL Fe²⁺. Samples above the linear range of the assay were diluted 1:10 with acid lysis buffer.

### 3.4.5 Quantitative reverse transcriptase PCR

Total liver RNA was extracted using Trizol according to the manufacturer’s recommendations (Life Technologies). Briefly, snap frozen liver segments (~100 mg) were added to 20 volumes of Trizol and rapidly homogenized using a PowerGen 700 homogenizer (Fisher Scientific – power “4”) and disposable hard tissue generators (Omni International). cDNA was synthesized with the Bio-Rad iScript reverse transcriptase kit using random hexamers. Control (-)RTase reactions were included for each sample. Quantitative PCR was performed on a Bio-Rad iQ5 using the SsoFast Evagreen PCR supermix. Primers designed to amplify hepcidin mRNA (5’-3’) were generated using the NCBI Primerblast software and were forced to cross an intronic boundary. Primers were tested for linearity over 3 logs of dilution and (-)RT reactions consistently yielded no amplified product.
4. Loss of Bpnt1 in the liver impairs hepatocyte maturation and leads to the accumulation of immature progenitors

4.1 Introduction

Throughout life, the liver is capable of undergoing a number of remarkable transformations. During embryogenesis, the liver is the predominating organ of hematopoiesis and is composed of both hepatic and hematopoietic progenitors. Later in development, these hematopoietic stem cells migrate to the bone marrow and are gradually replaced by immature parenchymal and non-parenchymal lineages (88). In rodents, the liver continues developing postnatally, possessing a high rate cell division that gradually decreases until shortly after weaning (89). A change in diet from milk to carbohydrates during weaning appears to induce a number of changes in the immature hepatocytes including zonation (expression of mature functionalized hepatocyte transcripts in different acinar zones) and polyploidization that are essential for the development of the mature liver (90).

Following normal postnatal development, the liver maintains many aspects of its transformative capacity. Indeed, the liver is renowned for its ability to regenerate following toxic insult is able to completely regenerate its entire mass from just 25% of its original size (91, 92). Additionally, the liver is able to tightly regulate this regeneration and regrow only what is necessary to reach either its original size or in the case of a transplant, to the size of the host’s original liver (91). Thus, the liver possesses a high
degree of plasticity and is capable of adapting to a variety of environments. Despite knowledge of the liver’s regenerative potential since early civilizations – as demonstrated by stories of Prometheus and the eagle from Greek mythology – the cellular components that provide such regenerative potential remain largely unclear. Classically, it was thought that the parenchymal liver cell, the hepatocyte, which accounts for 90% of the adult liver by mass, was maintained in a growth-arrested state. Then, upon injury, hepatocytes would receive a signal either from other hepatocytes or non-parenchymal cells and re-enter the cell cycle only to exit again once the predetermined liver size was reached (93). Further, it was thought that nearly all injuries induced the same signaling pathways necessary for regeneration. However, recent evidence has suggested that different types of injury result in distinct mechanisms of regrowth (91).

The first substantial evidence for an involvement of non-parenchymal liver cell types in liver regeneration was provided by studies that simultaneously inhibited the replication of hepatocytes during injury to the liver (94). This previously unclassified mobilized population of cells, termed oval cells for their high nuclear to cytoplasmic ratio, was only seen in response to certain types of injuries, including choline-deficient/ethionine-supplemented and acetyl aminofluorene/partial hepatectomy and was a striking contrast to the hepatocyte-specific proliferation following partial hepatectomy alone (95). In uninjured liver, quiescent oval cell populations are believed
to represent only a small fraction of total liver cell types and to reside in the Canal of Hering, a structure joining the bile duct to the hepatic plates (96).

While a number of distinct cell types reside in the Canal of Hering, one population of cells expressing the Sry (sex determining region Y)-box 9 (Sox9) transcription factor have recently been proposed to play an important role in mediating the regenerative response to a number of types of hepatic injury including bile duct ligation and methionine-choline deficient diet (97). These bipotent progenitor cells are stimulated in response to hepatic injury and migrate outward to replace the damaged biliary cells or hepatocytes.

Here we report that mice deficient for the cytoplasmic 3’-nucleotidase bisphosphate 3’-nucleotidase 1 (Bpnt1) are defective in normal hepatocyte maturation and define a novel type of hepatic injury. Bpnt1 null livers fail to undergo polyploidization and are unable to upregulate the genes normally found in mature hepatocytes. They also develop chronic liver damage resulting in a massive increase in immature hepatocyte progenitor cells. Further, we are able to demonstrate that these defects are due to loss of 3’-nucleotidase activity in hepatocytes through the use of liver-specific Bpnt1 knockouts. Together our findings suggest that Bpnt1 null mice develop hepatic insufficiencies as a result of PAP-induced toxicity in mature hepatocytes and impaired development of mature hepatocellular functions.
4.2 Results

4.2.1 Bpnt1 null livers fail to undergo normal maturation processes

Many of the hepatic defects in Bpnt1 null mice are processes enriched in mature hepatocytes, including albumin production, nitrogen metabolism, and urinary protein expression. Therefore, we wondered whether the loss of Bpnt1 might be affecting the ability of hepatocytes either to mature or to maintain a mature state. To address this, we first measured the amount of accumulated PAP in Bpnt1 null animals at different times after weaning. While 25-day-old Bpnt1 null livers contain approximately 12-fold more PAP than wild-type animals (100 vs. 8 nmol/g), we found that hepatic PAP concentrations increased gradually to a maximum of ~200 to 250 nmol/g, suggesting that the accumulation of PAP might be dependent on a process only present in mature hepatocytes (Figure 24).
Figure 24: Bpnt1 null animals accumulate PAP in their livers after weaning.

While little is known about the physiological cues that promote or the precise mechanism by which hepatocyte maturation occurs, it is widely accepted that in mice, hepatocytes undergo a significant transition at the time of weaning. To address this possibility, we examined a number of properties unique to mature hepatocytes at different developmental times in wild-type and Bpnt1 null mice.

