Sustained protein synthesis and reduced eEF2K levels in TAp73$^{-/-}$ mice brain: a possible compensatory mechanism

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Abstract

The transcription factor p73 is a member of the p53 family, of which the transactivation domain containing isoform (TAp73) plays key roles in brain development and neuronal stem cells. TAp73 also facilitates homoeostasis and prevents oxidative damage *in vivo* by inducing the expression of its target genes. Recently, we found that in addition to its role in regulation of transcription, TAp73 also affects mRNA translation. In cultured cells, acute TAp73 depletion activates eEF2K, which phosphorylates eEF2 reducing mRNA translation elongation. As a consequence, there is a reduction in global proteins synthesis rates and reprogramming of the translatome, leading to a selective decrease in the translation of rRNA processing factors. Given the dramatic effects of Tap73 depletion in vitro it was important to determine whether similar effects were observed in vivo. Here, we report the surprising finding that in brains of TAp73 KO mice there is a reduced level of eEF2K, which allows protein synthesis rates to be maintained suggesting a compensation model. These data provide new insights to the role of TAp73 in translation regulation and the eEF2K pathway in the brain.

Introduction

TAp73 is the longest isoform encoded by the *p73* gene, a member of the p53 family of transcription factors (1-3), which plays important roles in tumour suppression (4-8) and cellular homeostasis by promoting the expression of genes to regulate metabolism (9-17). The p53 family also includes p53 and p63 that share with p73 the capability of promoting cell cycle arrest and apoptosis following DNA damage (18-24). P53 is mainly known for its powerful tumour suppressor capability (25-29) and for the frequent mutations observed in cancer (approximately 50%) (30-34) that can interfere with the physiological function of all the three family members (35, 36). P63 has an exclusive predominant role in developmental of epidermis and stratified epithelia (37-42), fundamentally contributing to(43) this process with a wide range of downstream targets and interactors (44-50). Part of p63 function is also mediated by its contribution on the cellular metabolism (45, 51-55). TAp73 plays a peculiar function in brain development (56-58) and TAp73 knockout mice exhibit significant neurodevelopmental defects, including hippocampal dysgenesis with truncation of the lower blade of the dentate gyrus (4, 59-61). Concurrent loss of TAp73 and the shorter isoform ΔNp73 results in even more severe neurological phenotype; p73 full KO mice, in addition to defective hippocampus, display reduced cortical thickness and hydrocephalus (62). Recent studies have highlighted a critical contribution of p73 on the process of ciliogenesis. TAp73 appears necessary for basal body docking, axonemal extension, and motility during the differentiation of multiciliated cell progenitors, by transcriptionally controlling expression of key regulators of this process, FoxJ1, Rfx2, Rfx3, and miR34bc (63-65). This recent novel insight into the *in vivo* biological function of p73 might unify the complex phenotype displayed by p73 mutant mice (66).

The most well studied functions TAp73 have been related to its transcriptional activates and induction of target genes through binding promoter elements that are highly similar to those of p53 (67). Despite extensive studies of TAp73 functions in maintaining homeostasis and in particular, in protecting against oxidative stress, we still do not understand the full spectrum of TAp73 cellular functions.

Recently, we identified a new and surprising TAp73 function, regulation of mRNA translation (68). Regulation of protein synthesis is a mechanism for cells to readapt to stress conditions and cope with reduced energy/nutrient supply, attempting to optimise the cellular resources (69-71). Our study indicated that TAp73 depletion is accompanied by increased activity of eukaryotic elongation factor 2 kinase (eEF2K), a negative regulator of mRNA translation elongation, and reduced translation elongation. This results in a reprogramming of the translatome and using gene knock-down in cell culture and polysome profiling, we found that TAp73 promotes the translation of ribosomal RNA processing factors under resting conditions and of mitochondrial proteins under oxidative stress. Indeed, TAp73 depleted cells exhibit reduced protein synthesis under resting conditions and reduced ATP levels, decreased mitochondrial activity and increased cell death following oxidative stress. These findings show that TAp73 not only regulates mRNA transcription but that it is an important regulator of mRNA translation as well. Here we addressed whether TAp73 regulates protein synthesis and/or translation elongation *in vivo*.

