Metabolic pathways regulated by TAp73 in response to oxidative stress

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ABSTRACT

Reactive oxygen species are involved in both physiological and pathological processes including neurodegeneration and cancer. Therefore, cells have developed scavenging mechanisms to maintain redox homeostasis under control. Tumor suppressor genes play a critical role in the regulation of antioxidant genes. Here, we investigated whether the tumor suppressor gene TAp73 is involved in the regulation of metabolic adaptations triggered in response to oxidative stress. H_2O_2 treatment resulted in numerous biochemical changes in both control and TAp73 knockout (TAp73-/-) mouse embryonic fibroblasts, however the extent of these changes was more pronounced in TAp73-/- cells when compared to control cells. In particular, loss of TAp73 led to alterations in glucose, nucleotide and amino acid metabolism. In addition, H_2O_2 treatment resulted in increased pentose phosphate pathway (PPP) activity in null mouse embryonic fibroblasts. Overall, our results suggest that in the absence of TAp73, H_2O_2 treatment results in an enhanced oxidative environment, and at the same time in an increased pro-anabolic phenotype. In conclusion, the metabolic profile observed reinforces the role of TAp73 as tumor suppressor and indicates that TAp73 exerts this function, at least partially, by regulation of cellular metabolism.

INTRODUCTION

The maintenance of redox homeostasis is a crucial task for the cell, as different levels of reactive oxygen species can induce different biological responses, often associated with pathologies such as cancer and neurodegeneration [1-10]. High levels of ROS are detrimental, whereas at low levels, ROS sustains differentiation and proliferation, therefore acting as signaling molecules [11-21]. Indeed, cells can produce hydrogen peroxide (H_2O_2) in order to modulate biological processes as diverse as proliferation, differentiation and migration [22-26]. On the other hand, excessive production of ROS leads to the deleterious oxidative damage [27-31]. Hence, cells have developed numerous ROS scavenging mechanisms [32-36], most notably GSH [37, 38], catalase and superoxide dismutase and, of note, most of them are regulated by different tumor suppressor genes to safeguard cellular redox homeostasis counteracting excessive ROS production [39-41]. Among the tumor suppressor genes, the p53-family (p53, p63 and p73 proteins) [42-50] has a key role in controlling antioxidant gene expression [51-54]. Indeed, p53 regulates the expression of numerous antioxidant genes, including, sestrins, *TIGAR* and glutaminase-2 (*GLS2*) [55-58], thus contributing to ROS homeostasis.

Recent studies have also demonstrated an essential role for p73 and p63 in regulation of oxidative metabolism. In fact, deletion of the long TAp73 isoform of p73 increases ROS production and oxidative stress by

affecting electron flux during mitochondrial oxidative phosphorylation and flux through the oxidative arm of the PPP [59-61]. Similarly, p63 contributes to the maintenance of a balanced redox state in keratinocytes and lung cancer cells through the regulation of *GLS2*, cytoglobin, hexokinase-II and *REDD1* [62-65].

The aim of this study was to identify the differences in global biochemical responses to oxidative stress between wild-type and TAp73 knock-out (TAp73-/-) mouse embryonic fibroblasts (MEFs), with the held hypothesis that TAp73 controls oxidative metabolism and response to oxidative stress. H_2O_2 treatment resulted in numerous biochemical changes in both WT and TAp73-/- cells, but the number and extent of these changes was more robust in TAp73-/- cells as compared to WT control. Overall, it appears that in the absence of TAp73, H_2O_2 treatment results in an enhanced oxidative environment, possibly promoted by an increased nucleotide catabolism, concomitant to a decreased apoptotic biochemical profile as compared to TAp73-proficient cells.

RESULTS

H_2O_2 induced-oxidative stress and glutathione recycling is potentially greater in TAp73-/- versus WT MEFs

In order to explore the metabolic role of TAp73 in oxidative stress, MEF derived from TAp73-/- and control mice were treated with H_2O_2 and then subjected GC-MS and LC-MS-MS platforms for metabolomics studies as previously described [66]. The total numbers of significantly or nearly-significantly altered biochemicals are reported in Table S1.

The tripeptide glutathione (gamma-glutamyl-cysteinylglycine) functions as one of the major antioxidants in cells [67]. Both reduced and oxidized glutathione (GSH and GSSG) levels were increased following the H_2O_2 treatment time course in the WT and TAp73-/- cells, but these increases were greater in TAp73-/- cells

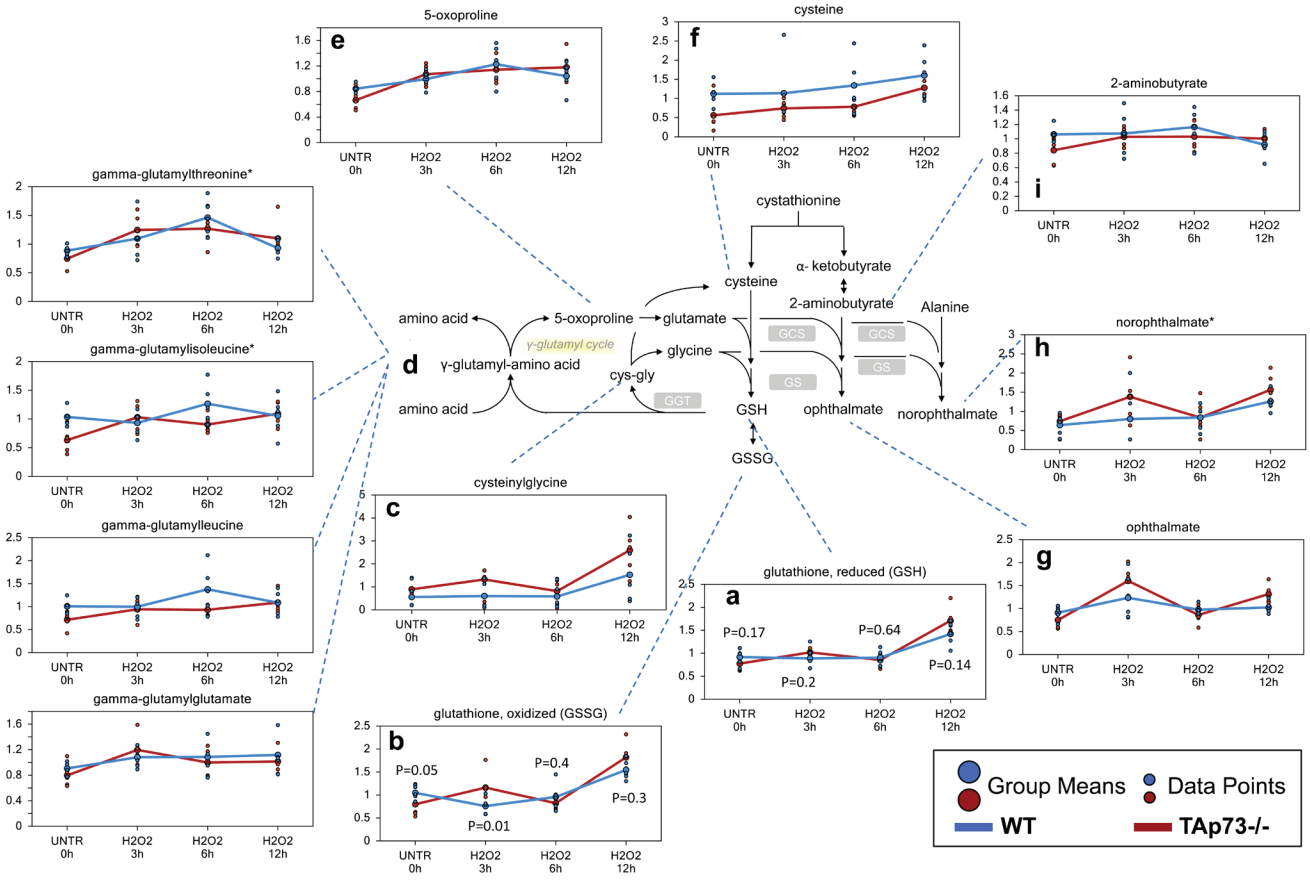

Figure 1: Glutathione recycling is potentially greater in TAp73-/- *versus* WT MEF. GSH is a key antioxidant molecule within the cell. The availability of the amino acid precursor, cysteine, and the activity of the rate-limiting enzyme, glutamate cysteine ligase, are the key factors in GSH synthesis. **a.-i.** Levels of the indicated metabolites were evaluated as described in material and methods. Anova contrasts *t*-tests were used to identify biochemicals that differed significantly between experimental groups (n = 5 for each time point). P-values for reduced and oxidized glutathione are also reported for each time point.