As hepatocytes mature, a multitude of ‘immature genes’ are downregulated while transcripts encoding more specialized functions are upregulated. Alpha fetoprotein (Afp), which is a functional homolog of albumin that is expressed by immature hepatocytes is replaced by the production of albumin (Alb) following weaning. To examine whether the normal transition from Afp to Alb expression was intact, we measured the levels of Afp and Alb transcripts by quantitative reverse
transcriptase PCR (qRT-PCR) in 45-day-old wild-type and Bpnt1 null livers. In agreement with the previously described reduction in total serum albumin (see Chapter 2), we found that mature Bpnt1 null livers contained a small but consistent reduction of approximately 50% in their albumin mRNA as compared to wild-type mice (Figure 25A). In contrast, Bpnt1 deficient livers expressed 10 to 100 fold more Afp mRNA (Figure 25B). This suggested that hepatocytes were both failing to repress Afp and unable to upregulate Alb mRNA.

![Graph A and B](image)

**Figure 25**: qRT-PCR of albumin and alpha fetoprotein in wild-type and Bpnt1⁻/⁻ livers.

In addition to the upregulation of Alb during normal hepatocyte maturation, murine livers also begin to produce a pheromone transport protein known as major
urinary protein 2 (Mup2). Mup2 binds to insoluble hydrophobic pheromones and transports them across the glomerular barrier into the urine where the molecules are then released. To determine whether Bpnt1 null livers were able to produce Mup2, we collected urine from male wild-type and Bpnt1+/− mice and analyzed the protein content by SDS-PAGE. Remarkably, we found that urine from Bpnt1 null mice contained undetectable levels of Mup2 (Figure 26A). To confirm that this was a defect in the production of Mup2 by the liver, we measured Mup2 transcript levels in the livers of male mice from 24 to 114 days postnatal. While wild-type mice increase the quantity of hepatic Mup2 mRNA 100-fold from weanlings to adults, Bpnt1 null livers failed to upregulate Mup2 expression after weaning (Figure 26B).
Figure 26: Bpnt1 null mice fail to produce the mature hepatocyte marker Mup2.

(A) Silver stained SDS-PAGE of male Bpnt1+/+ and Bpnt1−/− urine. The Mup2 band is visible as negative contrast by silver stain. (B) qRT-PCR of Mup2 transcript levels from wild-type and Bpnt1 null total liver RNA. Note that Bpnt1−/− livers do not upregulate Mup2 mRNA during normal hepatocyte maturation.

In addition to Mup2, we wondered whether the expression of other known markers of mature hepatocytes might also be inhibited in Bpnt1 null livers. To test this possibility, we examined three genes known to be expressed only in mature livers: hydroxy-delta-5-steroid dehydrogenase 3 beta, Hsd3b5; elongation of very long chain
fatty acids 3, Elovl3; and solute carrier family 1 member 2, Slc1a2. Remarkably, the levels of all three transcripts were significantly repressed in Bpnt1 null livers (Figure 27). Together, these results suggested that Bpnt1 null hepatocytes were unable to activate the normal transcriptional program of maturing hepatocytes.
Figure 27: Failure of Bpnt1 null livers to express various mature hepatocyte specific mRNA.

qRT-PCR of various transcripts specific to mature hepatocyte from total liver RNA of wild-type and Bpnt1−/− mice: (A) hydroxy-delta-5-steroid dehydrogenase 3 beta,
Hsd3b5; (B) Elongation of very long chain fatty acids 3, Elovl3; and (C) Solute carrier family 1 member 2, Slc1a2.

In addition to alterations to the transcriptional landscape, hepatocytes also undergo significant changes their genomic composition, transitioning from mononucleated diploid cells to a combination of binucleated tetraploid, mononucleated tetraploid, and other cells containing multiple higher ploidy states (Figure 28A). Because hepatocytes are often the first mechanism of defense against cytotoxic and genotoxic xenobiotics, polyploidization is believed to play an essential role in safeguarding their genomic integrity by ensuring that functional copies of the genome are maintained. In addition, it has recently been suggested to play a role in hepatocyte genetic variation. To assess whether Bpnt1 null hepatocytes were able to undergo normal polyploidization, we purified intact nuclei from wild-type and Bpnt1 null livers at three different times of development and measured their DNA content by flow cytometry. While wild-type hepatocytes significantly increased their DNA content between weaning and 45-days-old, Bpnt1 null livers failed to accumulate any tetraploid nuclei (Figure 28B and Figure 28C).
Figure 28: Bpnt1 null hepatocytes are defective in polyploidization after weaning.

(A) Schematic depicting the normal pathway for hepatocyte polyploidization in mice following weaning. Mononuclear diploid cells undergo a single round of failed cytokinesis endoduplication to become binucleated tetraploid hepatocytes. These binucleated cells then undergo normal mitosis to generate mononuclear tetraploid cells. (B) Histograms of DNA content from isolated hepatocyte nuclei of 20, 33, and 45 day old mice. Note that Bpnt1 null hepatocytes display slower kinetics of tetraploid generation.

4.2.2 Loss of Bpnt1 is toxic to mature hepatocytes and results in an accumulation of Sox9(+) progenitors

The persisting expression of immature hepatocyte transcripts in addition to the evidence of impaired maturation suggested two potential mechanisms: (i) loss of Bpnt1 in immature hepatocytes results in specific defects in the maturation program or (ii) loss of Bpnt1 is toxic only to mature hepatocytes, which results in consistent hepatocellular death and subsequent repopulation by immature progenitors. In order to delineate these possibilities, we looked for evidence of repeated hepatic insult, which is known to result in collagen deposition and the development of fibrosis. Picro-sirius red staining in 42 and 114-day-old mice revealed a striking accumulation of collagen fibers in Bpnt1 null livers (Figure 29). Of note, the accumulation of collagen was significantly greater in older Bpnt1 null livers, suggesting a chronic cycle of hepatocellular injury and scarring.
Figure 29: Bpnt1 null livers accumulate collagen.

