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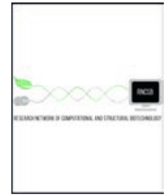

# Metazoan stringent-like response mediated by MESH1 phenotypic conservation via distinct mechanisms

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ABSTRACT

All organisms are constantly exposed to various stresses, necessitating adaptive strategies for survival. In bacteria, the main metabolic stress-coping mechanism is the stringent response, which is triggered by the accumulation of "alarmone" (p)ppGpp to arrest proliferation and reprogram the transcriptome. The level of (p)ppGpp is regulated by its synthetase RelA and its hydrolase SpoT. MESH1 is the metazoan homolog of bacterial SpoT that regulates the bacterial stringent response by degrading the alarmone (p)ppGpp. While MESH1, like SpoT, can also dephosphorylate (p)ppGpp, mammalian cells do not have significant levels of this metabolite, and the relevant enzymatic activities and function of MESH1 have remained a mystery. Through genetic and biochemical analyses, we have solved the long-held mystery and identified MESH1 as the first mammalian cytosolic NADPH phosphatase involved in ferroptosis. Furthermore, we discovered that MESH1 removal leads to proliferation arrest, translation inhibition, and a prominent transcriptional and metabolic response. Therefore, MESH1 knockdown triggers a novel stress response with phenotypic conservation with the bacterial stringent response via distinct substrates and molecular pathways. Here, we summarize the background of the MESH1, illustrate the striking conservation of phenotypes in different organisms during evolution and discuss remaining questions in the field.

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## 1. (p)pGpp, RSH Proteins, and the bacterial stringent response

All organisms are constantly exposed to various metabolic stresses and require coping mechanisms to maintain survival and homeostasis. One of the main bacteria coping strategies is the stringent response which relocates resources from proliferation towards dormancy during metabolic stresses to ensure metabolic homeostasis and stress survival. The stringent response is regulated by cellular levels of (p)ppGpp, also termed alarmone or "magic spots" [1]. The signaling molecule was identified in the 1960's in a bacterial strain that was capable of surviving amino acid deprivation [2]. The remarkable qualities of the phosphorylated molecule identified by autoradiograms gave the molecule the nickname "magic-spot" [2]. During metabolic stresses and nutrient deprivation, (p)ppGpp accumulates up to a concentration of ~ 1 mM and reprograms bacteria from proliferation to dormancy, including decreased cellular proliferation, reduced nucleotide synthesis, altered transcriptional profiles that promote survival, decreased protein translation, increased amino acid synthesis/import, and increased lipid metabolism [3,4]. Together, these changes allow bacteria to conserve the limiting resources and survive these metabolic stresses. Importantly, the stringent response is reversible. Once the metabolic stresses are resolved, the level of (p)ppGpp drops to the baseline and all the (p)ppGpp-mediated effects are relieved, and bacteria resume proliferation states. Therefore, this is a highly dynamic mechanism that allows the coupling of the bacterial phenotypes to the nutrient status.

Cellular levels of (p)ppGpp are controlled by the RelA/SpoT Homologue (RSH) superfamily of enzymes, which are found throughout the tree of life [5]. The RSH family is divided into three groups: Long RSHs, Small Alarmone Synthetases (SAS), and Small Alarmone Hydrolases (SAH) [5]. The Long RSH group of enzymes is the most well studied, as two long RSH proteins, RelA and SpoT, serve as the master regulators of the stringent response in *E. coli* [5]. Long RSHs proteins contain both enzymatic and regulatory domains [5]. Levels of (p)ppGpp are controlled through (p)ppGpp synthesizing and hydrolyzing domains that can act in a bifunctional manner where each domain can allosterically regulate the other [6]. In *E. coli*, RelA is the primary (p)ppGpp synthetase, and SpoT is the primary hydrolase. Long RSH proteins frequently contain regulatory domains that mediate inter and intramolecular interactions [5]. For example, these regulatory domains mediate an interaction between Long RSH protein and a stalled ribosome [7]. Long RSH enzymes are found throughout diverse species of bacteria as well as plant chloroplasts [5]. In contrast, SASs and SAHs are relatively small enzymes that contain only the synthesis or hydrolysis domain from long RSH enzymes and mediate the synthesis or hydrolysis of (p)ppGpp [5]. SAS enzymes synthesize (p)ppGpp by transferring a pyrophosphate from ATP to the 3′ hydroxyl group of GTP. SAS enzymes are found in bacteria, archaea, and several species of soil dwelling fungi [5], but are absent in metazoa [5]. SAH hydrolyzes (p)ppGpp using a manganese ion that coordinates a catalytic water molecule to perform a nucleophilic attack on the 3′ phosphate/diphosphate that dephosphorylates (p)ppGpp to form GTP/GDP. SAH enzymes are found in bacteria, archaea and eukaryotes [5]. It is hypothesized that SAS and SAH genes entered the genomes of archaea and eukaryotes via horizontal gene transfer [5]. Eukaryotes contains an SAH gene called MESH1 (Metazoan SpoT Homologue 1), which is widespread throughout metazoa [5].