Results and Discussion

To test whether TAp73 regulates protein synthesis *in vivo* we used TAp73 KO and WT mice and focused on the brain, since there is a clear brain phenotype in TAp73 KO mice (4) namely, hippocampal dysgenesis (62). Using qRT-PCR we confirmed that TAp73 is expressed in several regions of the brain (**Fig. 1A**) and analysing published data confirmed that p73 is also expressed in human brain (**Fig. 1B**).

To measure protein synthesis in mouse brain we used brain slices obtained from TAp73 KO and WT mice and puromycin labelling. The amount of puromycin incorporated proteins in the brain lysates was determined by Western blot and anti-puromycin antibodies (72, 73). Unexpectedly, given our previous in vitro data (where acute depletion of TAp73 resulted in a large decrease in global protein synthesis (68), we found no significant differences in the rate of protein synthesis between TAp73 KO and WT brains (**Fig. 2A**).

We then hypothesised that there could be a compensation mechanism allowing TAp73 KO brains, which have developed in conditions of chronic TAp73 depletion, to maintain translation. Such a compensation mechanism has been described in cell lines that were engineered to exhibit defects in rRNA processing and, despite having a ribosomal defect, maintain protein synthesis rates through a mechanism involving reduction of eEF2K levels (74). To address this possibility, we analysed the levels of eEF2K and, as a read-out for eEF2K activity, those of phosphorylated eukaryotic elongation factor 2 (eEF2), in lysates obtained from brains of TAp73 KO and WT mice. In line with our hypothesis, we found a striking reduction in eEF2K protein levels that correlate with reduced phosphorylation of eEF2 (**Fig. 2B-C**). We did not observe significant differences in the phosphorylation levels of eukaryotic initiation factor 2 alpha (eIF2α) (**Fig. 2B-C**), a marker of the ER stress pathway, suggesting that the proposed compensation occurs specifically through the translation elongation pathway. Mining published data revealed that there is a high correlation between the expression of p73 and eEF2K in human brain (**Fig. 1C**) supporting our premise that TAp73 is involved in regulation of translation elongation pathway in brain as well as in culture.

In previous studies, it has been proposed that to compensate for defective rRNA processing and reduced mRNA translation, cells reduce the expression of eEF2K (74, 75). In agreement with these data, acute TAp73 depletion in cultured cells inhibits the synthesis of nucleolar proteins, rRNA processing and protein synthesis (68). However, not all cases of defects in rRNA processing lead to reduced protein synthesis. Acute depletion of glutamate-amonia ligase (GLUL) in cell culture resulted in aberrant rRNA processing but sustained protein synthesis (62). How cells maintained protein synthesis in this case is not known. The transcriptional factor TAp73 was implicated in the metabolism of the glutamine. In particular, our previous studies report that TAp73 transcriptionally controls expression of the glutaminase-2 (GLS-2) a key metabolic enzyme in the hydrolysis of the glutamine in glutamate. The TAp73/GLS-2 axis appears to have multiple implications for neuronal differentiation as well as for the capability of the cancer cells to survive under nutrient deprivation (76, 77). Whether the connection between the function of TAp73 in the glutamine metabolism is connected with its capability of influencing rRNA processing and mRNA translation has not been not clarified. The capability of the cells to cope with oxidative stress is crucially influenced by mechanisms supporting mitochondrial health (78-82). Dysfunctions in the cellular metabolism result in reduced antioxidant capacity and increased susceptibility to oxygen radicals (83-86). TAp73 critically contributes to mitochondrial biology and cellular metabolism and under TAp73 acute depletion cells suffers of increased susceptibility to oxidative radicals in connection with a reduced protein synthesis capacity. Overall the current data are suggestive of a connection between TAp73 function on cellular metabolism, including regulation of mitochondrial activity, protein synthesis and oxidative defence. Consistently a readaptation in TAp73 KO mice of the cellular metabolic functions might underline the readaptation of the protein synthesis defect.

In conclusion, our data strongly suggests that chronic TAp73 depletion *in vivo* results in reduced eEF2K protein levels that may represent a compensation mechanism for reduced translation capacity (**Fig. 3**), further implicating TAp73 in regulation of protein synthesis through interaction with the eEF2K pathway.

Materials and Methods

Western blot

Brains were homogenised with RIPA buffer containing phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail; Roche), and protease inhibitors (cOmplete Protease Inhibitor Cocktail; Roche). Equal amounts of proteins were run in SDS-PAGE gels, transferred to nitro cellulose membranes (Life Technologies) and probed using the following antibodies: Cell Signalling: eEF2K, Phospho-eEF2, eEF2, Phospho-eIF2α; Santa Cruz: β Tubulin Antibody (H-235); p73: our home made antibody as described (87); Puromycin (Merk, clone 12D10).