Picrosirius red stain of wild-type and Bpnt1−/− liver tissue demonstrating the progressive development of fibrosis and collagen accumulation. In wild-type livers collagen is predominately found surrounding the portal triads and central veins. Bpnt1 null livers display significant collagen accumulation throughout the entire tissue.

Following hepatotoxic insult, one potential source of replacement hepatocytes is a recently described pool of bile duct-derived Sry (sex determining region Y)-box 9 (Sox9) positive bipotent progenitors that, depending on the type of injury, are capable of repopulating the entire liver. Because we suspected that loss of mature hepatocytes
might coincide with the accumulation of immature hepatocyte progenitors, we investigated whether Sox9(+) cells were over-represented in Bpnt1 null livers. Indeed, livers from 42-day-old Bpnt1 null mice contained significantly greater numbers of Sox9(+) cells surrounding the portal triads and central veins (Figure 30). Further, livers from 114-day-old Bpnt1 null animals revealed the presence of a significantly greater number of widely distributed Sox9(+) cells, suggesting that as a result of chronic insult to Bpnt1 null hepatocytes, Sox9(+) bile duct cells are mobilized and repopulate the liver with immature hepatocyte progenitors.
Figure 30: Bpnt1 null livers accumulate Sox9(+) progenitor cells.

Immunohistochemistry for the transcription factor Sox9 in wild-type and Bpnt1 null liver tissue. Note that in wild-type mice relatively few Sox9(+) cells are found surrounding the portal triads and central veins whereas. In contrast, Bpnt1 null livers contain greater numbers of Sox9(+) cells both surround veins and distributed throughout the tissue.

4.2.3 Loss of Bpnt1 in the liver phenocopies the liver defects of global knockout mice

Although our evidence supported a model in which the loss of Bpnt1 in the liver resulted in PAP accumulation and hepatic dysfunction, we were interested to determine whether the defects in maintenance of mature hepatocyte phenotypes were intrinsic to
hepatocytes or the result of a secondary effect from other liver cells or organ systems. To address this question, we generated mice deficient for Bpnt1 in hepatocytes by crossing Bpnt1 floxed animals (described previously) with mice expressing Cre recombinase under the control of the albumin promoter. Mice expressing both a floxed allele of Bpnt1 and Albumin-Cre displayed significantly reduced levels of Bpnt1 in liver tissue, with the remaining detectable protein likely due to its expression in other liver-resident cell types (Figure 31A). Importantly we did not detect any expression differences in other organs including the kidneys or small intestine (Figure 31B).
Figure 31: Generation of mice deficient for Bpnt1 specifically in the liver.

(A) Western blot of Bpnt1 and B-tubulin from wild-type, heterozygous, and Bpnt1 null livers. (B) Immunohistochemistry for Bpnt1 in liver, kidney, and small intestines of Bpnt1\(^{+/\text{liv}}\) and Bpnt1\(^{-/\text{liv}}\) animals. Note the specific decrease in staining intensity only in the livers of Bpnt1\(^{-/\text{liv}}\) mice.

We also measured the concentrations of PAPS and PAP in the livers, kidneys, and small intestines of Bpnt1\(^{-/\text{liv}}\) mice. Relative to wild-type, Bpnt1\(^{-/\text{liv}}\) and global knockout mice accumulated similar amounts of PAPS and PAP and we did not detect any changes to the levels of PAPS and PAP in the kidneys or small intestines (Figure 32).
Figure 32: Liver-specific Bpnt1 null mice accumulate PAP only in the liver and not in the kidneys or small intestine.

In order to determine whether loss of Bpnt1 in hepatocytes was sufficient to impair serum protein production, we analyzed serum from wild-type and Bpnt1−/liv mice by SDS-PAGE and for clinical chemistries. By 45 days postnatal, Bpnt1−/liv animals displayed significantly reduced total serum protein content, including decreases in albumin as well as alpha 2 macroglobulin and other globulins (Figure 33A-D). Bpnt1−/liv mice also presented with low levels of total serum cholesterol (approximately 45% of wild-type) and significantly reduced quantities of Mup2 in urine (Figure 33E and Figure 34). Further, clinical chemistries revealed elevations in the markers of hepatocellular damage ALT, AST, and ALKP (Figure 35). Together, these results suggested that the defects observed in global Bpnt1 null mice were the direct result of loss of Bpnt1 in hepatocytes and not due to secondary effects from other liver-resident cells or organ systems.
Figure 33: Hypoproteinemia and hypocholesterolemia in liver-specific Bpnt1 null mice.

(A) Coomassie stained SDS-PAGE of total serum proteins from both global and liver-specific Bpnt1 knockouts demonstrating the hypoproteinemia due to loss of Bpnt1 in the liver. (B, C, D) Quantification of total serum protein, albumin, and globulins as measured by clinical chemistry analysis. (E) Total serum cholesterol of liver-specific Bpnt1 null mice.

Figure 34: Liver-specific Bpnt1 null mice do not produce Mup2 in urine.

Coomassie stained SDS-PAGE of urine proteins from male Bpnt1+/liv and Bpnt1-liv animals.
Figure 35: Liver-specific Bpnt1 knockouts develop significant liver damage.

Hepatocellular damage in liver-specific Bpnt1 null mice as measured by clinical chemistries: (A) Aspartate aminotransferase, AST; (B) Alanine aminotransferase, ALT; and Alkaline phosphatase, ALKP.
4.3 Discussion

Our data demonstrate an important role for Bpnt1 in the maintenance of mature differentiated hepatocytes. Mice deficient for Bpnt1 globally or specifically in hepatocytes displayed defects in normal hepatocyte maturation and evidence of substantial age-dependent hepatocellular toxicity. In addition, Bpnt1 null livers are repopulated with immature Sox9(+) progenitors cells as a result of chronic hepatocyte injury. Together our results describe a novel connection between cytoplasmic 3’-nucleotide hydrolysis and hepatocyte maturation and suggest that the physiological defects of Bpnt1 null mice might be due loss of mature hepatocytes.

