Reviewer's report

Title: ID3 Contributes to Cerebrospinal Fluid Seeding and Poor Prognosis in Medulloblastoma

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Reviewer: Janusz Rak

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The manuscript by Phi et al describes a link between the overexpression of the ID3 transcription factor and metastatic seeding, aggressiveness and poor prognosis in medulloblastoma (MB). These investigators have previously identified changes in the expression of another member of the inhibitor of differentiation gene family, ID1, along with other molecular alterations in a group of patients with embryonal brain tumours comprising MB and PNET (Phi et al m2010). In the present paper this analysis is extended to all four ID genes (1-4) and leads to the finding that ID3 is markedly upregulated in those MB patients who exhibit the evidence of seeding. Through experimentation with MB cell lines, such as DAOY and especially D283 cells, Phi and colleagues interrogated the functional impact of ID3 on cellular aggressiveness in vitro and in vivo. Silencing of ID3 in these cells using siRNA and shRNA approaches revealed diminished survival, proliferation and migration of MB cells (but not senescence). Intracranial modelling of tumour growth and dissemination in vivo (in mice) resulted in similar primary growth to controls, but reduced spinal seeding of MB cells with reduced levels of ID3 expression. Several migration/invasion genes were investigated and found to be altered in ID3 silenced cells, including upregulation of TIMP3, COL12A1, ITGB4, ADAMTS8, and downregulation of TNC, CTGF and ICAM1. Moreover, the paper provides statistical evidence that both seeding and more than 5 fold upregulation of ID3, both co-segregate with poor prognosis in the cohort of 30 patients, including in a multivariate analysis.

Overall, this is an intriguing study on an interesting group of genes previously linked to several progenitor cell populations, but not in MB, and thereby of interest in embryonal-type tumours. For the most part, the findings are well documented (with some qualification below). The paper is clearly written and informative. However, the authors should consider several revisions to make their work more convincing.

Major compulsory revisions

1. The paper is designed and executed in a manner that is almost completely detached from the emerging consensus as to the molecular diagnosis, prognostication and classification of MB (as described in several recent publications including by Pfister et al 2010; Parsons et al 2011; Gibson et al 2010; Kool et al 2012 etc). This classification distinguishes 4 molecular subtypes of MB, which could effectively be regarded as separate diseases associated with
unique pathogenetic mechanisms, prognosis and survival data. Moreover, metastasis in MB appears to be dictated by a unique pattern of mutations, which are present in a minority of cells and barely detectable in the primary tumour (Wu et al 2012). Surprisingly these important developments are hardly mentioned throughout the present paper and only touched on in passing in the last few sentences of the discussion (e.g. the paper by Northcott et al 2012). This is a significant weakness and should be remedied in the following manner:

(i) The emerging molecular classification of MB should be brought into view in the Introduction and Discussion of the paper;

(ii) The authors should examine the published datasets related to molecular subtypes of MB and determine whether the expression of ID genes in general, and ID3 in particular (or its targets mentioned in the study), are a part of this classification. This is important since DAOY cells are derived from the SSH type MB and this could be one determinant of their ID expression. Something could also be said about the possible reasons for ID3 upregulation in view of recent sequencing data in MB which might contain information of potential relevance for ID3 regulation (Jones et al 2012; Pugh 2012).

(iii) It would also be of interest if it could be determined whether the ID gene status is linked to seeding in all, or only in some MB subtypes. None of this is investigated, even in silico, or discussed.

2. The nature of the link between the ID3 status and seeding should also be clarified in some detail. For example, the reader would like to know why did the authors choose the 5 fold increase in ID3, as a cut off in the analysis of patient survival? There is also certain imbalance in this part of the study with regards to patient numbers, since twice as many patients have been included in the high-ID3 group compared to the low ID3 group. It is also notable in Table 1 that seeding appears to be a stronger predictor of poor prognosis then ID3, which raises the question as to other regulators of both seeding and ID3 expression. The prognostic significance of ID3 is not entirely clear, especially in comparison to other factors, such as the molecular subtype of the disease or the completeness of surgical resection, among other variables. While the study may not be powered to address all these questions and is, understandably, focused on the role of ID3, the reported observations are presently discussed somewhat out of context.

3. There are some experimental questions that need additional clarification. It is interesting that downregulation of ID3 leads to reduced survival, migration and invasion in vivo in one MB cell line, but it is less clear what does this mean from the biological standpoint. Notwithstanding the predominant use of one cellular model of MB (D283 cells), it is not entirely clear what survival mechanisms are controlled by ID3. Is the reduced survival the reason for reduced migration? Do tumours emerging from ID3 down regulated MB cells, or low ID3 lesions in MB patients also contain higher fraction of apoptotic cells or is this number reduced due to in vivo selection (if so is ID3 involved)? Some of the protein expression patterns shown in Fig 4C seem paradoxical. For example, cells expressing ID3 in
some of the tissue sections seem to be different than those in which changes occur in levels of putative ID3 targets, such as TNC, which is mainly present in what looks like tumour stroma (while ID3 expression is more ubiquitous). Since ID proteins are transcriptional repressors one wonders what are links between ID3 and genes downregulated in conjunction with ID3 knock-down. In general, the genes altered through ID3 manipulations are not validated as ID3 targets and one would like to know whether at least some of them are direct targets of this transcriptional regulator. At least some of these questions should be addressed experimentally and by more specific comments in the text.

4. The authors should provide information (data) as to the specificity of the siRNA and shRNA reagents they used to downregulate ID3. In particular, it is important to document how many independent sequences were used to bring down the levels of ID3, and whether reversal experiments have been carried out to prove that these are true on target effects.

Minor essential revisions

5. Data presented in Figure 1 A-D suggests that while ID3 expression exhibits the most pronounced alterations in the subset of MB patients with evidence of seeding, versus the non-seeding counterparts, in some patients seeding also correlates with changes in ID1 and ID2. It is also unclear whether any of these patients had tumours with multiple ID gene alterations. Moreover, it is striking that the majority of patients in the seeding (+) category exhibited no change in ID3 expression levels. What is the interpretation of this finding, and has this been confirmed at the protein level? Have the putative ID3 target genes (e.g. TNC) been examined in these patients and what are the results? It is possible that other regulatory networks could have substituted for ID3 in generation of the metastatic phenotype in MB. Is there any common molecular denominator of seeding?

6. In Fig. 3F the survival curve for mice with ID3 downregulated tumours does not reach 30 days with the majority of these mice surviving beyond the experimental endpoint. What is the fate of these mice if left in the experiment longer? Do they eventually develop metastatic disease? The authors should provide the complete set of data in this regard, such that readers could understand whether the effect of ID3 is durable, or transient, and whether ID3 it is obligatory for metastasis in this system. Do cells isolated from spinal infiltrates of MB cell lines exhibit higher levels of ID3 then their counterparts in the primary tumour?

Discretionary revisions

7. It would be helpful if the figures were labelled in a more self-explanatory manner, since the numbers included in many of the line graphs are heavily derivatised through calculations, and often the actual values obtained in these assays are not particularly clear (have to be extracted from legends and the text).

8. There is considerable variability in the array expression data. What is the reason for this and could it be avoided?
9. The authors might consider some edits in the paper. For example, “corresponding authors” should be converted to singular (page 20).

10. The references in the paper are somewhat outdated and the authors should include more recent review and original papers, as indicated earlier.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Acceptable

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**

I declare that I have no competing interests