In 2010, MESH1 was identified and enzymatically characterized [8]. The Chung group found that MESH1 was capable of hydrolyzing (p)ppGpp to form GTP/GDP [8]. Additionally, the group solved a crystal structure of apo MESH1 (PDB: 3NR1)[8]. The crystal structure revealed that MESH1 consists of ten α-helices and two β-strands and that the catalytic pocket consists of a His-Asp-Box motif, which coordinates the placement of a catalytic Mn ion [8]. The structure was remarkably similar to the hydrolase domain of the previously solved crystal structure of *Streptococcus dysgalactiae* long RSH protein, Rel(seq) (PDB: 1VJ7) [6]. The hydrolase domain of Rel(seq) aligned to a 2.1 Å (R.M.S deviation) with the alpha carbons in human MESH1[8]. Attempting to understand the biological function of MESH1, the group removed *MESH*1 in *Drosophila* and found significant transcriptional changes in larvae including the up-regulation of various stress response genes, which shared significant similarity with the bacterial stringent response [8]. Additionally, *mesh1*-null larvae had impaired ability to survive starvation, demonstrating its important role in the stress survival of *Drosophila* [8]. Based on the transcriptional and phenotypic response to MESH1 removal, the group proposed the novel idea that the stringent response may exist in metazoa. However, this provocative idea was not supported by the identification of relevant substrates in *Drosophila* [8]. While MESH1 can catalyze the dephosphorylation of (p)ppGpp, metazoan genomes don't contain homologues of (p)ppGpp synthetase enzymes. Additionally, various groups, including us, have attempted to identify the (p)ppGpp metabolite in metazoa organisms without success [9]. Although a recent study used LC MS/MS (Liquid Chromatography Tandem Mass Spectrometry) to quantify the alarmone and was able to detect ppGpp in germ-free HeLa cells and in Drosophila larvae at low concentrations: ~40 picomol per gram of fresh weight in germ-free HeLa cells and between 50 and 250 picomol per gram of fresh weight in *Drosophila* [10]. Additionally, the study showed that the loss of function of MESH1 resulted in seven-fold higher levels of ppGpp in *Drosophila* larvae [10]. The enzymatic origins the ppGpp metabolite in metazoa is unknown. The concentration of ppGpp in Hela cells was calculated to be ~ 10,000 fold lower than the concentration of ppGpp in bacteria and the physiological relevance of the metabolite at described concentrations remains to be established [10]. The discovery of MESH1 prompted the question: what are the potential function and relevant substrates of MESH1 in mammalian cells? Recently, our group has been studying this question. This review will center around our findings so far about the function and substrates of MESH1, especially about the evolutionary conserved phenotypes between MESH1 knockdown and bacterial stringent response.