(Figure 1a and 1b). In addition, biochemicals associated with increased glutathione recycling (cysteinylglycine, gamma-glutamyl-amino acids, and 5-oxoproline) were also more elevated in the TAp73-/- cells, suggesting an increased rate of glutathione turnover occurring in the TAp73-/- cells over the course of H_2O_2 treatments (Figure 1c-1e). Cysteine, which is the rate-limiting precursor to glutathione [68], showed increased levels in both WT and TAp73-/- cells during the H_2O_2 treatment and this increase was more pronounced and reached statistical significance in TAp73-/- cells. However, the absolute levels of cysteine remained consistently lower in the TAp73-/- cells, suggesting reduced cysteine precursor for glutathione biosynthesis (Figure 1f). The increased glutathione levels in both WT and TAp73-/- MEFs during the time course suggest that cysteine biosynthesis is enhanced by H_2O_2 in order to fuel the supply of glutathione. It should be noted that, in untreated cells (UNTR) the levels of cysteine were significantly lower in TAp73-/- as compared to WT, and remained such throughout the H_2O_2 time course. In keeping with the reduced cysteine levels in TAp73-/- cells, we identified increased levels of the tripeptides opthalmate (gamma-glutamyl-alpha-aminobutyrylglycine) (Figure 1g) and norophthalmate (gamma-glutamyl-alanylglycine) (Figure 1h) in knockout cells as compared to WT controls following H_2O_2 treatment. 2-aminobutyrate and alanine replace cysteine during the synthesis of ophthalmate and norophthalmate respectively (Figure 1i). Thus, the increase in ophthalmate and norophthalmate could suggest either adaptation to limiting cysteine levels or to augmented glutathione synthetase (GCS) activity, triggered by oxidative environment. Increased levels of the oxidative by-product of sterols, such as oxysterols, 7-ketocholesterol and 7-beta-hydroxycholesterol further support an increased oxidative environment in the TAp73-/- cells as compared to WT cells (Table S1).

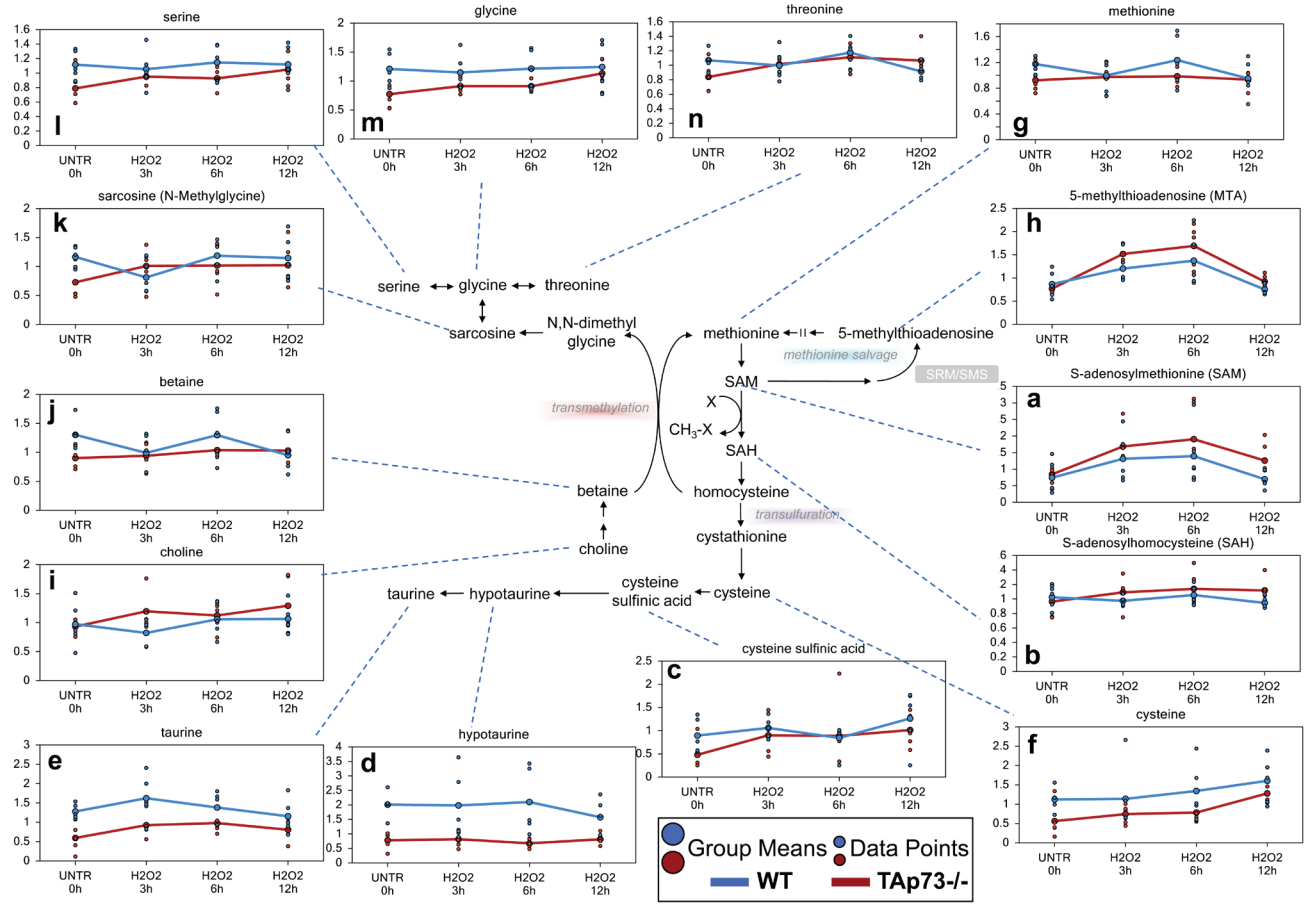

Figure 2: Loss of TAp73 enhances methionine metabolism following H_2O_2 treatment. Methionine is the initiating amino acid in the synthesis of eukaryotic proteins. Methionine metabolism begins with its activation to SAM by methionine adenosyltransferase. **a.-n.** Levels of the indicated metabolites were evaluated as described in material and methods. Anova contrasts *t*-tests were used to identify biochemicals that differed significantly between experimental groups (n = 5 for each time point).