Mice

TAp73 WT and KO mice were maintained as described (60). All animal work conformed to UK regulations and institutional guidelines and was performed under the authority of a project license granted by the UK Home Office. Western blot analysis of mice hippocampus was performed as described (60). *In situ* translation measurements were performed as described (73).

RNA isolation and qPCR

Total RNA from cortex, hippocampus and cerebellum was isolated using Trizol according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Qiagen). Total RNA was reverse transcribed using RevertAid H Minus First Strand cDNA synthesys kit (Fermentas). qPCR was performed using qPCR Mastermix with SYBR green (Applied Biosystem). The expression of TAp73 was defined from the threshold cycle (Ct), and relative expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method after normalization with the housekeeping GAPDH and relative to cortex.

Figure legends

Figure 1. TAp73 is expressed in brain. (**A**) mRNA levels of TAp73 in the indicated regions of the brain isolated from wild-type mice. KO is used as negative control (RNA derived from the brain of p73 KO mice). (**B**) The expression of p73 in different regions of human brain was determined using R2.

Figure 2. TAp73 KO mice maintain translation in the brain. (**A**) Brain slices obtained from the indicated genotypes were pulsed with puromycin (45 min; 5 μg/ml). Puromycin incorporation in the brain lysates was determined using Western blot and anti-puromycin antibodies as described [25]. Scanned lanes were quantified using ImageJ. n=3; *p<0.05. (**B**) The levels of the indicated hippocampal proteins and their modifications in lysates obtained from the indicated genotypes were determined using Western blot. (**C**) Protein levels were quantified using ImageJ. n=3; *p<0.05.(**D**) The correlation between the expression of p73 and eEF2K in brain was determined using R2.

Figure 3. Proposed model for TAp73 interaction with the translation pathway. Under normal conditions, TAp73 promotes the translation of nucleolar proteins which process rRNA and thus promoting global protein synthesis. Acute TAp73 depletion results in reduced translation of nucleolar proteins, increased activity of eEF2K and reduced global protein synthesis. TAp73 KO *in vivo* triggers a compensation mechanism in the brain where eEF2K expression is reduced and global protein synthesis is maintained.