## 2. MESH1 was identified in genome-wide functional genomic screens of ferroptosis

With our interests in understanding the nutrient requirements for cancer cells, we have treated each amino acid as a "gene" to be removed in a nutri-genetic screens to identify the resulting phenotypes of cancer cells with particular oncogenic mutations [11]. From these screens, we have identified that glutamine was essential for triple-negative breast cancers [12], a finding forming the basis for developing glutaminase inhibitors for triple-negative breast cancer. We also found that methionine was essential to maintaining the epigenetic landscape and gene expression patterns in aging and regeneration [13]. Most striking, such a screening identified a profound cystine addiction of the renal cell carcinoma [14], triple-negative breast cells [15] and ovarian cancer cells [16]. Cystine deprivation triggers ferroptosis, a newly recognized form of cell death characterized by iron-mediated oxidative damage triggering lipid peroxidation [17]. Ferroptosis can be induced with the small molecule erastin, which inhibits the xCT complex, an antiporter which transports cysteine into the cell [17]. Imported cysteine is used to generate glutathione, a major antioxidant that protects cells from reactive oxygen species [17]. Oxidized glutathione is reduced via a glutathione reductase enzyme that uses NADPH as a cofactor. Both cystine deprivation

and erastin can trigger profound ferroptosis [17]. To understand the genetic determinants of ferroptosis, we have performed multiple functional genomic screens [18,19]. MESH1 was found during a genome-wide RNAi screen in RCC4 cells deprived of cystine [18]. The knockdown of MESH1 robustly rescued ferroptosis in all tested cells up to one week. While MESH1 is found to be essential for ferroptosis, its relevant substrates and biochemical activities were a mystery.

## 3. Discovery of human substrate for MESH1

Recently, our group made a significant advance by identifying NADPH as the relevant substrate of MESH1-regulated ferroptosis [20]. We found that MESH1 is capable of cleaving the 2′phosphate off of NADPH to form NADH [20]. NADPH is similar to (p)ppGpp in regard to the fact that they both center around a purine moiety, however, NADPH differs from (p)ppGpp in respect to it having the cleavable phosphate at the 2′ location instead of the 3′ of the ribose. MESH1 has a catalytic efficiency ($k_{cat}/K_M$) of $14.4 \times 10^3$ $M^{-1}$ $s^{-1}$ when hydrolyzing NADPH, comparable to the MESH1 (p) ppGpp catalytic efficiency of 9.46 x$10^3$ $M^{-1}$ $s^{-1}$[8,20]. Corroborating this biochemical observation, the structure of the catalytic inactive MESH1-NADPH complex was captured by crystallography, which provides a detailed description on how MESH1 recognizes and binds to NADPH (PDB: 5VXA) [20]. The crystal structure also explains the mechanism underlying the NADPH phosphatase activity [20]. The observation of the NADPH phosphatase activity of MESH1 has been validated in a independent study by Mak et al. in a MESH1 homologue from *Caenorhabditis Elegans* [21]. MESH1′s ability to hydrolyze both (p)ppGpp and NADPH indicates that it possesses broad substrate specificity. However, the cellular concentration of NADPH is far higher than (p)ppGpp in metazoa. For example, the cytosolic concentration of NADPH in rat liver was calculated to be $\sim$ 370 μM, which is orders of magnitude higher than the concentration of (p)ppGpp [22]. It is possible that MESH1 may dephosphorylate the smaller cellular pool of (p)ppGpp. However, based on the similar catalytic efficiencies of MESH1 towards (p) ppGpp and NADPH, but the vastly different concentrations it is probable that the NADPH enzymatic activity is more physiologically relevant in metazoa.

Interestingly, NADPH has been found to be a predictor of ferroptosis sensitivity during the pharmacogenomics analysis of a large panel of cell lines [23]. Our group found that *MESH1* silencing mitigated the reduced NADPH during ferroptosis, which in turn increased the reduced glutathione and protection against ferroptosis [20]. This ferroptosis protection phenotype could be reversed via the simultaneous silencing of *NADK*, an NAD kinase that can generate NADPH [20]. Collectively, this study highlighted the role of MESH1/NADPH enzymatic activity in regulating oxidative stress that occurs during ferroptosis [20].