Methionine metabolism is enhanced following H_2O_2 treatment predominately in TAp73-/- cells

Cysteine biosynthesis

As previously stated, cysteine levels were elevated in both WT and TAp73-/- cells over the H_2O_2 treatment time course, but these increases were more robust in TAp73-/- cells. The major source for cysteine biosynthesis is through methionine metabolism [69]. H_2O_2 treatment induced significant increases in the methionine metabolite, S-adenosylmethionine (SAM), in both the WT and TAp73-/- cells, with TAp73-/- cells having more robust changes (Figure 2a). In addition, S-adenosylhomosysteine (SAH), which is formed when SAM participates in methylation events, demonstrated a trend of increasing levels in TAp73-/- over the H_2O_2 time course, but this increase did not reach significance, while SAH was unchanged over time in WT (Figure 2b). One possible explanation for why SAH showed non-significant increases in TAp73-/- and was unchanged in WT could be due to increased metabolism to homocysteine and subsequently to cystathionine to fuel cysteine biosynthesis. The previously described increase in cysteine supports this possibility. Not only can cysteine be metabolized to glutathione, but it can also be oxidized to cysteine sulfinic acid, which can be further metabolized to hypotaurine and taurine. This metabolic route further depletes the cells of cysteine for glutathione synthesis. While cysteine sulfinic acid was increased in WT cells depending upon the H_2O_2 time point investigated, this increase never reached significance, and neither hypotaurine nor taurine were significantly changed in WT (Figure 2c-2e). In contrast, cysteine sulfinic acid was significantly elevated in the TAp73-/- cells following H_2O_2 treatment as compared to UNTR cells (Figure 2c), and although hypotaurine was unchanged, taurine was also significantly elevated in the TAp73-/- cells over the H_2O_2 time course (Figure 2d and 2e). The lack of change in hypotaurine in TAp73-/- cells may reasonably result from subsequent metabolism to taurine. Thus, the already lower pool of cysteine in TAp73-/- cells (Figure 2f) appears to be further decreased by conversion to cysteine sulfinic

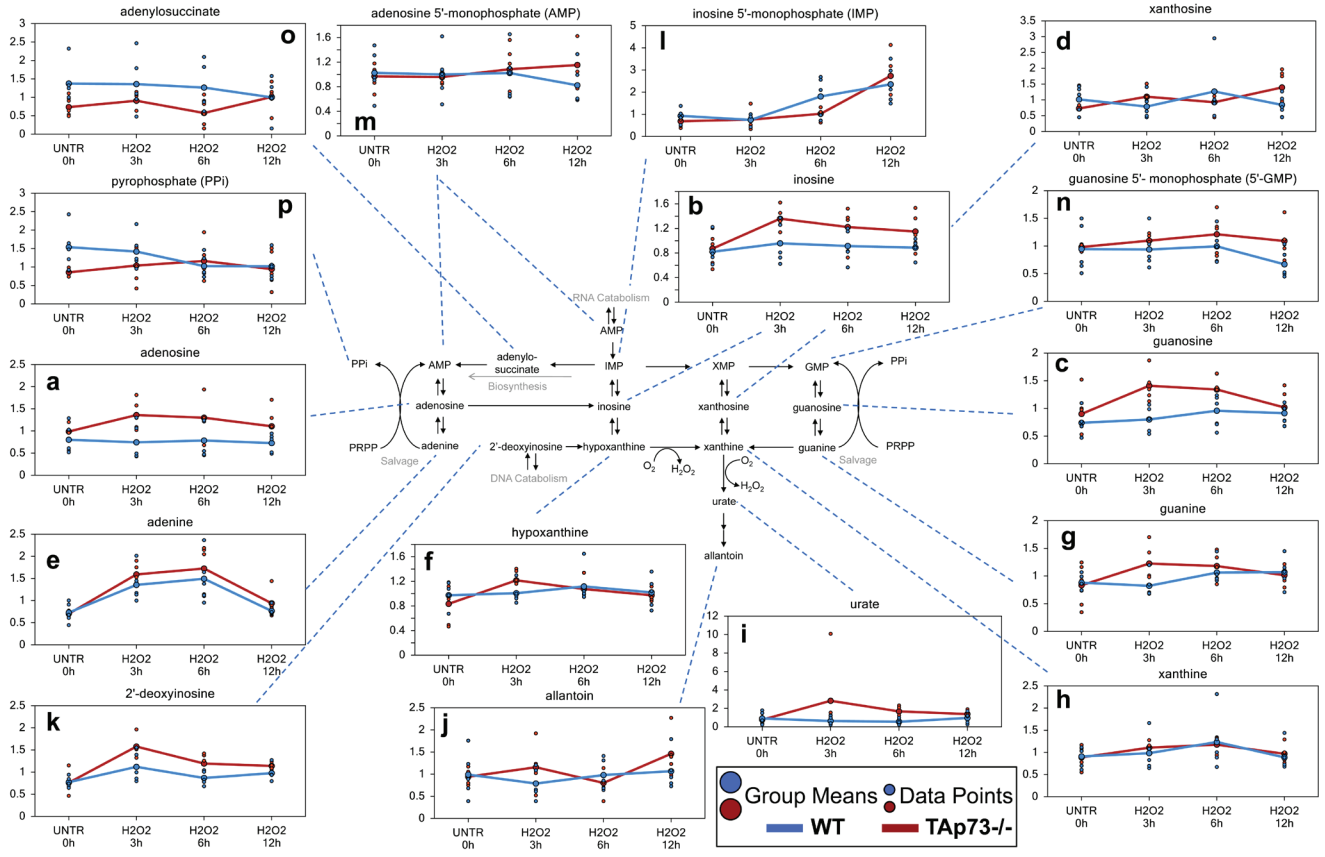

Figure 3: Purine associated metabolites. a.-p. Levels of the indicated metabolites were evaluated as described in material and methods. Anova contrasts *t*-tests were used to identify biochemicals that differed significantly between experimental groups ($n = 5$ for each time point).

acid at a higher rate than that seen in WT and may have adverse effects on the synthesis of glutathione and thus compromise redox homeostasis in the TAp73-/- cells.

Methionine salvage and transmethylation

Although there appeared to be an increase in methionine metabolism in both the WT and TAp73-/- cells, methionine levels were only significantly lower at the 12 hour H2O2 time point in the WT cells and were unchanged throughout the H_2O_2 treatments in the TAp73-/- cells (Figure 2g), which may suggest increased methionine salvage at the earlier time points in WT and TAp73-/- cells. Increased methionine salvage was supported by the significant increase in 5-methylthioadenosine (MTA) in both WT and TAp73-/- cells following 3 and 6 hours H_2O_2 treatment, and this treatment-induced increase was greater in the TAp73-/- cells as compared to WT cells (Figure 2h). In addition to increased methionine salvage, it is possible that increased transmethylation following H_2O_2 treatment also contributed to the lack of change in methionine levels in the TAp73-/- cells. Elevated transmethylation in H_2O_2-treated TAp73-/- cells was supported by changes in choline, betaine, and sarcosine, glycine, serine and threonine in the TAp73-/- cells (Figure 2i-2n). Briefly, choline can be oxidized to betaine, and betaine can be further metabolized to N,N-dimethylglycine by functioning as a methyl source for the transmethylation of homocysteine back to methionine. Although N,N-dimethylglycine was below the level of detection in TAp73-/- cells, its metabolite sarcosine was increased in the TAp73-/- cells throughout the H_2O_2 treatment time course, further supporting increased transmethylation. Sarcosine is rapidly degraded to the amino acids glycine, and glycine can be further metabolized to either threonine or serine. Thus, the increases observed in these amino acids support increased transmethylation activity. It is possible that the increase in sarcosine in the TAp73-/- cells is a *consequence* of increased glycine, which can be methylated to sarcosine, and thus would suggest increased amino acid uptake in the TAp73-/- cells is responsible for the increased sarcosine. Although increased amino acid uptake is possible, due to the additional changes observed for methionine metabolism, increased transmethylation remains a likely explanation for the observed changes in methionine-associated biochemicals.