The normal development of hepatocytes involves a number of major transitions. Initially, the liver is populated with hematopoietic cells that serve as the major source of red blood cells during early and mid developmental stages. Then, in late embryonic and early postnatal development the hematoblasts are replaced with young hepatocytes that are highly proliferative and express only low levels of the functionalized metabolic transcripts found in normal hepatocytes. After weaning, hepatocytes slow their proliferation and upregulate a wide array of genes essential for adult liver physiology. In this study, we found that loss of Bpnt1 leads to a disruption in this process and results in a failure of young livers to mature properly. In particular, Bpnt1 null mice fail to
repress the fetal albumin homolog Afp and express lower levels of Alb. In addition, the expression of other previously reported mature hepatocyte genes that we examined including Mup2, Hsd3b5, Elovl3, and Slc1a2 were significantly repressed relative to wild-type.

We also examined other aspects of normal hepatocyte maturation in wild-type and Bpnt1 null livers. One of the best described, but least understood changes that occurs in maturing hepatocytes is an increase in their DNA content. Immediately after weaning, wild-type hepatocyte nuclei are more than 99% diploid. However, within 2 weeks, hepatocyte nuclei are roughly 50% 2N and 50% 4N or greater. In contrast, Bpnt1 null hepatocyte nuclei remain predominately 2N (80%) up to 45 days postnatal. Thus, hepatocytes deficient for Bpnt1 are unable to undergo normal polyploidization, limiting potentially sensitizing them to genotoxic stress from endogenous or xenobiotic molecules.

One potential mechanism by which Bpnt1 null livers might fail to express markers specific to mature hepatocytes is if loss of Bpnt1 is toxic only to mature cells and thus results in their death and removal exclusively. If this hypothesis was correct, then we would have expected evidence of chronic hepatocellular damage and turnover. Indeed, we detected a striking age-dependent accumulation of collagen in Bpnt1 null livers. We also observed significant elevations in the serum concentrations of the normally intracellular enzymes ALT and AST (see Chapter 2), indicative of
hepatocellular death and lysis. In order to maintain adequate hepatic function as a result of increased hepatocyte turnover or death, the liver appears to possess two potential mechanisms of regrowth. In partial hepatectomy models of liver regeneration, it has been well documented that differentiated hepatocytes themselves re-enter the cell cycle. However, in other models of injury, such as CCl₄ injection or bile duct ligation, the mobilization of a population of bile duct-derived, Sox9(+) cells appears to be more relevant. In our model of liver injury we observed a significant accumulation of Sox9(+) cells that increased with age, suggesting that loss of Bpnt1 stimulates a ductal proliferation response. Hopefully future studies will be able to determine the various factors necessary to direct the damaged liver down one regenerative pathway or the other.

In order to address whether the defects that we observed were intrinsic to hepatocytes or the result of a secondary effect from other liver cell types or organ systems, we generated liver-specific Bpnt1 knockout mice. These mice presented with hypoproteinemia, hypocholesterolemia, substantial hepatocellular damage, and repressed Mup2 expression. While the complete characterization of Bpnt1⁻/⁻ mice will be the work of future studies, our preliminary data suggest that the hepatic insufficiencies are a direct result of loss of Bpnt1 in hepatocytes.
4.4 Materials and Methods

4.4.1 Animals

Bpnt1 floxed mice were generated using a standard homologous recombination approach as described above (Figure 14). To obtain liver-specific knockouts, we first crossed B6.Cg-Tg(Alb-cre)21Mgn/J mice (The Jackson Laboratory) expressing Cre recombinase under the control of the albumin promoter with Bpnt1+/− animals to obtain Bpnt1+/− Alb-Cre+ double heterozygotes. These animals were then crossed to Bpnt1+/− mice to generate Bpnt1+/−, Bpnt1−/−, Bpnt1+/liv, and Bpnt1−/liv experimental mice. Wild-type and conventional knockout Bpnt1 alleles were genotyped by multiplex PCR using the following primers: (a) 5’-cctatagtcctagcacttgagagg-3’; (b) 5’-accaaagaacggagccggttgccgtggc-g-3’; and (c) 5’-aggtcgaacccctgttctagtc-3’. Floxed Bpnt1 alleles were genotypes by PCR using the following primers: (a) 5’-cttgtagtttggggtgccgaccccttag-3’ and (b) 5’-ctctagcccagctagcatgtcag-3’. Albumin-Cre expression was determined by PCR using the following primers: (a) 5’-gccccctagtaaaacttc-3’ and (b) 5’-gtgaaacagctattgctgtcactt-3’. All animals unless otherwise noted were maintained on Purina 5058 natural products chow. Animal care and experiments were performed in accordance with the National Institutes of Health guidelines and approved by the Duke University Institutional Animal Care and Use Committee.
4.4.2 Serum and urine analysis and clinical chemistries

Mice were sacrificed by CO₂ exposure and blood collected by cardiac stick from the right ventricle. Blood was collected into serum separator tubes (Becton Dickinson) and allowed to clot before centrifugation and removal of the hematocrit. Clinical chemistries of liver-specific Bpnt1 null mice was performed by Antech Diagnostics. For urinary protein analysis, individual mice were housed temporarily in a clean, dust free cage. Urine was collected over the course of an hour by manual observation. Urine and serum proteins were separated by SDS-PAGE and stained with coomassie brilliant blue by standard procedures.