## 4. Phenotypic similarity to stringent response: Stress survival

There are interesting parallels between the bacterial stringent response and the mechanisms underlying MESH1-mediated ferroptosis. Both iron levels and oxidative stress can induce the stringent response in bacteria. Iron starvation can activate the stringent response in *Bacillus Subtilis* and *E. coli* [24,25]. Additionally, oxidative stresses can activate the stringent response in *Francisella tularensis* and *Staphylococcus aureus* and the removal of the alarmone makes the bacterial cells more susceptible to oxidative stresses such as $H_2O_2$[26,27]. The bacterial stringent response alters the expression of antioxidant defense mechanisms. *Staphylococcus aureus* contain a bacillithiol (BSH) redox defense mechanism that is very similar to the glutathione defense mechanism. Both consist of small molecules derived from cysteine and can be oxidized/reduced to provide redox homeostasis. *Staphylococcus aureus* lacking (p)ppGpp had higher levels of oxidized BSH and impaired ability to survive oxidative stresses [26]. Additionally, the transcriptome of *Staphylococcus aureus* lacking (p)ppGpp had significant changes to genes associated with iron metabolism which resulted in higher cellular levels of free iron [26]. The increased free iron contributed to greater oxidative damage [26]. Overall, both the bacterial and metazoan stringent response seem to play a significant role in iron and redox homeostasis, and in humans this is demonstrated in the context of ferroptosis.

## 5. Phenotypic similarity to stringent response: IF2α and reduced translation

The stringent response in bacteria is associated with extensive transcriptional changes which allow the bacteria to survive periods of metabolic stress [1]. In fact, over one-third of bacterial genes can be differentially expressed upon the induction of the stringent response [28]. There also appears to be extensive transcriptional changes that occur in metazoa during a stringent response. Genetic knockdown of MESH1 in starved *Drosophila* larvae resulted in the upregulation of genes associated with stress response such as heat shock proteins [8]. Recently, we used RNA-seq to profile changes in the transcriptome upon the knockdown of MESH1 in a human cancer cell line (GEO: GSE114282) [29]. MESH1 knockdown was associated with the upregulation of all three branches of the unfolded protein response (UPR)/endoplasmic reticulum (ER) stress pathways including the integrated stress response (ISR) pathway [29]. The ISR is a signaling pathway in eukaryotes that is activated during periods of stress and causes decreased protein synthesis until cellular homeostasis is restored [30].

The ISR pathway centers around the phosphorylation of Eukaryotic Translation Initiation Factor 2A (eIF2α) [30]. Phosphorylated eIF2α activates the transcription factor, activating transcription factor 4 (ATF4), which induces extensive transcriptional changes [30]. Knockdown of MESH1 resulted in increased levels of phosphorylated eIF2α [29]. Additionally, ATF4 accounted for $\sim$ 30% of transcriptome change that occurs during MESH1 knockdown [29]. The importance of the enzymatic activity of MESH1 in regulating the ISR was revealed by concurrent knockdown of NADK, which abolished upregulation of genes in the ISR pathway and highlighted the importance of MESH1-NADPH activity [29]. However, a detailed mechanism of how NADPH accumulation regulates an integrated stress response remains a mystery.

Besides ATF4, other branches of the ER stress responses are also activated upon MESH1 knockdown. For example, the RNA-sequencing data revealed the activation of the Inositol-requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6) branches of the UPR pathways [29]. It was recently shown in *C. elegans,* that the MESH1 homolog, RSH-1, also regulates the activation of the ATF-6 branch of the UPR [21]. The study found that the enzymatic activity of RSH-1 regulates the m-TOR (mammalian/mechanistic target of rapamycin) signaling pathway which then activates an UPR response by increasing the expression of XBP-1 (X-Box Binding Protein-1)[21]. These findings suggest that MESH1 regulation of ER proteostasis exists throughout metazoa. Both bacterial and metazoan stringent responses center around adapting to metabolic stresses to maintain homeostasis to adapt to environmental changes. Homeostasis is restored in part by the decrease of in protein synthesis. Interestingly, bacterial and metazoan stringent responses have similar mechanisms to inhibit protein synthesis. In human cell lines, the knockdown of *MESH1* results in the phosphorylation of eIF2α, which causes a block in cap-dependent translation, thus reducing general protein synthesis [29]. However,