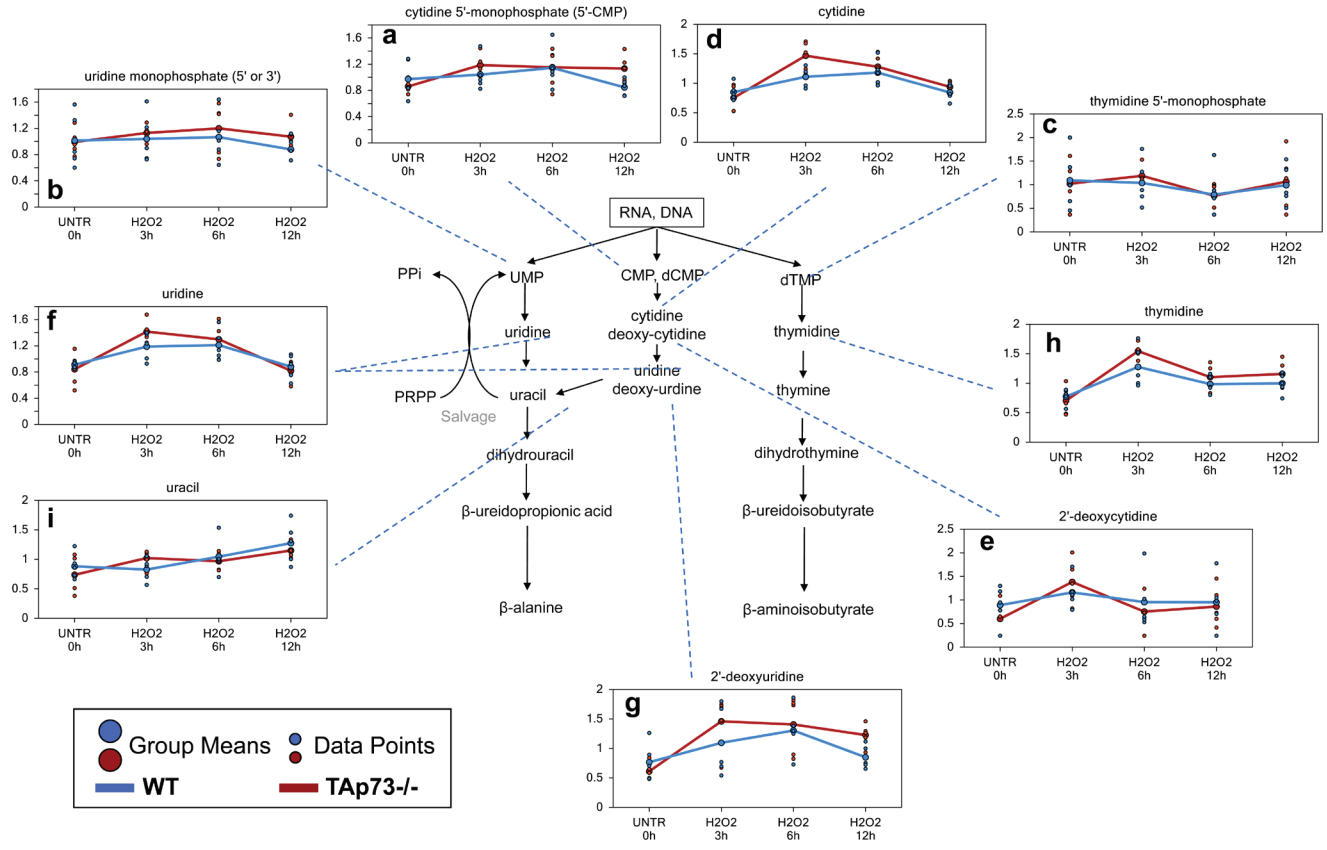

Figure 4: Pyrimidine associated metabolites. a.-i. Levels of the indicated metabolites were evaluated as described in material and methods. Anova contrasts *t*-tests were used to identify biochemicals that differed significantly between experimental groups ($n = 5$ for each time point).

Effects on nucleotide metabolism by H_2O_2 treatment of TAp73-/- are more profound compared to WT

Purine associated metabolites

Interestingly, only the TAp73-/- cells demonstrated significant or trending increases in the purine nucleosides (adenosine, inosine, and guanosine, and xanthosine) (Figure 3a-3d) and nucleobases (adenine, hypoxanthine, and guanine) following 3 hours H_2O_2 treatment (Figure 3e-3g). Both hypoxanthine and guanine can be metabolized to xanthine (Figure 3h), which is subsequently metabolized to urate (Figure 3i) and allantoin (Figure 3j). Xanthine increase initiated at 3 hours of treatment and reached significance at 6 hours in the TAp73-/- cells. We also identified a large, but not significant, increase in urate in TAp73-/- cells following H_2O_2 treatment, and this probably fuelled the significant increase in allantoin at the 12 hours H_2O_2 treatment time point. The increases in urate and allantoin would suggest that purine catabolism further increased H_2O_2 levels in the TAp73-/- cells potentiating the effects of H_2O_2 treatment in these cells. In contrast, in WT cells, only xanthine showed an increase, which only trended towards significance. Probably xanthine increase was fuelled by direct conversion of its precursor 2'-deoxyinosine (Figure 3k), which was significantly increased at 3 hours in both WT and TAp73-/- cells. Once again this increase was not only larger in the TAp73-/- cells, but 2'deoxyinosine was also significantly elevated in TAp73-/- cells throughout the entire H_2O_2 time course. These changes suggest increased purine catabolism following H_2O_2 treatment is more severe in TAp73-/- cells.

In addition to increases in purine catabolites, there was also an increase in the nucleotides, inosine 5'monophosphate (IMP), adenosine 5'-monophosphate, and guanosine 5'-monophosphate (5'-GMP) in the TAp73-/- cells (Figure 3l-3n), although only the increment in IMP reached significance at the 12 hours H_2O_2 time point. AMP was unchanged at 3 hours, but a non-significant increase at the 6 and 12 hours H_2O_2 treatment time points was