4.4.3 Staining and histological analysis

For histology, mice were sacrificed using the above method and then perfused transcardially with 30 mL of phosphate-buffered saline pH 7.4. Tissues for histology were fixed in 10% formalin (VWR) for 2 days then embedded in paraffin, cut into 5 μm sections, and stained for H&E by the Duke University Medical Center Immunohistology Research Laboratory. Sections were stained for picro-sirius red (Sigma) according to standard procedures (IHC World). For immunohistochemistry, sections were blocked, stained, and visualized with DAB according to standard procedures. Primary antibodies recognizing Bpnt1 (York lab - 2296) or Sox9 (Millipore) were incubated at 4°C overnight. All slides were imaged on a Nikon TE2000 inverted microscope.
4.4.4 Tissue PAPS and PAP analysis

Tissue PAPS and PAP levels were measured using a combination of two previously published protocols (59, 60). Briefly, frozen liver slices (~150 mg) were boiled for 3 minutes in 5 µL of PAP isolation buffer [50 mM glycine (pH 9.2)] per mg of tissue and disrupted using a PowerGen 700 homogenizer (Fisher Scientific – power “4”) and disposable hard tissue generators (Omni International). This process was repeated once more before transferring the samples to ice. Homogenates were clarified by at 16,100 x g, 4°C for 20 minutes. Following addition of 0.2 volumes of CHCl₃, mixtures were shaken vigorously and then centrifuged at 16,100 x g, 4°C for 20 minutes. Finally, the upper aqueous phases were collected. The final extract was stable at -80°C for at least 3 months.

To quantify PAP levels, we developed a simple colorimetric microplate absorbance assay in which recombinant mouse Sult1a1-GST is used to transfer a sulfate group from p-nitrophenyl sulfate to 2-naphthol, using PAPS or PAP as a catalytic cofactor. Briefly, 10 µL of the tissue lysate or PAP standard was incubated with 190 µL of PAP reaction mixture [100 mM bis-tris propane (pH 7.0), 2.5 mM β-mercaptoethanol, 2.5 mM p-nitrophenyl sulfate, 1 mM β-naphthol, and 1 ug of PAP-free recombinant mouse GST-Sult1a1]. Reactions velocities were determined by monitoring the production of 4-nitrophenol at 400 nm. Unknown concentrations of PAP in lysates were interpolated from Michaelis-Menten one-site binding curves of initial velocity vs. PAP concentration via Prism 5 software (GraphPad).
4.4.5 Quantitative reverse transcriptase PCR

Total liver RNA was extracted using Trizol according to the manufacturer’s recommendations (Life Technologies). Briefly, snap frozen liver segments (~100 mg) were added to 20 volumes of Trizol and rapidly homogenized using a PowerGen 700 homogenizer (Fisher Scientific – power “4”) and disposable hard tissue generators (Omni International). cDNA was synthesized with the Bio-Rad iScript reverse transcriptase kit using random hexamers. Control (-)RTase reactions were included for each sample. Quantitative PCR was performed on a Bio-Rad iQ5 using the SsoFast Evagreen PCR supermix. Primers designed to amplify target genes were generated using the NCBI Primerblast software and were forced to cross an intronic boundary. The following primers were used for analysis: Primers were tested for linearity over 3 logs of dilution and (-)RT reactions consistently yielded no amplified product.

4.4.6 Western blotting

For duodenal protein analysis animals were sacrificed by pentobarbital and blood was collected via cardiac stick. The first 2 cm of the small intestine was rapidly excised, cut open along its length, rinsed vigorously in ice-cold PBS, and snap frozen in liquid nitrogen.

4.4.7 Analysis of hepatocyte nuclear DNA content

For hepatocyte nuclei isolation, mice were sacrificed by CO₂ exposure and perfused with 30 mL of phosphate-buffered saline pH 7.4. Whole livers were excised, the
gall bladder removed, and then weighed and transferred to a Dounce glass-glass tissue homogenizer containing 3 volumes of ice-cold isotonic STKM buffer (250 mM sucrose, 50 mM Tris-Cl pH 7.4, 25 mM KCl, 5 mM MgCl₂). Livers were first reduced using a loose pestle and then homogenized using a tighter pestle with 20 strokes each. The homogenate was filtered through a 70 µm cell strainer into a 50-mL conical tube. The crude lysate was then centrifuged at 800 x g, 4°C for 5 min. The supernatant was removed and the crude nuclear pellet resuspended in the same volume of isotonic lysis buffer. The centrifugation and removal of supernatant was then repeated 1 additional time. The final nuclear pellet was resuspended and kept on ice until the remaining samples were finished. For analysis, excess propidium iodide and RNase A were added to the suspension of nuclei and incubated at room temperature for 10 min. The fluorescent intensity of the nuclei was quantified by flow cytometry using a Becton Dickinson FACScan analyzer.
5. Identification and characterization of a novel 3’-phosphorylated nucleotide

5.1 Introduction

Bisphosphate 3’-nucleotidase 1 (Bpnt1) is a member of a metal dependent, structurally related family of small molecule phosphatases (53). Mammalian genomes encode 7 family members, two of which, Bpnt1 and Golgi resident PAP phosphatase (gPAPP) hydrolyze the common substrate 3’-phosphoadenosine 5’-phosphate (PAP) to produce 5’-AMP. PAP phosphatases (3’-nucleotidases) are conserved from bacteria to humans and are involved in a number of cellular processes (see Chapter 1). Intriguingly, the substrates hydrolyzed by 3’-nucleotidases are not identical across the varying species and examples of either more selective (gPAPP) or more flexible (bacterial CysQ) enzymes are found across evolution (98). Murine Bpnt1 has been shown to prefer PAP as a substrate, but has been demonstrated to hydrolyze PAPS, PCP, PGP, PTP, and PUP, in addition to IP3 and IP2 (30). While the evidence presented in Chapter 2 has clearly demonstrated that PAP is the most likely the relevant mediator for the physiological defects, we had not thoroughly examined the possibility that additional substrates might exist. Because of its in vitro promiscuity, we were interested to determine whether we could find other small molecules hydrolyzed by Bpnt1 in vivo.

Here we report that genetic inactivation of Bpnt1 in mice results in the accumulation of three identifiable nucleotides, PAP, PAPS, and the previously undescribed 3’-phosphoadenosine 5’-diphosphate (PAPP). Using a combination of
analytical and biochemical techniques, we were able to identify and characterize PAPP as a novel substrate of Bpnt1 that accumulates in multiple tissues. Although the importance of PAPP to the development of physiological deficiencies in Bpnt1 null mice remains unclear, our results provide the basis for future studies aimed at understanding its origin and function in mammalian cells.

