Response to Reviewer 3 Comments

Point 1: Although the findings are interesting and of importance, the authors fail to mention that two EGR1 mRNA variants were detected in astrocytomas, one that contains N-methyl-D-aspartate-receptor (NMDA-R)-responsive element (https://www.ncbi.nlm.nih.gov/pubmed/18489490). These findings suggest that EGR-1 could be regulated in a sequence-dependent manner in human astrocytomas.

Response 1: We agree with the referee on the importance of the polyadenylation mechanism in the regulation of EGR1 function. In this regard, we included the following sentences in the text of the introduction section: “In addition, both in mouse and human, EGR1 is regulated at a post-transcriptional level by alternative polyadenylation, a mechanism that generates two EGR1 mRNA variants differing for a potential NMDA-R-responsive CPE sequence [44,45]. No additional molecular mechanism has been characterized for EGR1 functional control beyond regulation of transcription, alternative polyadenylation, mRNA and protein stability, and post-translation modifications.”

Point 2: It would be interesting in the context of previous reports to validate the presence/localisation etc. of the newly identified splicing events in neuronal cells. It seems likely that the mentioned changes and occurrence of various transcripts on mRNA level described in the Mittelbroon et al, 2009 paper or the isoforms reported here divergent on the protein level may be even associated with many diseases and possibly triggered by the onset of these diseases etc.

Response 2: We agree with the referee regarding the importance of a better characterization of a plethora of features of the EGR1 Δ141-278 in neuronal cells or another cell type. However, this falls outside of the main aim of our paper that is focused on a preliminary characterization of the difference between the canonical and the alternative isoform on the transcriptional regulation, and on the identification of an exon that encodes for a specific activation domain, in accordance with the analysis of Gashler and colleagues (1995).

Point 3: Unfortunately, the authors choose HEK293T cells as a model system to determine the localisation patterns etc. of different splicing variants. While this is an easy system to test with it only provides limited information regarding complex EGR1 interactions that are associated with many different cells/tissues etc. Therefore, in my view, the approach seems to be a bit oversimplified to provide reliable evidence regarding differences between 2 splicing variants isolated from a significantly different cell type than used in described experiments. I believe the paper would benefit greatly from considering testing these splicing variants in wider spectrum of cells including neuronal cultures.

Response 3: The main aim of this paper was a preliminary molecular characterization of the EGR1 Δ141-278 isoform. We identify this isoform in the differentiated SH-SY5Y where the transcript level of the isoform was relatively low. In addition, these cells are generally considered hard to transfect, therefore, we believe the HEK293T cell line was a suitable model to collect preliminary information on the alternative isoform considering that HEK293T are extensively used in transfection-based experiments. In fact, our approach was based on the use of an expression vector bearing the canonical and alternative EGR1 coding region and iper-expression experiments in order to understand whether the deletion characterizing the EGR1 Δ141-278 isoform affected the ability of the protein to translocate into the nucleus. Since the nuclear translocation ability of this isoform was not affected, we investigated the ability of this isoform to regulate transcription in comparison with the full-length isoform. This was of interest considering the analysis made by Gashler et al. (1995) who characterized
a protein domain involved in the transcriptional activation that we demonstrated to be encoded by the transcript sequence removed in EGR1 Δ141-278 mRNA. From a molecular and evolutionary perspective, this is an interesting result considering the idea that this exitron may encode for a specific protein domain. Although we performed investigations on the splicing event in other cell lines, we noted a relatively low expression level of the EGR1 Δ141-278 isoform, as demonstrated for the SH-SY5Y. These results are in accordance with the analysis of Marquez et al. (2015), who observed that this splicing event has low efficiency. This is the main reason why we made the following statement in the discussion section “In this regard, it is likely that other unexplored conditions are needed to increase the expression of this splicing isoform”. We agree with the referee on the importance of searching and analysing the splicing isoform expression in other cell lines and/or conditions, but this falls outside of the main aim of the paper.

**Point 4:** lines 103-110; Figure 2. WB Figure 2 shows higher level of expression of delta141-278 EGR1 compared to EGR1 (especially in nuclear fraction) which has been normalised against housekeeping genes. It seems to be contradictory to further statement in lines 159-161: “We explored the occurrence of the splicing event that generates the EGR1 alternative isoform during SH-SY5Y differentiation; the alternative EGR1 isoform shared a similar expression pattern but its levels were significantly lower when compared to the canonical isoform (data not shown).”

**Response 4:** We agree with referee about our confusing statement “We explored the occurrence of the splicing event that generates the EGR1 alternative isoform during SH-SY5Y differentiation; the alternative EGR1 isoform shared a similar expression pattern but its levels were significantly lower when compared to the canonical isoform (data not shown).” We modified the sentences as follows’ “We explored the occurrence of the splicing event that generates the EGR1 alternative isoform during SH-SY5Y differentiation; the alternative EGR1 transcript isoform shared a similar expression pattern but its levels were significantly lower when compared to the canonical mRNA isoform (data not shown).”, specifying that both isoforms were analyzed at transcript level.

**Point 5:** Lines 160-162: it would be interesting to actually see these results: how big is the difference? Over which time the differentiation was monitored? Were mRNA levels used to determine these differences or WB? WB would be helpful as the mRNA stability etc. may not reflect the protein level

**Response 5:** The comparison between the two isoforms during RA-induced differentiation was made at early (0.5h, 1h) and late (6h, 24h, 48h) time points after stimulation. We preferred not to report this expression analysis in detail since, as stated in the discussion section, the mRNA level of the splicing isoform was significantly lower when compared to the canonical mRNA isoform. The conclusion is that data on the expression of the alternative isoform under the conditions we tested, are only interesting considering their agreement with the observations of Marquez regarding the generally low level and low efficiency of the exitrons. We decided to not show these data in the article since they do not add further information that fits within the scope of the paper.