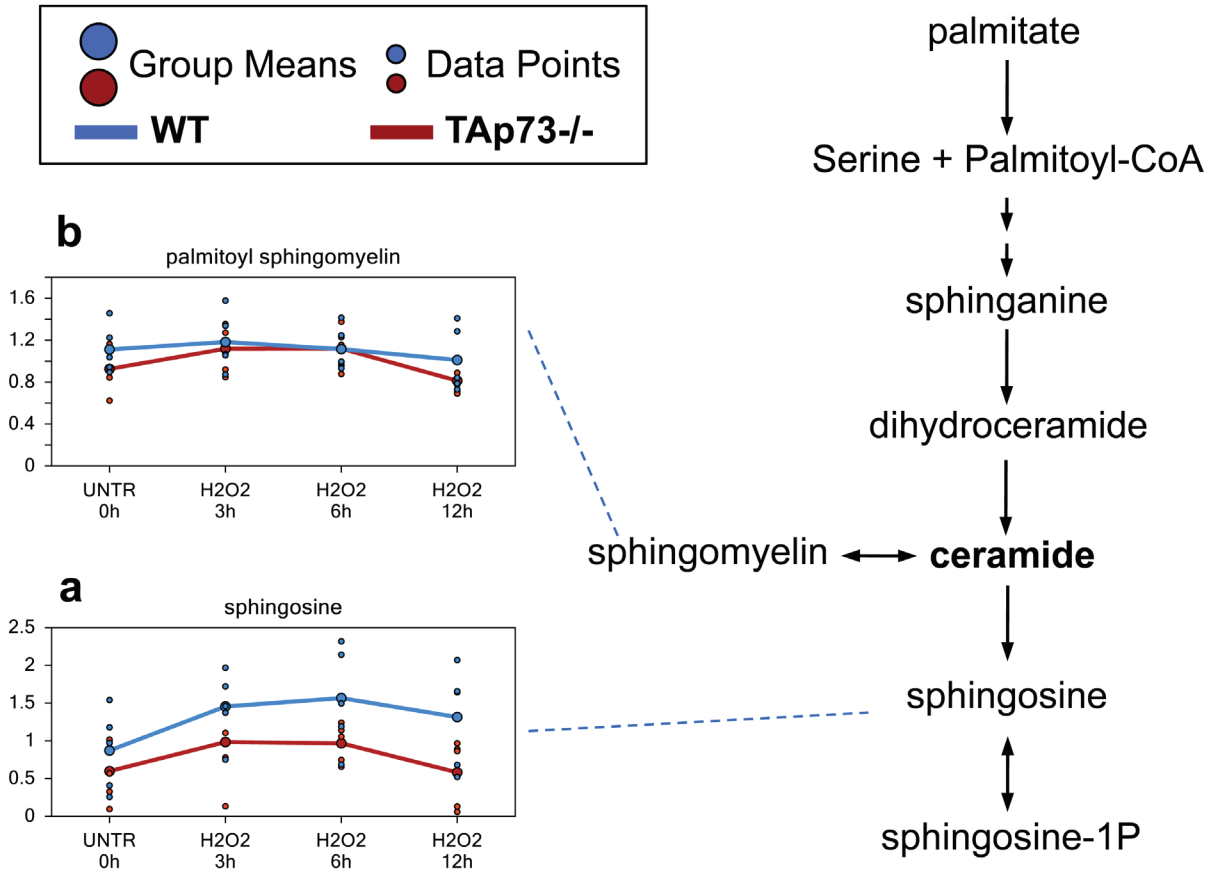

Figure 5: Sphingosine and ceramide metabolism. Ceramide is a sphingolipid which functions as bioactive signaling molecule. Ceramide plays key roles in a variety of cellular responses, including regulation of cell growth, viability, differentiation, and senescence. **a.-b.** Levels of the indicated metabolites were evaluated as described in material and methods. Anova contrasts *t*-tests were used to identify biochemicals that differed significantly between experimental groups ($n = 5$ for each time point).

observed in TAp73-/- cells. In contrast, 5'-GMP levels were increased throughout the H_2O_2 treatments in TAp73-/- cells, but these changes never reached significance. The changes in IMP and AMP were greatest at 12 hours H_2O_2, while the majority of the previously discussed purine catabolites were increased the greatest at 3 hours. Thus, the changes in IMP and AMP may represent either increased purine salvage or biosynthesis to compensate for the increased purine catabolism in the TAp73-/- cells following H_2O_2 treatment. The trending increases in pyrophosphate (PPi) (Figure 3o), which is formed from phosphoribosyl pyrophosphate (PRPP) during salvage and biosynthesis, and the purine biosynthesis intermediate adenylosuccinate potentially support a late increase in both purine salvage and biosynthesis in TAp73-/- cells (Figure 3p). Nonetheless, we cannot formally rule out that RNA breakdown could contribute to changes in the nucleotide pool.

In contrast to what was observed in TAp73-/- cells, IMP levels were significantly elevated only at the later H_2O_2 time points in WT cells, but PPi, adenylosuccinate, and AMP levels trended downwards in the WT cells. Thus, there does not appear to be increased purine salvage or biosynthesis in the WT cells, and the increase in IMP may rather represent enhanced purine catabolism at a later H_2O_2 time point in WT cells compared to TAp73-/- cells.

Pyrimidine associated metabolites

A number of pyrimidine catabolites, likely associated with increased DNA and RNA breakdown, were increased following H_2O_2 treatment in both WT and TAp73-/- cells and these changes were in general greatest at the 3 hour time point and in the TAp73-/- cells. These changes included early increases in the pyrimidine nucleotides (cytidine 5'-monophosphate, uridine monophosphate, and thymidine 5'monophosphate) (Figure 4a-4c), and nucleosides (cytidine, 2'-deoxycytidine, uridine, 2'-deoxyuridine, and thymidine) and the pyrimidine base (uracil) (Figure 4d-4i). Although PPi is also generated by pyrimidine salvage and/or biosynthesis,

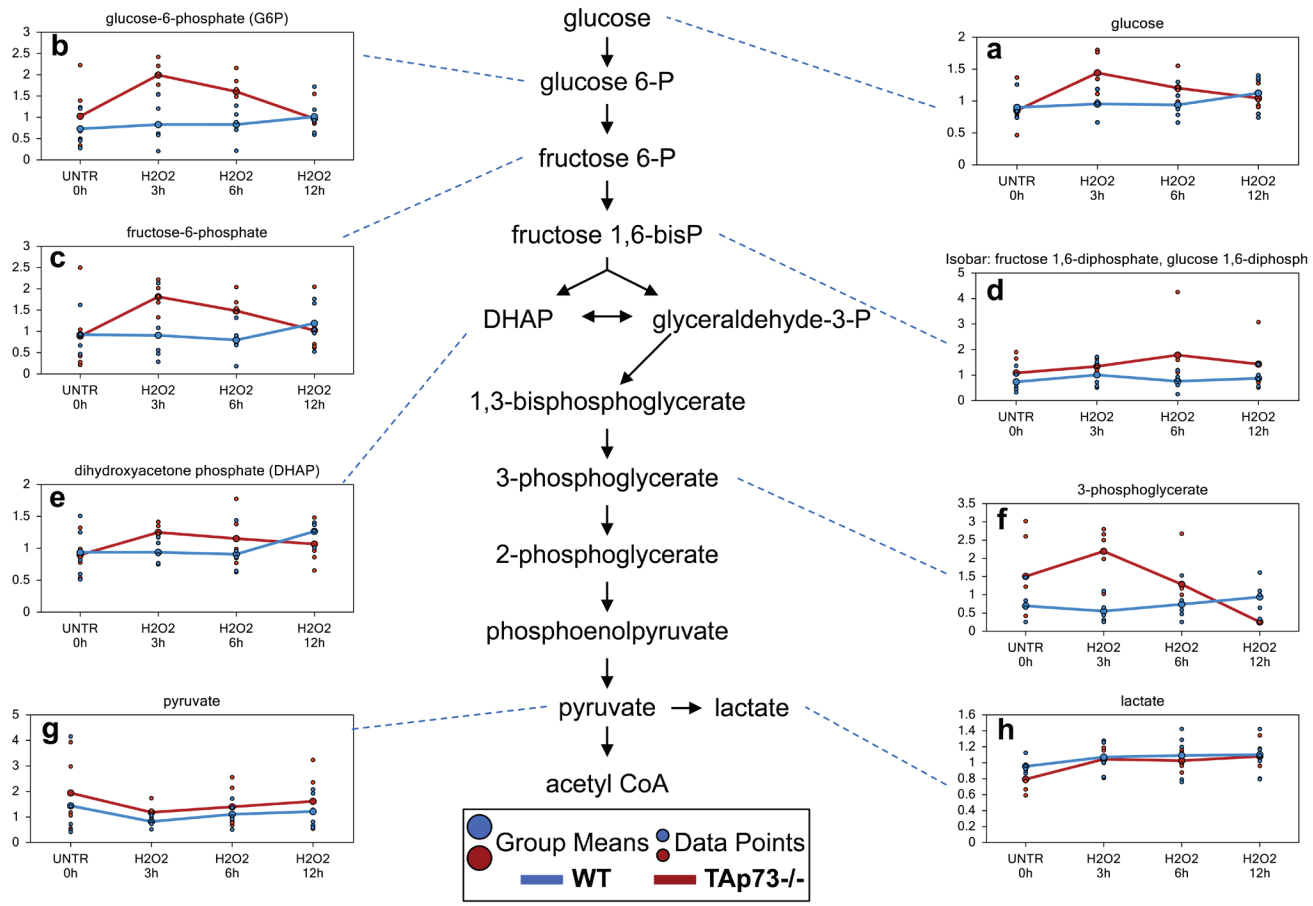

Figure 6: Glycolysis is increased early only in TAp73-/-following H_2O_2 treatment. Glycolysis is the metabolic pathway that converts glucose into pyruvate. **a.-h.** Levels of the indicated metabolites were evaluated as described in material and methods. Anova contrasts *t*-tests were used to identify biochemicals that differed significantly between experimental groups ($n = 5$ for each time point).

the observed changes in pyrimidine-associated metabolites do not indicate this is occurring. Rather, the observed changes suggest increased pyrimidine catabolism following H_2O_2 treatment, and this is more severe in the TAp73-/- cells.