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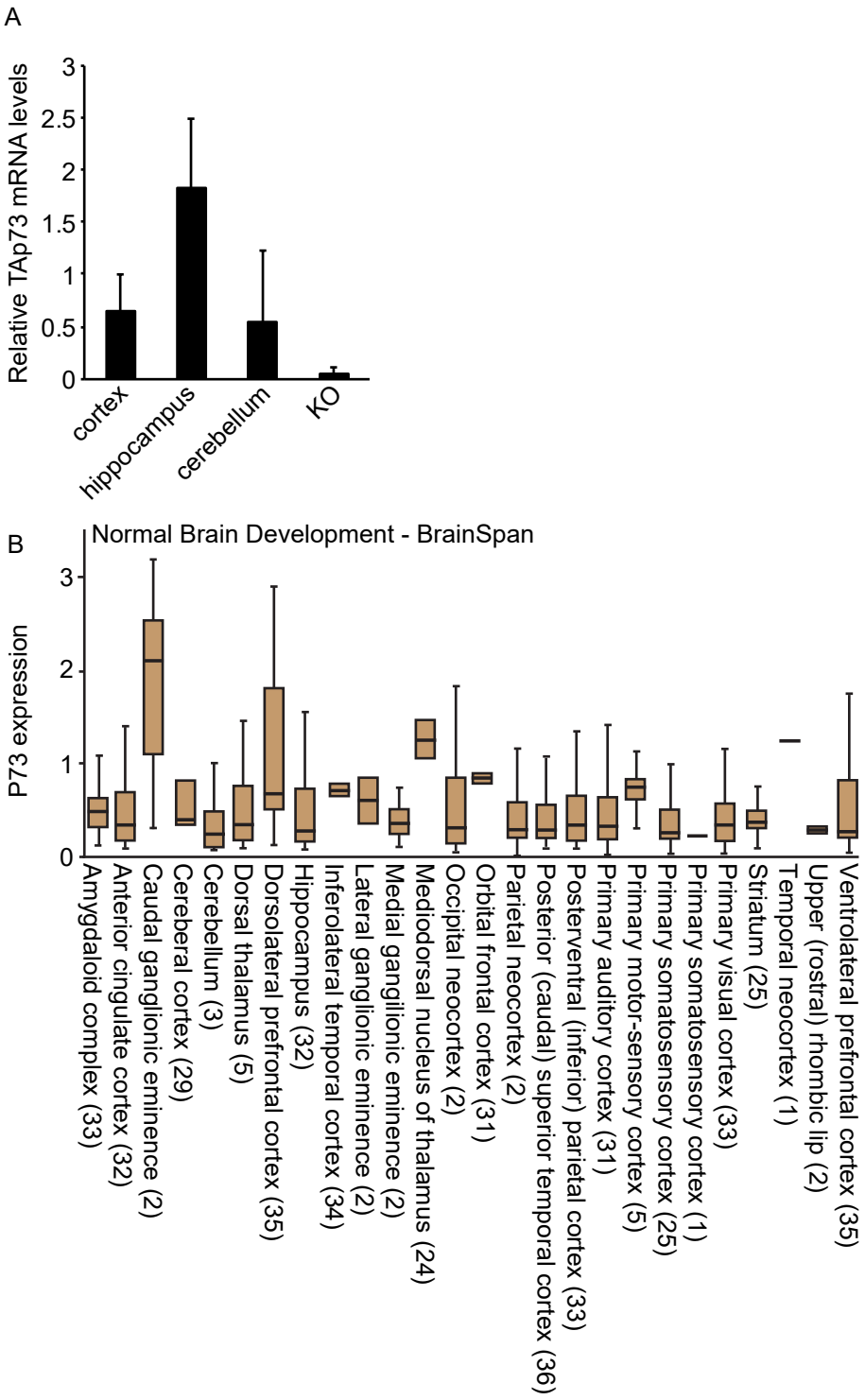
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Cerebellum (3)
Dorsal thalamus (5)
Dorsolateral prefrontal cortex (35)
Hippocampus (32)
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Occipital neocortex (2)
Orbital frontal cortex (31)
Parietal neocortex (2)
Posterior (caudal) superior temporal cortex (36)