5.2 Results

Murine Bpnt1 is known to hydrolyze with varying efficiencies a number of 3’-phosphorylated nucleotides in vitro including PAPS, PAP, PCP, PGP, PTP, and PUP (30). However, it was unclear whether PAPS and PAP were the only substrates of Bpnt1 in vivo. To identify additional potential substrates, we isolated small molecule acid extracts of wild-type and Bpnt1 null liver tissue and analyzed the homogenates by strong anion exchange HPLC coupled to UV detection at 259 nm. In addition to the previously described accumulation of PAPS and PAP (Chapter 2), examination of chromatograms from Bpnt1 null liver extracts revealed the presence of an unknown anionic molecule eluting at 70 min between ATP and GTP (Figure 36). The time of elution suggested that the molecule possessed significant negative charge while its detection by UV absorption indicated that it contained an aromatic ring sufficient to absorb light at 259nm.
Figure 36: Bpnt1 null livers contain an unknown anionic small molecule.

Strong anion exchange HPLC-UV chromatogram of wild-type and Bpnt1-/- liver acid extracts demonstrating the accumulation of an unknown anionic small molecule in Bpnt1 null livers.

Because we detected the unknown molecule in Bpnt1 null livers, we wondered whether we might be able to detect it in other tissues in which PAPS and PAP are accumulated. To examine this, we isolated small molecule extracts from 25-day-old livers and 40-day-old kidneys of Bpnt1 null mice. Indeed, the unknown peak was
detectable in both tissues, although at a significantly lower concentrations than the 40-day-old knockout liver. This suggested that the molecule likely accumulated over a long period of time and that it might be produced in vivo only at very low levels.

Figure 37: The accumulation of the mystery peak is present in multiple tissues and correlates with the appearance of PAP.

(A) Small molecule extracts from 25 and 40 day old livers. Note that the concentrations of both PAP and the mystery peak are elevated in older animals. (B) Small molecule
extracts from 40-day-old kidneys demonstrating the accumulation of both PAP and the unknown molecule.

In order to shed light on the identity of the unknown peak, we analyzed the UV absorption spectrum of Bpnt1 null liver extract separated by SAX-HPLC (Figure 38A). Surprisingly, the mystery peak possessed an identical UV absorption to that of ATP with absorption maxima at 207 and 260 nm. Because each nucleotide possesses a unique UV absorption spectrum, we were able to conclude that the unknown molecule was a nucleotide with an adenine base.
Figure 38: UV absorption spectrum of Bpnt1 null liver extracts.

(A) 3D chromatogram of small molecule extracts from Bpnt1 null liver demonstrating that the mystery peak absorbs similar UV wavelengths as ATP. (B) UV absorption spectrum of ATP, GTP, and the mystery peak. ATP and the mystery peak display identical absorption spectra.

Given the elution time of the unknown adenosine molecule between ATP and GTP, we hypothesized that the molecule might contain three phosphates at the 5’-ribose position. To test this, we utilized T4 polynucleotide kinase (T4 PNK) to transfer gamma
phosphate from available nucleotide triphosphates in a Bpnt1 null liver extract to the 5’-hydroxyl group of 3’AMP. Surprisingly, while ATP and GTP were able to donate their 5’-phosphates to 3’AMP, the levels of the unknown adenosine peak were unchanged (Figure 39, top panel relative to middle panel). Thus, the molecule did not contain a conventional 5’-triphosphate arrangement.

Figure 39: The adenosine-based peak is not a substrate for T4 PNK.

Small molecule extracts from Bpnt1 null livers were treated with T4 PNK in the presence of 3’ AMP. While both ATP and GTP are able to transfer a gamma phosphate to 3’ AMP, the mystery peak cannot be used as a substrate.

The resistance to T4 PNK gamma phosphate transfer led us to consider a number of alternative possibilities: (i) the strong anionic charge might be provided by groups other than phosphate; (ii) the phosphate groups might be arranged in a way that is
distinct from conventional nucleotide triphosphates. To examine the first possibility, we treated small molecule extracts from Bpnt1 null livers with calf intestinal phosphatase (CIP), which is known to hydrolyze phosphate groups from a diverse array of substrates. Treatment of extracts with CIP degraded all detectable nucleotide peaks including the unknown adenosine peak between ATP and GTP, suggesting that the molecule is indeed triphosphorylated. Because we discovered the molecule in tissues deficient for 3'‐nucleotidase activity, we wondered whether the unknown molecule contained a 3' or 2' phosphate. To test this, we treated the same extracts with two 3'‐nucleotidases, recombinant mouse Bpnt1 and its bacterial ortholog CysQ, which has a more inclusive substrate profile. Treatment with 1 µg of recombinant mouse Bpnt1 hydrolyzed PAP and PAPS but did not appreciably degrade the unknown adenosine peak. CysQ however, was able to efficiently hydrolyze PAP, PAPS, and the unknown molecule. Remarkably, upon treatment of liver extracts with CysQ, we observed that the area of the ADP peak increased by the same amount as the mystery adenosine peak decreased. Thus, hydrolysis of the mystery peak with a 3'‐nucleotidase resulted in the production of ADP and suggested that the identity of the molecule was 3'‐phosphoadenosine 5’‐diphosphate (PAPP).
Figure 40: The triphosphorylated adenosine peak is sensitive to CysQ and Bpnt1 hydrolysis.

Small molecule extracts from Bpnt1 null livers were treated with various phosphatases. Note that in the mBpnt1 treated lysates only PAP and PAPS are efficiently degraded while in CysQ treated samples the PAP, PAPS, and the mystery peak are all hydrolyzed.