Difference in sphingosine levels may reflect decreased ceramide in TAp73-/- cells

Increases in ceramide have been associated with growth arrest, differentiation, senescence, and H_2O_2-induced apoptosis [70-72]. Although we failed to detected ceramide directly, its metabolite sphingosine had a trending increase at the 3 hours H_2O_2 treatment time point and was significantly increased at 6 hours in both WT and TAp73-/- cells (Figure 5a). At the 12 hours, the increase in sphingosine was maintained only in the WT cells, while the levels went back to control levels in TAp73-/- cells. Furthermore, overall levels of sphingosine were consistently lower in TAp73-/- cells compared to WT cells at any time point investigated. The differences in sphingosine levels suggest increased ceramide in WT cells compared to TAp73-/- cells following H_2O_2 treatment. The reason for this increase in sphingosine remains unclear. Indeed, although H_2O_2 is known to increase sphingomyelinase activity and *de novo* ceramide synthesis, we did not observe changes in palmitoyl sphingomyelin, hence excluding increased sphingomyelinase activity. Therefore, we believe that *de novo* synthesis is the most reasonable explanation for the higher sphingosine levels (Figure 5b).

Glycolysis is increased early only in TAp73-/- cells following H_2O_2 treatment

One of the biggest differences between WT and TAp73-/- cells following H_2O_2 treatment was observed in glucose metabolism. Upon cell entry, glucose is immediately phosphorylated to glucose-6-phosphate which then can either be shunted to the PPP for NADPH production and nucleotide biosynthesis or continue through glycolysis generating pyruvate and subsequently acetyl-CoA to supply to the tricarboxylic acid (TCA) cycle for oxidative energy metabolism. We identified concerted, albeit non-significant, increases in glycolytic intermediates glucose-6-phosphate, fructose-6-phosphate, fructose 1,6-diphosphate (observed as an isobar with glucose 1,6-diphosphate), dihydroxyacetone phosphate and 3-phosphoglycerate (Figure 6a-6f) in WT H_2O_2-treated cells compared to WT UNTR cells at the 12 hour time point. In contrast, glucose and the glycolytic intermediates were increased earlier in the TAp73-/- cells at 3 and 6 hours, but returned to or fell below UNTR levels at 12 hour H_2O_2 treatment. Notwithstanding these changes, pyruvate levels in both WT and TAp73-/- (Figure 6g) cells were lower at the 3 hour treatment compared to their

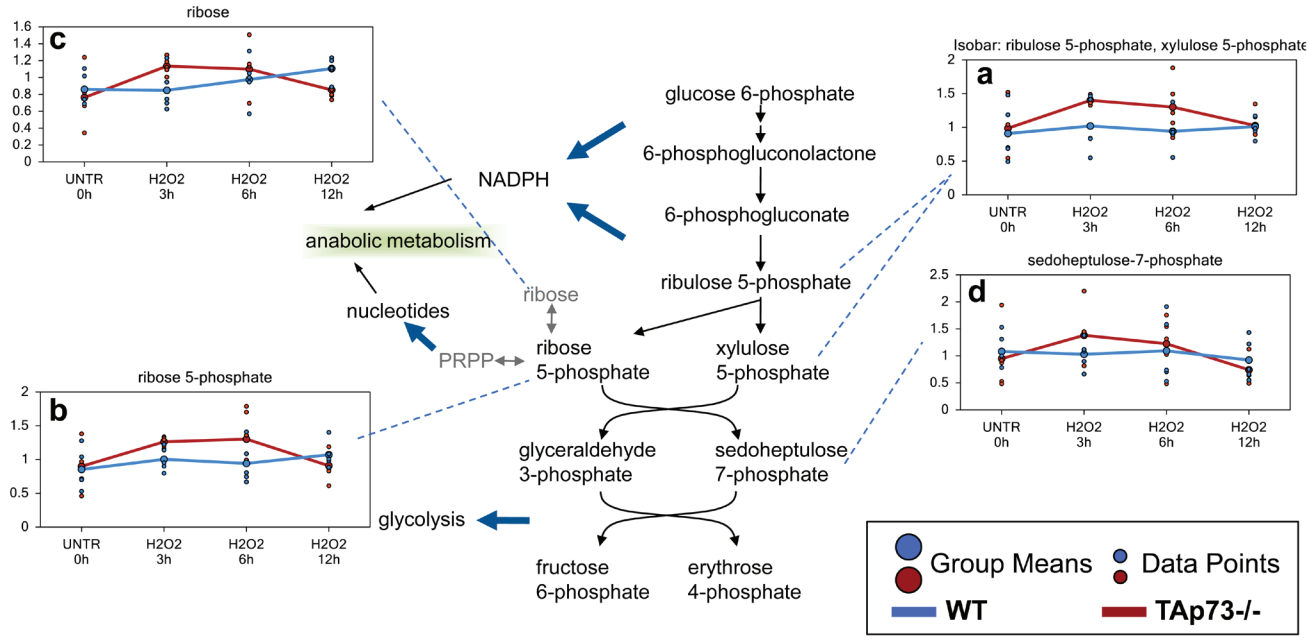

Figure 7: Enhanced PPP in TAp73-/- MEF after H_2O_2 treatment. The PPP is a metabolic pathway that generates NADPH and ribose for nucleotide biosynthesis. **a.-d.** Levels of the indicated metabolites were evaluated as described in material and methods. Anova contrasts *t*-tests were used to identify biochemicals that differed significantly between experimental groups ($n = 5$ for each time point).

respective UNTR cells and subsequently began to trend upwards back to UNTR levels following 6 and 12 hour H_2O_2 treatments in both WT and TAp73-/-. In summary, H_2O_2 treatment results in an early response in TAp73-/- cells with enhanced glycolysis, while this activity is unchanged until 12 hours following H_2O_2 treatment in WT cells.

Increased glycolysis induced by H_2O_2 in TAp73-/- cells sustains enhanced PPP

As previously discussed, one potential consequence of an early increase in glycolysis in the TAp73-/- cells following H_2O_2 treatment could be an increase in the PPP for NADPH production and nucleotide biosynthesis. Through sequential enzymatic steps, glucose 6-phosphate can be metabolized to ribulose 5-phosphate (observed as an isobar with xylulose 5-phosphate). Ribulose 5-phosphate is subsequently processed to ribose 5-phosphate, which can be metabolized to the end product ribose or to PRPP for nucleotide synthesis. All these metabolites were increased in TAp73-/- MEFs at 3 hour and 6 hour treatment (Figure 7a-7c). The PPP-associated biochemical sedoheptulose 7-phosphate was also increased in early H_2O_2-treated TAp73-/- cells, although these changes did not reach significance (Figure 7d). The changes clearly indicate that increased glycolysis in TAp73-/- cells following H_2O_2 treatment results in increased PPP activity that does not occur in WT cells.