eIF2α phosphorylation also favors the cap-independent translation and increases the translation of activating transcription factor 4 (ATF4), which transactivates a transcriptional program as an important component of the transcriptional response to *MESH1* knockdown [29]. Interestingly, the bacterial stringent response can also regulate translation initiation through IF2[31]. (p)ppGpp is capable of binding to translational initiation factor 2 (IF2) and the binding can disrupt the formation of the bacterial initiation complex, thus disrupting bacterial protein synthesis [31]. Overall, MESH1 inhibition leads to the activation of the integrated stress response in human cell lines in a similar manner to transcriptional changes that occur in bacteria during the stringent response.

## 6. Phenotypic similarity to stringent response – Proliferation arrest and dNTP depletion

Transcriptome profiling of human cancer cell lines also revealed that MESH1 knockdown resulted in the down-regulation of several genes associated with cell cycle progression including CDKC (Cell Division Cycle) and RRM (Ribonucleotide Reductase) genes. As was the case in *Drosophila*, MESH1 knockdown in human cancer cell lines significantly decreased levels of BrdU incorporation- indicating that MESH1 regulates cellular proliferation [8,32]. The MESH1 knockdown depleted cellular levels of dNTP by downregulating genes associated with dNTP synthesis, such as RRM1 and RRM2 [31]. MESH1′s ability to influence cell cycle led us to explore the role of MESH1 in cancer. Patient data revealed that MESH1 expression is higher in tumors and associated with poorer patient outcomes [32]. MESH1 knockdown reduced the size of tumor spheres and slowed the growth of xenografts in mice [32].

MESH1 knockdown was consistently associated with the down-regulation of the mRNA but not protein levels of TAZ (Transcriptional coactivator with the PDZ binding motif) [32]. TAZ and its paralog YAP (Yes associated protein) are the master regulators of the HIPPO pathway which regulates cellular proliferation [32]. Overexpression of wild-type MESH1 but not enzymatically dead MESH1 were capable of restoring TAZ mRNA levels [32]. Overexpression of NADK could also restore TAZ levels, highlighting the importance of the MESH1-NADPH phosphatase activity in regulating TAZ [32]. Overexpression of TAZ could reverse the cellular proliferation and dNTP level phenotypes caused by MESH1 knockdown [32]. Transcriptome profiling revealed that TAZ repression was responsible for one third of transcriptional changes that occur during MESH1 depletion including the downregulation of cell cycle genes such as RRM2 and CDC6 [32]. MESH1 knockdown is associated with the hypoacetylation of the H3K27Ac in the promoter and enhancer regions of the TAZ promoter/enhancer region and H3K27Ac serves as an epigenetic regulator of TAZ expression [32]. The Hippo signaling pathway exerts profound effects on cellular proliferation, survival, cell death, and organ sizes. YAP and TAZ, two Hippo effectors, are usually tightly co-regulated by the phosphorylation of the kinase cascade of MST1/2, LATS1/2 and RASSF family proteins. However, the regulation of TAZ by MESH1 occurred at the mRNA instead of the post-translational level, indicating the role of MESH1 to maintain the *TAZ* mRNA expression, the activity of the HIPPO pathway and thus cellular proliferation [32]. Interestingly, many components of the HIPPO pathway (including YAP and TAZ) first emerge in cnidarians, a very ancient group of metazoans [33]. All major domains of YAP and TAZ are also conserved between cnidarians and mammals [33]. Given that MESH1 also shares the conserved domains with the bacterial hydrolase SpoT, it is tempting to speculate the functional convergence between MESH1 and the HIPPO pathway as they both appear in metazoans during evolution.