5.3 Discussion

Together our data provide the first identification and characterization of PAPP, a novel substrate of 3’-nucleotidases. PAPP is a triphosphorylated adenosine molecule that does not contain a gamma phosphate and is sensitive to 3’-nucleotidase activity. We
were able to detect accumulated PAPP in the liver and kidney, tissues previously demonstrated to harbor high levels of PAPS and PAP. Surprisingly, PAPP is a relatively poor substrate for mouse Bpnt1 but is readily hydrolyzed by its more promiscuous ortholog bacterial CysQ, suggesting that its normal cellular concentration might be quite low. Our results suggest three possibilities that might explain how a molecule that is a relatively poor substrate for Bpnt1 can accumulate to such high intracellular concentrations: (i) PAPP is normally degraded by Bpnt1 but is produced only sparingly as a byproduct; (ii) other enzymes in the cell are able to compensate for the absence of Bpnt1 and partially prevent its accumulation; and (iii) PAPP is normally degraded by another phosphatase whose activity is inhibited by the accumulation of PAPS or PAP. Hopefully, future studies will be able to shed light on these potential explanations and provide insights into the origin and function of PAPP in mammalian cells.

5.4 Materials and Methods

5.4.1 Isolation of small molecule extracts

Extracts were isolated either by TCA or PCA acid precipitation. For TCA extraction, liquid nitrogen frozen tissue segments (~150-250 mg) were weighed and transferred to 5 volumes of TCA in a 2.0-mL cryovial (Corning). The tissue was rapidly homogenized using a PowerGen 700 homogenizer (Fisher Scientific – power “4”) and disposable hard tissue generators (Omni International) for 30 sec. Extracts were then mixed with 1 volume of water-saturated diethyl-ether and shaken vigorously. The
aqueous lower layer was then extracted and the process repeated two additional times to remove the TCA and neutralize the solution. Aqueous lysates were then clarified by centrifugation at 16,100 x g, 4°C for 10 min. For PCA extracts, samples were isolated, weighed, and homogenized as described above with the only exception being the substitution of PCA for TCA. Following homogenization, extracts were centrifuged at 16,100 x g, 4°C for 10 min. Equal volumes of supernatant were transferred to clean microcentrifuge tubes and the homogenates were neutralized with 5 M K2CO3. Nucleotides were then separated by HPLC (Waters) using a 5 µm partisphere SAX column (Whatman) on an 80-min linear gradient from 10 mM NH4H2PO4 (pH 3.7) to 500 mM KCl, 250 mM NH4H2PO4 (pH 4.5). Eluted nucleotides were detected by inline UV absorption at 259 nm, or using 3D data collection absorption spectrum were collected from 200 nm to 300 nm (Waters).

5.4.2 Biochemical analysis of tissue extracts

For biochemical assays, extracts of Bpnt1 null liver were isolated as described above. Equal volumes of extract were then transferred into separate microcentrifuge tubes. For T4 PNK reactions, 10x T4 PNK buffer (NEB) was added to the lysates for a final concentration of 1x. 5 µL of T4 PNK or 1mg/mL BSA was added and the reaction was allowed to proceed for 30 min at 37°C. For experiments involving CIP, CysQ, or mouse Bpnt1, reactions were carried out in 1x HEKM buffer (50 mM NaHepes pH 7.5, 100 mM KCl, 1 mM EGTA, 3 mM MgCl2). 100 µL of each extract was incubated with 1
μg of recombinant Bpt1 or CysQ or 50 units of CIP (NEB) at 37°C for 30 min. All reactions were quenched by addition of 0.6 M PCA and subsequent neutralization with 5 M K₂CO₃. Completed reactions were analyzed by strong anion exchange HPLC-UV as described above.
6. Concluding remarks and future directions

Over the course of this dissertation I have attempted to provide a comprehensive examination of the findings that I have contributed to the field of 3’-nucleotidase biology. My studies have focused on the cytoplasmic 3’-nucleotidase Bpnt1 and its roles in mammalian physiology. Despite two decades of investigation since the discovery and initial descriptions of 3’-nucleotidase activity in yeast, relatively little has been learned about their role in mammals. Here we report that Bpnt1 is involved in a host of different cellular processes occurring in a number of tissues. Through the study of mice deficient for Bpnt1 globally, in the intestine, and in the liver, we have uncovered unexpected connections between loss of 3’-nucleotidase activity and hepatic protein production, hepatocyte maturation, intestinal iron absorption, and nucleolar architecture. In addition, we have determined that the observed physiological defects of Bpnt1 null mice are dependent on the accumulation of its substrate PAP. Despite these significant advances, there are a number of important questions that remain to be answered and would serve as excellent starting points for subsequent studies.

1) Why do the liver, kidney, and small intestine accumulate high levels of PAP while other tissues that express similar levels of Bpnt1 do not?

It is very interesting that despite relative consistent levels of Bpnt1 protein expression in multiple tissues, the accumulation of PAP is only seen in a relatively few number of tissues including the liver, small intestine, and kidney. To address the
possible reasons for this discrepancy it would be useful to have a better understanding of what tissues experience high rates of PAPS production. Because we hypothesize that toxicity due to loss of Bpnt1 is only seen in tissues with PAP accumulation and that the synthesis of PAPS is the rate-limiting step, we propose a thorough examination of the distribution of the various enzymes involved in PAPS synthesis including, sulfate transporters and PAPS synthase 1/2. Apart from the relative expression levels of the synthesizing enzymes, the availability of inorganic sulfate to the cell might be relevant. Thus, it would be interesting to determine the relative sulfate burden in various tissues. In addition, we would like to prevent the synthesis of PAPS by feeding mice PAPS synthase inhibitors (chlorate) or depleting them of sulfate through dietary modifications.

2) What is the molecular basis for the condensed nucleoli that we have observed in hepatocytes, enterocytes, and proximal tubule epithelium?