Posterventral (inferior) parietal cortex (33)
Primary auditory cortex (31)
Primary motor-sensory cortex (5)
Primary somatosensory cortex (25)
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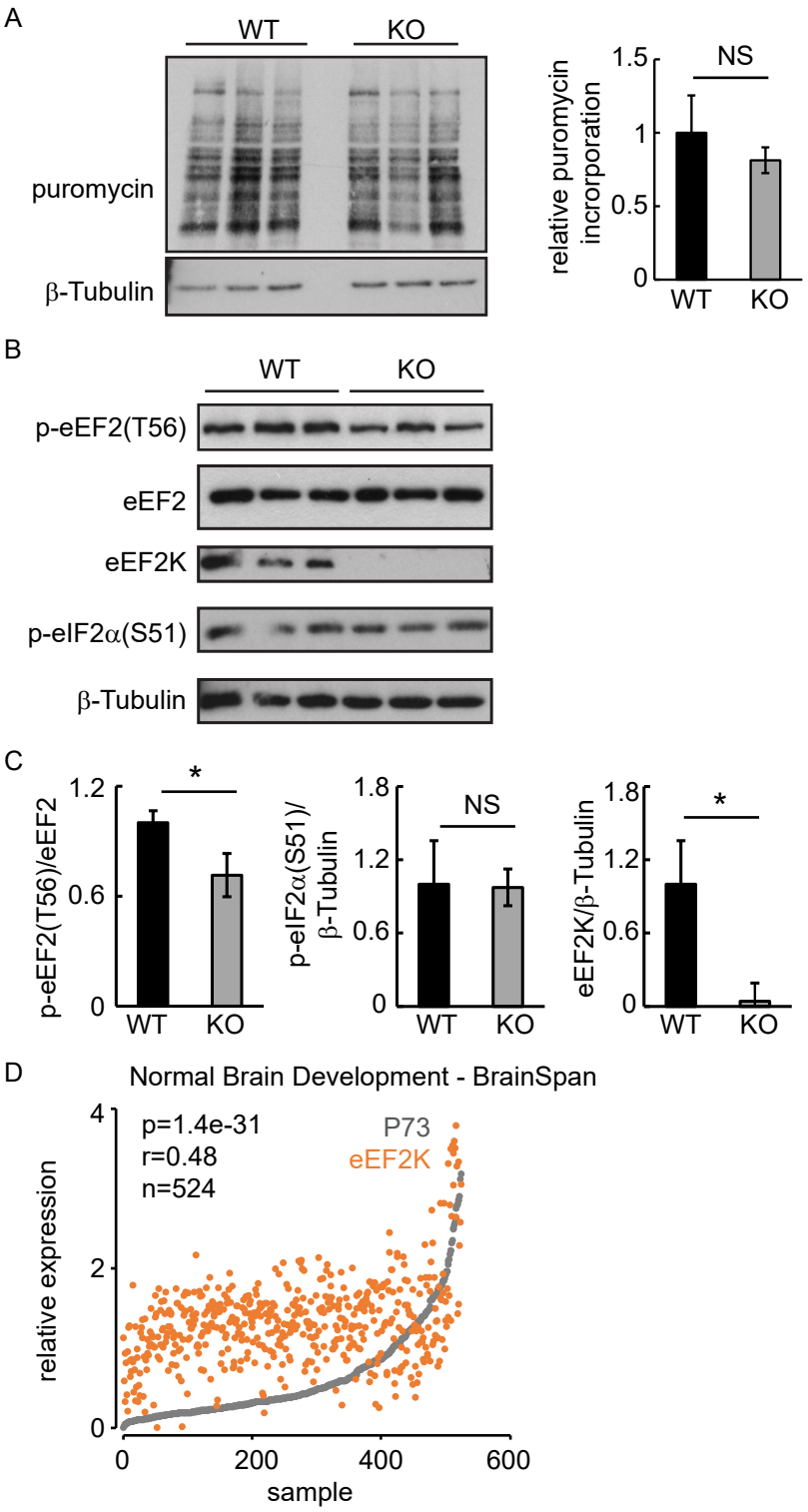
Ventrolateral prefrontal cortex (35)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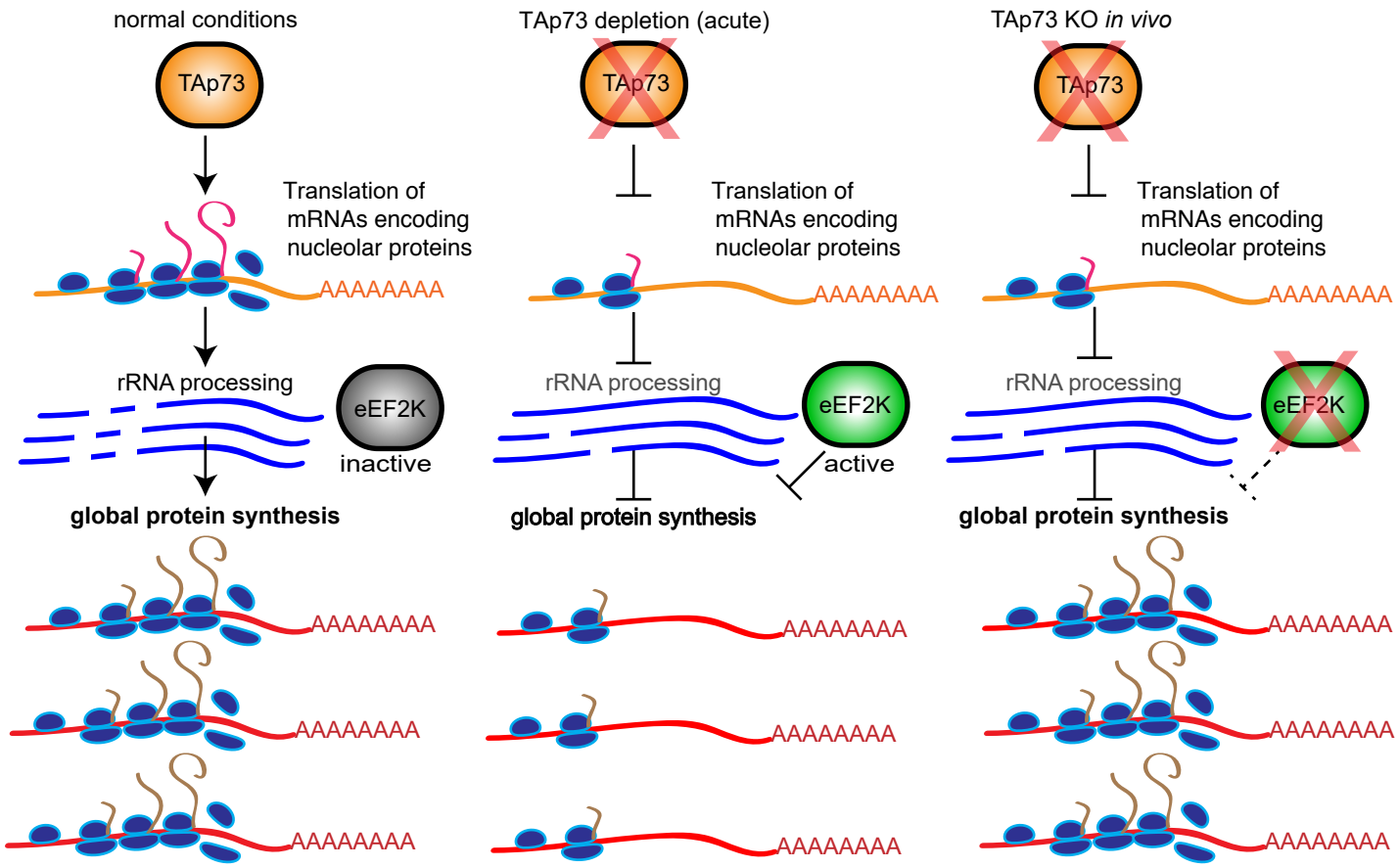
Cell Cycle Fig. 3