Urea cycle associated biochemicals are altered in cells treated with H_2O_2

We identified changes in urea cycle-associated biochemicals compatible with anaplerotic production of TCA cycle intermediates fumarate. The urea cycle functions to convert toxic ammonia to urea during amino acid catabolism [73]. The amino acid aspartate enters the urea cycle by condensing with citrulline to produce argininosuccinate. Argininosuccinate is then cleaved to

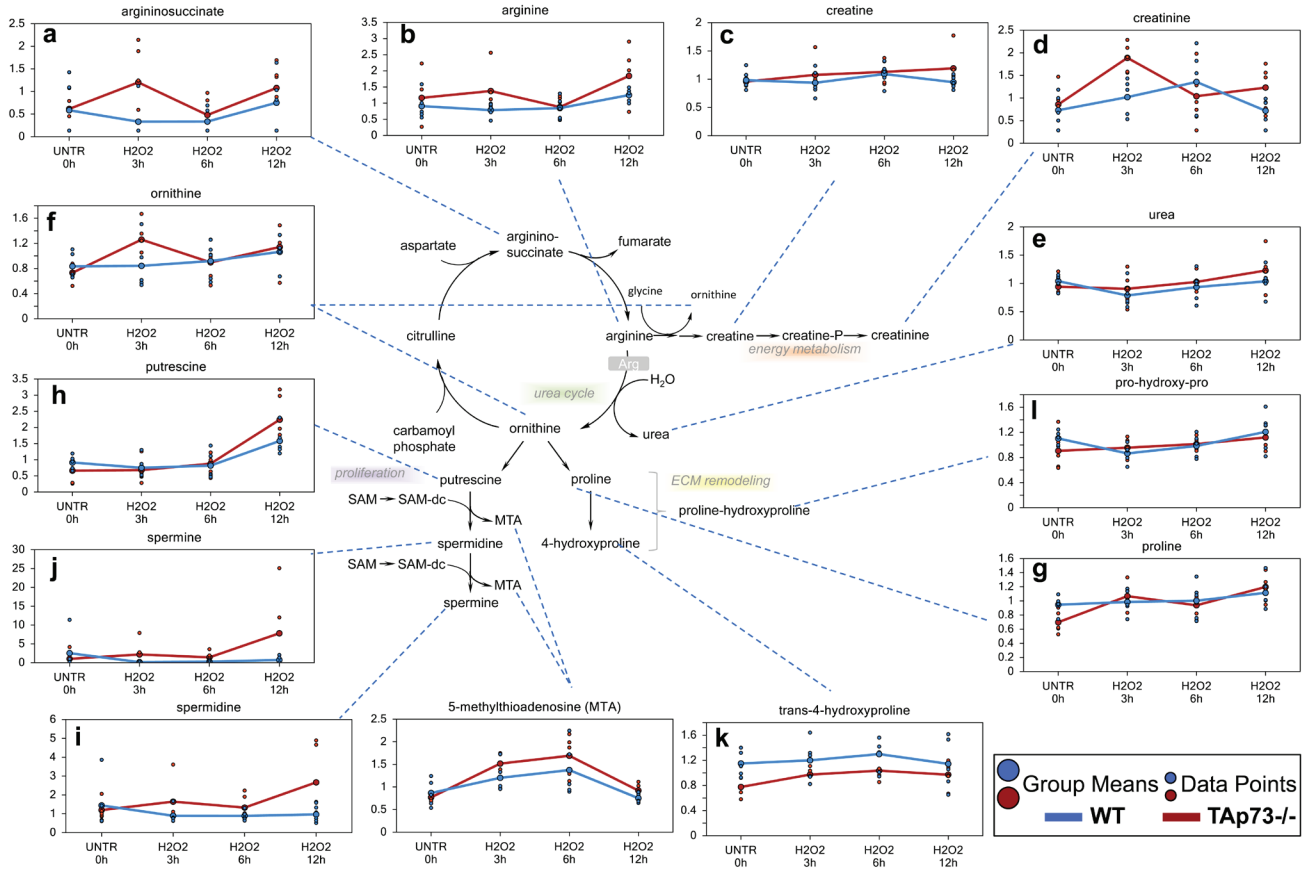

Figure 8: Urea cycle. In the urea cycle, ornithine combines with ammonia to form citrulline. Then, a second amino group is transferred to citrulline from aspartate to form arginine the immediate precursor of urea. Arginine is hydrolyzed to urea and ornithine; thus ornithine is regenerated in each turn of the cycle. **a.-l.** Levels of the indicated metabolites were evaluated as described in material and methods. Anova contrasts *t*-tests were used to identify biochemicals that differed significantly between experimental groups ($n = 5$ for each time point).

form fumarate and arginine. In general, both significant and trending increases in aspartate, argininosuccinate and arginine (Figure 8a and 8b) were observed in WT and TAp73-/- cells upon H_2O_2 treatment, which correlated with significant increased levels of fumarate and malate (all H_2O_2 treatment time points in TAp73-/- cells and only 12 hour H_2O_2 time point in WT cells). Arginine can be further metabolized to creatine, which can be phosphorylated to creatine-phosphate, an energy storage compound. Interestingly, TAp73-/- cells showed trending increase in creatine levels over the H_2O_2 treatment time points that reached significance at 12 hours, an effect not observed in WT cells (Figure 8c). Changes in the levels of the spontaneous creatine-phosphate breakdown product, creatinine, also were observed during various time points of the H_2O_2 treatment time course (Figure 8d). The exact consequence of these changes is not clear, but an intriguing possibility is that creatine phosphate metabolism in both WT and TAp73-/- cells following H_2O_2 treatment acts as a potential survival mechanism.

Since ornithine is also produced during the metabolism of arginine to creatine, this may account for the increase in ornithine at the earlier time points in TAp73-/- cells, even though urea was unchanged. Ornithine can be further metabolized to proline (Figure 8g), whose metabolites contribute to synthesis of collagen or to the polyamines (putrescine, spermidine and spermine) (Figure 8h-8j). While no changes were observed in metabolites associated with extracellular matrix remodeling and collagen breakdown (proline, trans-4-hydroxyproline or pro-hydroxy-proline) in WT cells, all three of these biochemicals were significantly increased following H_2O_2 treatment in TAp73-/- cells depending upon the time point investigated (Figure 8k and 8l). In addition, the proliferation-associated polyamines were increased in the TAp73-/- cells over the H_2O_2 treatment time course and these increases reached significance at 12 hours. Overall, these changes in urea cycle metabolites in WT and TAp73-/- cells suggest that oxidative stress caused an early increase in biochemicals associated with the urea cycle in TAp73-/- cells that were not observed in WT cells. Such changes in TAp73-/- cells supports enhanced energy metabolism and anabolic activity in these cells.

