OTX2 is a novel regulator of alternative splicing in Group 3 medulloblastoma

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INTRODUCTION

Brain tumors are among the most prevalent forms of childhood cancer and account for nearly 20% of all new pediatric cancer diagnoses. Medulloblastoma (MB) is the most common malignant primary pediatric brain tumor and is currently divided into 4 molecular subgroups that exhibit different genomic alterations, gene expression profiles and response to treatment. Group 3 MB tumors account for 25% of MB cases, are highly metastatic, and exhibit the worst prognosis. Overexpression/amplification of OTX2 is a molecular hallmark for Group 3 MB. To date, studies have primarily focused on it's role in controlling tumor growth via cell cycle regulation. Our recent work uncovered a novel role for OTX2 in regulating protein synthesis through mTORC1 signalling, thus significantly expanding the regulatory scope of OTX2. Here we sought to further explore the non-canonical functions of OTX2 in Group 3 MB tumor progression and report a novel role for OTX2 in regulating alternative splicing (AS). These findings demonstrate the multifaceted role for OTX2 in orchestrating multiple steps in protein-coding gene expression from transcription to mRNA translation in the progression of Group 3 MB tumorigenesis.

RESULTS

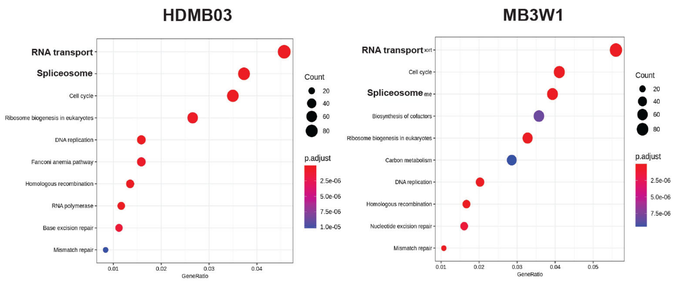

Figure 1: OTX2 silencing is associated with significant splicing alterations in Group 3 MB tumorspheres. KEGG pathway analyses on OTX2-downregulated genes. Genes associated with the spliceosome and RNA transport (highlighted) are significantly downregulated following OTX2 silencing in HDMB03 (left) and MB3W1 (right) tumorspheres suggesting that OTX2 may play a role in regulating mRNA processing.

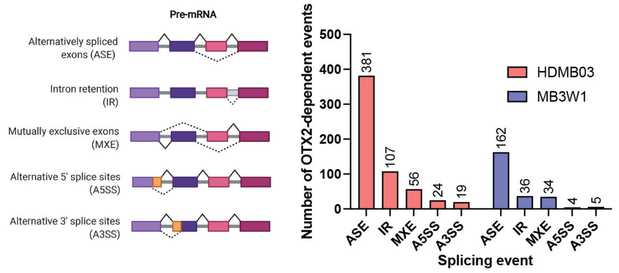

Figure 2: Alternatively spliced exons are the most frequent splicing event in OTX2 silenced tumorspheres. Evaluation of AS events revealed that OTX2 silencing is significantly associated with a large number of splicing alterations in MB tumorspheres. Alternatively spliced exons (ASE) were the most frequent event identified in OTX2 silenced tumorspheres. ASE: alternatively spliced exons, IR: intron retention, MXE: mutually exclusive exons, A5SS, alternative 5'splice sites, A3SS, alternative 3'splice sites.

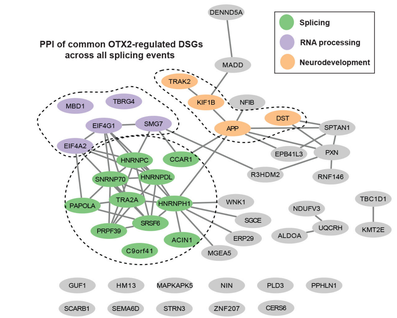

Figure 3: 48 differentially spliced genes (DSG) are common to both HDMB03 and MB3W1 OTX2 silenced tumorspheres. Interaction map showing protein-protein interactions (PPI) (edges) between protein products (nodes) of 48 common OTX2-regulated significantly DSG across all splicing events (ASE, IR, MXE, A5SS and A3SS). Shared DSG are typically associated with RNA processing (purple nodes), splicing (green nodes) and neurodevelopment (orange nodes).

TABLE 1: 11 DSG shared between tumorspheres and Group 3 patient tumors

PPHLN1	***MADD***	*MBD1*	*EPB41L3*
HM13	*KIF1B*	*KMT2E*	*MGEA5*
PLD3	*RNF146*	*TRA2A*	

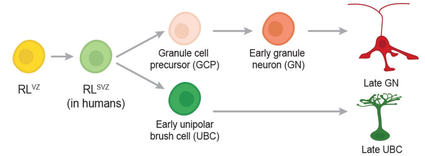

Figure 4: Schematic of the lineages descending from the rhombic lip during human cerebellar development. The human rhombic lip (RL) splits into a stem cell enriched ventricular zone (RL^{VZ}) and a progenitor enriched subventricular zone (RL^{SVZ}) which gives rise to both unipolar brush cells (UBC) and granule cell precursors (GCP) residing in the external granular layer.