The reduction of cellular proliferation/DNA replication is a defining feature of the bacterial stringent response. Mechanistically, the decrease in DNA replication in bacteria is caused by (p)ppGpp binding to several proteins responsible for DNA replication. For example, in *E. coli,* (p)ppGpp binds to and modulates the function of DnaG, a DNA primase, which is essential for DNA replication [34]. Additionally, accumulation of the alarmone in *E. coli* leads to transcriptional decrease in DnaA, a replication initiation ATPase [35]. Decreased production of dNTP is another hallmark feature of the bacterial stringent response. The alarmone is capable of inhibiting several enzymes associated with nucleotide synthesis such as PurF [35]. PurF, an amidophosphoribosyl transferase that is essential for the *de novo* synthesis of purine nucleotides [35]. In conclusion, both the bacterial and metazoan stringent responses regulate cellular proliferation, DNA replication, and nucleotide synthesis and in humans the metazoan stringent response may play a critical role in tumor biology.

## 7. Summary and outlook

The evolutionarily ancient stringent response provides the primary means by which bacteria survive metabolic stresses. RSH proteins regulate the stringent response by controlling the cellular levels of (p)ppGpp. Increased levels of (p)ppGpp cause the bacterial cells to decrease proliferation and induce changes in the transcriptome and allow the bacteria to survive stress. The metazoan genome encodes an alarmone hydrolysis enzyme called MESH1. However, the human genome does not contain an alarmone synthesis enzyme and the presence of endogenous (p)ppGpp is a topic under debate. Our group has found that MESH1 can act as a phosphatase on NADPH, which may be the relevant substrate in mammals. Additionally, our group has demonstrated that the MESH1 deletion produces many of the similar phenotypes **(Figure)** as the bacterial stringent response. Similar to bacteria where inhibition of the alarmone hydrolase alters redox homeostasis, in humans MESH1 regulates the oxidative stress that occurs during ferroptosis. Accumulation of the alarmone causes changes in the bacterial transcriptome (for example: increased expression of amino acid and lipid synthesis enzymes) that allow the bacteria to survive stress. We have found that MESH1 knockdown upregulates all three branches of the ER stress pathways including the ISR and allows human cells to survive stresses such as ferroptosis. Finally, a hallmark feature of the stringent response is a decrease in cellular proliferation. We have found that MESH1 silencing triggers a decrease in cellular proliferation by halting the cell cycle. The striking similarities between the bacterial stringent response and MESH1 deletion phenotypes lead us to believe that the evolutionarily ancient stringent response remains in metazoa. We have termed this pathway the "metazoan stringent-like response" [36]. The metazoan stringent response may be an example of homologous proteins regulating similar phenotypes through distinct substrates and mechanisms. Enzymes are capable of evolving new functionality over time including developing increased specificity for a new substrate or broader substrate specificity [37]. For example, mutations in the active site of a monoamine oxidase could convert the enzyme to an *L*-amino-acid oxidase and P450 enzymes have evolved to possess broad substrate specificity towards xenobiotics [38]. Evolutionary changes in substrate preferences can coincide with the development of new signaling pathways. For example, eukaryotic kinases evolved new substrate preferences from their bacterial ancestors, and these new substrate preferences arose with the development of eukaryotic cell signaling pathways [39]. It is possible that after the gene loss of an RSH synthetase enzyme in metazoa, that MESH1 evolved to change substrate specificity. The change in substrate specificity could have coincided

with the evolution of new cell signaling pathways, which fulfills the function of the bacterial stringent response in the context of a multi-cellular organism.

Despite these advances, much remains unknown about MESH1 and the metazoan stringent like response. It is unknown what stresses and external stimuli could induce the stringent response in metazoa. Additionally, it is unclear if MESH1 could act upon any other substrates and if MESH1/(p)ppGpp activity is physiologically relevant. Additionally, the mechanisms that connect MESH1 enzymatic activity to phenotypes are largely unknown. For example, it is unclear how MESH1/NADPH activity can regulate the integrated stress response and epigenetic regulation of TAZ. MESH1 appears to play a significant role in tumor biology and MESH1 could prove to be a useful therapeutic target to treat various cancers. The field of the metazoan stringent response is in its infancy, and much is to be explored about this important signaling system.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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