Alterations to the nuclear and nucleolar architecture appears to be a commonality among the tissues phenotypically affected by loss of Bpnt1. In addition, we have consistently observed an accumulation (albeit relatively small) of unprocessed ribosomal RNA, a process mediated by the 5’-3’ exoribonuclease Xrn2. Defects due to loss of Xrn2 has not been well characterized in mammals and thus far has only been studied in cells treated with anti-Xrn2 shRNAs. Thus, to address the hypothesis that inhibition of Xrn2 leads directly to the condensation of Bpnt1 null
nucleoli, it would be necessary to generate a mouse deficient for Xrn2. This would allow the direct comparison of their respective phenotypes and by breeding Bpnt1 and Xrn2 null mice, to determine if Xrn2 is the only relevant mediator of the physiological defects in Bpnt1 null mice. While this candidate approach is justified based on the extensive evidence connecting PAP accumulation to impaired Xrn2 function in yeast (and now mice), it would also be interesting to pursue an alternative unbiased strategy. In order to examine the differences between wild-type and Bpnt1 null nucleoli, we would compare the proteomes and transcriptomes of purified nuclei from livers, intestines, and kidneys of wild-type and Bpnt1 null mice.  

3) Why have we been unsuccessful in accumulating PAP using an in vitro cell culture system? 

Over the course of this dissertation, we have attempted on numerous occasions to develop an in vitro system that can recapitulate defects observed in Bpnt1 null animals including: Bpnt1 null mouse embryonic fibroblasts; stable knockdown of Bpnt1 in multiple immortalized hepatocytes and primary hepatocytes; and inducible knockdown of Bpnt1 in immortalized hepatocytes, proximal tubule epithelial cells, and HEK293T cells. However, we have never detected any growth defects, morphological changes, or PAP accumulation. In order to overcome these obstacles, we propose studies aimed at increasing the throughput of the PAPS synthesis system. To do this we would create stable cell lines (hepatocytes, enterocytes,
proximal tubule epithelial cells) that overexpress sulfate transporters and PAPS Synthases 1 and 2, and an inducible Bpnt1. In addition, these cells could be grown in high sulfate or normal medium. Hopefully, by stimulating the flux of PAPS in the these cell lines we would be able to create a dependence on 3’-nucleotidase activity that could only be rescued by overexpressing Bpnt1.

4) What are the molecular targets of PAP, PAPS, or PAPP that when accumulated lead to cellular toxicity?

This is one of the most interesting but also most challenging questions to answer in mice. While strong evidence in vivo implicates Xrn2, it is likely that a number of additional proteins are affected by loss of Bpnt1 and subsequent PAP accumulation. However, because it has remained a challenge to detect any defects in a number of Bpnt1 deficient cell lines (see question 3), we have been limited to the use of traditional biochemical approaches from isolated animal tissues. If we are able to generate a cell culture system that displays growth inhibition as a result of an accumulation of PAP (question 3), then there are a number of high-throughput genetic screening technologies available that would help us to identify potential molecular interactors of PAP. For instance, using lentiviral vectors to overexpress various tissue cDNA libraries would allow us to determine targets of PAP whose activity is inhibited, while using shRNA knockdown libraries would identify pathways that might be stimulated by PAP.
5) Given the high expression level of Bpnt1 and detectable accumulation of PAP, is there a defect in Bpnt1 null kidneys?

In order to address this question, we propose the generation of mice specifically deficient for Bpnt1 in the kidney. Using our already validated floxed Bpnt1 allele, we would delete Bpnt1 in the kidney by crossing our mice with mice expressing Cre recombinase under the control of the kidney-specific cadherin 16 (Ksp1.3) promoter, which is activated in the developing nephrons, ureteric bud, and tubules. Because we hypothesize that the defects due to loss of Bpnt1 in the liver and intestine might mask more subtle kidney abnormalities, examining mice deficient for Bpnt1 only in the kidney would allow us to overcome this difficulty.

6) How is PAPP synthesized and degraded normally in the cell?

Although we have substantial evidence that Bpnt1 null tissues accumulate the novel small molecule PAPP, it is unclear how it is made or normally degraded. To address this we would like to take a traditional biochemical approach and look for proteins that bind to PAPP in protein extracts of Bpnt1 null tissues. Because PAPP is not commercially available, it would be first necessary to purify large amounts of PAPP from Bpnt1 null tissues. Pilot experiments have suggested that this is possible. Once purified, there are a number of methodologies available to conjugate various nucleotides to agarose or sepharose beads, which could then be used to probe lysates for potential interactors. We would then identify potential interactors by mass
spectrometry, express them recombinantly, and test them for binding to PAPP *in vitro*.

While these questions are beyond the scope of this investigation, it is my hope that future studies will be able to use these questions as steps towards the broader goal of understanding the roles of 3’-nucleotidases in mammalian cells.
References


90. Anonymous (!!! INVALID CITATION !!!).


Biography

Ben was born to Larry and Maxine Hudson on July 25, 1986 in Winter Park, FL. He attended the International Baccalaureate programme at Seminole High School where he met his now-wife Jessica (Stone) Hudson and graduated in the top 10% of his class. While in the IB programme, he competed in both athletic and academic pursuits including varsity Olympic weightlifting and the Chemistry Olympiad. During his senior year, Ben dual enrolled at the University of Central Florida (UCF) to take an advanced physics course not available through his normal curriculum. He then enrolled at UCF where he initiated a Physics and Chemistry double major. After the death of his father and role model, Larry Hudson, Ben accelerated his trajectory to pursue a newfound academic passion, Biology. Receiving the Outstanding Chemistry Student of the Year and graduating Magna Cum Laude with a B.S. in Chemistry in only three years, Ben then enrolled in the Cellular and Molecular Biology Program at Duke University where he worked with Dr. John York. Ben has taken a rigorous approach to his study of the role of 3’-nucleotidase Bpnt1 in mammals and has uncovered novel insights into their many functions in cells. He published two papers entitled “Roles for nucleotide phosphatases in sulfate assimilation and skeletal disease” and “A role for cytoplasmic nucleotide hydrolysis in hepatic function and protein synthesis,” with two additional manuscripts in preparation at the time of his defense. If nothing else, Ben hopes to be remembered as a dedicated scientist, an intense friend, and a loyal husband.