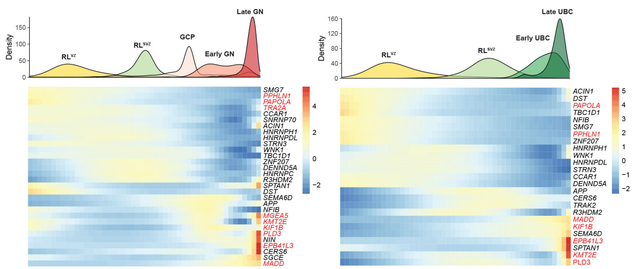

Figure 5: OTX2-regulated DSG map to human RL lineage genes of the developing cerebellum. Pseudotime mapping of the 48 OTX2-regulated DSG from tumorspheres reveals 30 and 25 genes overlap with the GN and UBC lineage genes respectively including a majority of the 11 OTX2-spliced genes shared between tumorspheres and Group 3 patient tumors (red). PPHLN1 represents an "early" RL linage gene while MADD represents a "late" RL lineage and both were chosen for further functional validation in Group 3 MB models.

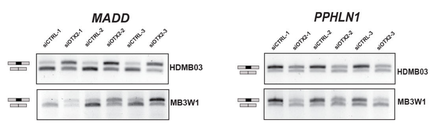

Figure 6: Exon retention and exclusion are favoured for MADD and PPHLN1 respectively in OTX2 silenced tumorspheres. RT-PCR shows exon retention (exon 26) is favoured for the "late" RL lineage gene MADD in HDMB03 and MB3W1 tumorspheres, while exon skipping (exon 6) is favoured for the "early" RL lineage gene PPHLN1 following OTX2 silencing.

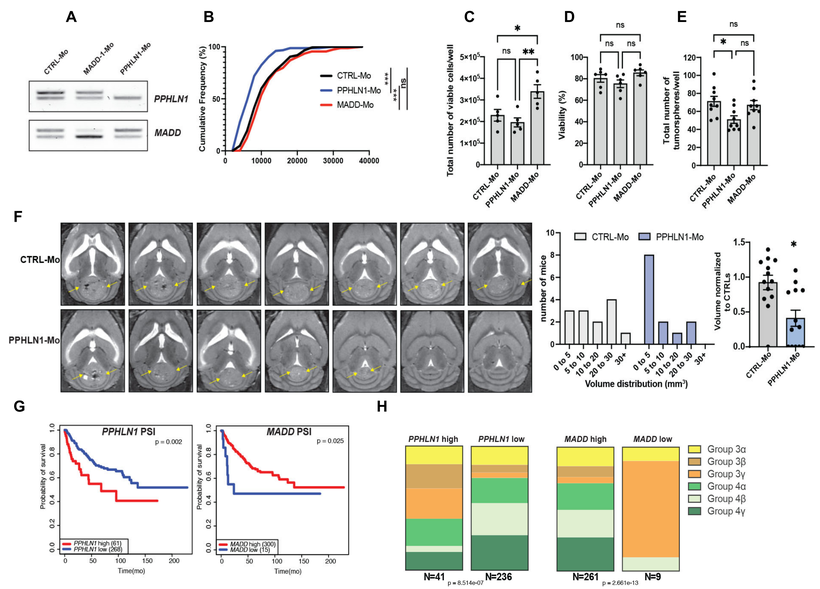

Figure 7: Alternative splicing of PPHLN1 and MADD significantly impacts tumorigenic properties of Group 3 MB. Splice blocking morpholinos drive MADD and PPHLN1 exon skipping (A). PPHLN1-Mo inhibits HDMB03 tumor properties and induces significant decreases in both tumorsphere size and number (B-E). MADD-Mo increases cell number while having no significant effect on other tumor properties. Treatment of HDMB03 cells with PPHLN1-Mo prior to intracerebellar injection is sufficient to significantly reduce tumor initiation capacity and growth (F). Analysis of PPHLN1 and MADD PSI in Group 3/Group 4 MB patient tumors reveals a small subset of PPHLN1 high (exon 6 retained)/MADD low (exon 26 skipped) PSI significantly associated with poor survival, and associated with the OTX2 "wild-type" splicing state. This PPHLN1 high/MADD low PSI signature is associated with the aggressive Group 3γ subtype (H), which arises from the primitive RL^{VZ}, that is comprised of stem cells.

CONCLUSIONS

We have uncovered an entirely new gene regulatory layer in Group 3 MB in which spliced variants of OTX2-regulated RL lineage genes significantly alter cell fate decisions in these highly aggressive tumors. The connections between OTX2-regulated exons and early cerebellar developmental states are consistent with our r ecently published findings demonstrating a critical role for OTX2 in retaining RL identity and suppressing a GN lineage program, favouring a developmentally stalled primitive cell state. Given the rapid tumor progression of our representative Group 3 MB models, the decreased tumor growth following one-time treatment with the PPHLN1-Mo is encouraging. Indeed, as the the PPHLN1 high/MADD low PSI signature is associated with the aggressive Group 3γ subtype, these results represent a promising new avenue for future studies that optimize direct PPHLN1-Mo delivery to the tumor site driving differentiation of neoplastic cells and further sustaining decreases in MB tumor growth of this highly aggressive subtype.

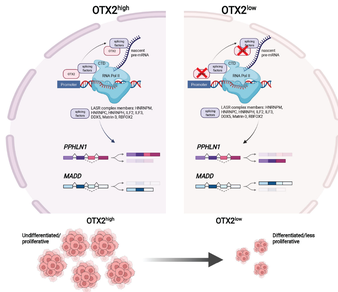

Figure 8: Proposed model of OTX2-mediated regulation of Group 3 MB AS. OTX2 affects this process indirectly through traditional DNA binding to alter expression of splicing factors and directly through protein interactions with the LASR complex (data not shown). This leads to AS of genes associated with cerebellar development such as PPHLN1 and MADD in favor of isoforms that perpetuate the undifferentiated stem cell state.

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