DISCUSSION

p73, together with p63 and p53, belongs to the well-established p53 gene family of transcription factors. Of these, p53 was discovered almost 40 years ago and still remains one of the most intensively studied tumor suppressor genes; as a consequence it shows very diverse, complex and articulated physiological functions, spanning from regulation of apoptosis, autophagy, mitochondria activity and oxygen radical homeostasis metabolism, DNA damage and repair pathways, maintenance of stem cell repertoire, as well as cell lineage determination [74-93]. Despite all these years of exciting investigations, many controversial issues remain to be fully clarified to elucidate the physiological and pathological roles of the p53. This wide complexity raises from different aspects and facts, including regulation by proteasomal degradation [54, 94-98] and micro-RNA [99-107] or the existence of numerous splicing variants [108-116]. Accordingly, significant efforts are under way to harness its potential practical application for human diseases, especially with regard to cancer [117-126]. On the other hand, p63 and p73 were discovered only circa 15 years ago [127-130], but already show a complexity comparable to p53, as well as a fascinating intricate interaction with p53 itself [49, 131-135]. Importantly, a certain degree of specificity characterizes p63 and p73. Indeed, p63 is pivotal for epidermal formation and homeostasis [136-144], as well as playing a role in cancer and metastasis [133, 145-156], and fertility [157-159], whereas p73 has peculiar roles in neuronal development [160-164] and fertility [165-168]. Additionally, TAp73 is a known tumor suppressor gene that regulates cell cycle progression, survival, genomic stability, hypoxia and angiogenesis [42, 45, 166, 169-178]

Numerous accruing findings indicate that TAp73 can also regulate cell metabolism [59, 61, 179-183]. Indeed, we recently showed that ectopic expression of TAp73 increases rate of glycolysis, and stimulated amino acid uptake, nucleotide biosynthesis and biosynthesis of acetyl-CoA [182, 183]. In addition, TAp73 plays an important role in the maintenance of redox homeostasis either by directly regulating the expression of the mitochondrial complex IV subunit cytochrome C oxidase subunit 4 (*COX4I1*) or by enhancing the PPP flux and hence NADPH biosynthesis [59-61]. Prompted by these findings, we sought to broaden our investigation onto whether TAp73-mediated regulation of metabolism contributes to the orchestration of cellular responses to external oxidative stress. Toward this end, we exposed TAp73 knockout and control MEFs to H_2O_2-mediated oxidative damage and assessed metabolic changes over a 12h time course. Overall, this study shows that a number of biochemical pathways are significantly altered following H_2O_2 treatment. While H_2O_2 induces a plausible oxidative stress response in both WT and TAp73-/- cells, the degree of response appears to be greater in TAp73-/- cells, suggesting increased susceptibility to oxidative stress in TAp73-/- cells, as previously demonstrated [59]. Notwithstanding this evidence, TAp73-/- cells probably decrease biochemicals associated with apoptosis (as demonstrated by sphingosine metabolism). Moreover, TAp73-/- cells shows changes in glucose metabolism and amino acid metabolism at earlier time points than WT cells, which may not only allow the cells to handle the oxidative stress through increased NADPH production, but may also result in pro-anabolic activity in the TAp73-/- cells. The increase in ribulose 5-phosphate/xylulose 5-phosphate in the TAp73-/- cells compare to WT cells, may suggest that an increased

pool of NADPH was available to reduce glutathione. In addition, the early increases in ribose 5-phosphate and ribose in TAp73-/- following H_2O_2 treatment may indicate both an increase in the metabolism of ribose 5-phosphate to ribose, but also an increased capacity for nucleotide biosynthesis. This finding deserves further investigation, but, in any case, the observed changes clearly indicate that increased glycolysis in TAp73-/- cells following H_2O_2 treatment boosts PPP activity, an adaptation not occurring in WT cells. Intriguingly, despite the increased PPP flux should lead to enhanced NADPH synthesis and therefore higher GSH levels, we failed to detect any increase in the reduced glutathione pool in TAp73-/- MEFs. The reason for this is unclear and an accurate measurement of NADPH/NADP dynamics in these cells might help explaining this apparent conundrum. Moreover, it is also possible that the severe oxidative environment caused by exposure to H_2O_2 might have blunted any change in GSH or that the reduced pool of cysteine in TAp73-/- cells could have limited glutathione biosynthesis compared to their WT counterparts. Interestingly, with regard to cysteine, we observed a higher increment triggered by H_2O_2 treatment in TAp73-/- cells compare to WT (Figures 1 and 2). It is tempting to argue that such increase could be fueled by the glycolytic intermediate 3-phosphoglycerate (3-PG). Indeed, 3-PG is used to produce serine *via* the reaction catalyzed by 3-PG dehydrogenase, phosphoserine aminotransferase and phosphoserine phosphatase. In turn, serine can produce cysteine *via* homocysteine. Homocysteine can be the precursor of cysteine in a two-step reaction, first the condensation between homocysteine and serine catalyzed by cystathionine-β-synthase, followed by cystathionine γ-lyase-mediated production of cysteine, ammonia, and α-ketobutyrate. This attempt to compensate for the reduce cysteine levels in response to oxidative damage might contribute to the dampened glycolytic flux observed in TAp73-/- cells.

We also observed changes in nucleotide metabolism compatible with increased DNA and RNA breakdown that is potentially a consequence of oxidative damage. Once again, this increase in nucleotide breakdown appears to be more severe in the TAp73-/- cells.

In summary, our results suggest that metabolic changes in TAp73-/- cells following H_2O_2 treatment may result in a pro-growth metabolic profile of cells that have undergone severe oxidative damage, rather than in promotion of a cell death response under these conditions. Hence, loss of TAp73 leads, at least under oxidative stress conditions, to a rewiring of the cellular metabolism that partially resembles metabolic changes observed in cancer cells [2, 184-188], such as increase of PPP flux. The findings presented here reinforce the role of TAp73 as tumor suppressor gene and indicate that the regulation of cellular metabolism by TAp73 contributes to its tumor suppressor function. It is also fascinating to speculate that such metabolic regulations might play a role in the p53-family regulation of stem cells, as described by several research groups [47, 49, 141, 163, 164, 189-195]. Similarly, recent findings, linking epithelial-mesenchymal transition to nucleotide catabolism [196], open additional scenarios whereby regulation of nucleotide metabolism, so prominent for p73, might regulate additional cancer-related phenotypes. These and other hypotheses await investigation and could be easily tested with the use of genetically modified animals or through the flourishing CRISPR/Cas9 technology [197-199].

MATERIALS AND METHODS

Mice

Generation and genotype protocol of TAp73 knock-out mice were described elsewhere [166]. Mice were bred and subjected to listed procedures under the Project License released from the UK Home Office. The experimental design met the standards required by the UK Coordinating Committee on Cancer Research guidelines [200].

Cell culture

Mouse embryonic fibroblasts (MEFs) were prepared as previously described [59]. Briefly MEFs were isolated from E13.5 littermate embryos and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2mM L-glutamine. Cells were treated with 0.25mM H_2O_2 for the indicated time.

All experiments were performed within the first 3 passages from MEFs generation to avoid ensuing senescence in primary mouse fibroblasts.

Metabolic analysis

Sample preparation

Cells were harvested after the treatment and cell pellet stored at -80°C. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions; one for analysis by LC and one for analysis by GC. The organic solvent was removed using a TurboVap® (Zymark). Each sample was then frozen and dried under vacuum.

Liquid chromatography/mass spectrometry (LC/MS)

Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS. The sample extract was split into two aliquots, dried and then reconstituted in acidic or basic LC-compatible solvents. One aliquot was analyzed using acidic positive ion optimized conditions and the other using basic negative ion optimized conditions in two independent injections using separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol both containing 0.1% Formic acid, while the basic extracts, which also used water/methanol, contained 6.5mM Ammonium Bicarbonate. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion. The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap mass analyzer.

Gas chromatography/mass spectrometry (GC/MS)

The samples for GC/MS analysis were re-dried under vacuum desiccation for a minimum of 24 hours prior to being derivatized under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA). The GC column was 5% phenyl and the temperature ramp is from 40° to 300° C in a 16 minute period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization.

Compound identification

Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards. The combination of chromatographic properties and mass spectra gave an indication of a match to the specific compound or an isobaric entity.

Statistical analysis

For these studies we perform various ANOVA procedures (e.g., repeated measures ANOVA). All results with $p < 0.05$ was considered significant.

Abbreviations

TAp73, Transcriptionally active p73; DMEM, Dulbecco minimal essential medium; FBS, fetal bovine serum; GC, Gas chromatography; MS, Mass spectrometry; LC, Liquid chromatography

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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