Detailed reply to the reviewer

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Sustained protein synthesis and reduced eEF2K levels in TAp73$^{-/-}$ mice brain: a possible compensatory mechanism

By: Rotblat I et al. (corresponding G. Melino)

We thank the Editor and the Reviewer for their positive consideration of our work and for their constructive criticisms. Here, we detail what is included in this revised version.

Referee 1

Referee: To support the mining data indicating a correlation between p73 and eEF2K mRNA levels in human brain, the authors should quantify eEF2k mRNA level in TAP73 KO brain.

Reply: We thank the reviewer for raising the point and for giving us the chance to clarify. We did indeed check expression level of eEF2k in TAp73 KO mice brain. This is shown as protein level in figure 2b. We measured protein level and not RNA as this obviously correlate more accurately with the function of the gene. RNA expression does not necessarily imply expression of the protein.

Referee: In Figure 2B the protein level of eIF2alpha should be included as well as the levels of other proteins of the translational machinery (4EBP1, eIF4E for instances)

Reply: We thank the reviewer for raising this point. We indeed tried to measure eIF2alpha protein (and related translational machinery factors) but in facts the commonly used antibodies do not work on the mouse protein. We are afraid due to technical limitations we are not able to address this point.

Referee 2

Referee: Fig 1 The authors should include also the expression of eEF2K at mRNA level in different brain tissues similarly to TAp73 mRNA, comparing WT and KO mice. This will give further information about the molecular mechanism of eEF2K down-regulation in TAp73KO brain. Also, it would be interesting to show by immunohistochemistry the reduced expression of eEF2K in TAp73 brain tissues.

Reply: We thank the reviewer for raising the point. As discussed in the first point of the reviewer 1 we did measure eEF2k in TAp73 KO mice brain, but we performed WB analysis as we believe this would better reflect the expression of the protein. The suggested IHC would indeed be just a confirmatory analysis of the WB.

***Referee**: We thank The authors should expand the data presented in Fig 2. By looking at the western blot it seems that eEF2K expression is completely abrogated. Analyzing other eEF2K substrates (ie. AMPK and alpha4; Lazarus et al. Cell Signal 2017) the authors should be able to demonstrate if eEF2K activity is totally compromised.*

Reply: We thank the reviewer for raising this point, but as discussed in the point 2 of the reviewer 1, antibodies of these singallings have poor affinity for mouse proteins, hecen due to technical limitations we are not able to address this point.

Referee: It has been reported that eEF2K knock-out has a role in maintaining genomic integrity by arresting cell cycle in response to stress (Liao Y, et al. JBC 2016). The authors should discuss this aspect in the discussion also considering how they could be linked to the TAp73KO brain phenotype. If possible, they should measure these specific features also in the TAp73KO brains.

Reply: We thank the reviewer for this suggestion. This part is indeed now been added to the discussion.