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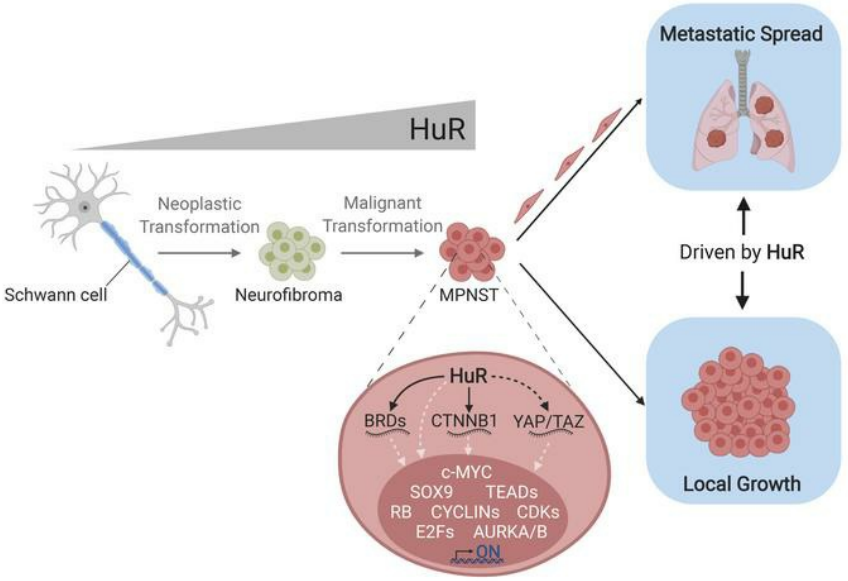
Graphical abstract



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HuR/ELAVL1 drives malignant peripheral nerve sheath tumour growth and metastasis

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ABSTRACT

Cancer cells can develop a strong addiction to discrete molecular regulators, which control the aberrant gene expression programs that drive and maintain the cancer phenotype. Here, we report the identification of the RNA-binding protein HuR/ELAVL1 as a central oncogenic driver for malignant peripheral nerve sheath tumours (MPNSTs), which are highly aggressive sarcomas that originate from cells of the Schwann cell lineage. HuR was found to be highly elevated and bound to a multitude of cancer-associated transcripts in human MPNST samples. Accordingly, genetic and pharmacological inhibition of HuR had potent cytostatic and cytotoxic effects on tumour growth, and strongly supressed metastatic capacity *in vivo*. Importantly, we linked the profound tumorigenic function of HuR to its ability to simultaneously regulate multiple essential oncogenic pathways in MPNST cells, including the Wnt/β-Catenin, YAP/TAZ, Rb-E2F and BET proteins, which converge on key transcriptional networks. Given the exceptional dependency of MPNST cells on HuR for survival, proliferation, and dissemination, we propose that HuR represents a promising therapeutic target for MPNST treatment.

INTRODUCTION

Malignant peripheral nerve sheath tumours (MPNSTs) are highly aggressive sarcomas with a strong metastatic potential, usually to the lung (1). MPNST patients are largely refractory to current treatments that include radical surgical resection and/or radiation and chemotherapy, and have very poor 5-year survival rates ranging between 15% and 50% (1). Half of MPNST cases arise in the context of Neurofibromatosis Type 1 syndrome (NF1), and these tumours are a leading cause of death in NF1 patients. In NF1, loss of the *NF1* tumour suppressor gene that encodes the Ras GTPase-activating protein neurofibromin leads to the development of benign neurofibromas that are located on the skin (cutaneous neurofibromas) or can be deep-seated in large peripheral nerves (plexiform neurofibromas). Plexiform neurofibromas can transform into MPNSTs, which can also occur spontaneously (sporadic MPNSTs) or after radiotherapy. There is general acceptance that cells of the Schwann cell lineage are the crucial neoplastic cells in MPNST (1,2).

A number of mutations that drive MPNST pathogenesis have been identified, with a surprising degree of overlap in NF1-associated and sporadic forms. These include molecular variants of the *NF1* tumour suppressor gene that are present in all NF1 patients, and in a majority of sporadic and radiation-induced MPNSTs (2,3). Other ancillary, yet essential, cancer-driving genetic aberrations include loss of genes *CDKN2A*, *TP53*, *RB*, *PTEN*, or the genes encoding the PRC2 components *SUZ12* or *EED*, and amplification of *PDGFRA*, *EGFR* or *MET* (4,5). In addition, recent studies have shown that activation of multiple signalling pathways that include the PI3K/AKT/mTOR, RAS/RAF-MEK-ERK, WNT/β-Catenin and HIPPO-YAP/TAZ pathways, and other less ubiquitous molecular alterations involving aurora kinases and transcription factors, such as SOX9, also contribute to MPNST pathogenesis (1,3,6).

Gene dysregulation is a hallmark of cancer cells. Genetic alterations in cancer cells invariably lead to a global remodelling of their transcriptome allowing them to acquire advanced functional capabilities for survival, proliferation and dissemination. MPNSTs have a unique transcriptomic signature that is clearly distinct from normal or even neurofibroma-derived primary Schwann cells or tumours (7,8), and that is strongly associated with key Schwann cell developmental programs, including control of survival and proliferation. Targeting dysregulated gene expression programs in cancers has emerged as a promising therapeutic strategy, and there is an intense focus on identifying the key molecular regulators that govern these programs (9,10). In particular, RNA-binding proteins (RBPs) are increasingly recognised as attractive targets because of their ability to regulate the type and abundance of hundreds of transcripts by modulating every aspect of their post-transcriptional life – splicing, transport, localization, translation, stabilization and decay. Furthermore, each RBP can bind to multiple overlapping groups of functionally related RNAs, forming “RNA regulons” that control many biological functions (11).

We have previously shown that the ubiquitously expressed RBP HuR/ELAVL1 was highly expressed in immature Schwann cells, a stage of development characterized by a peak in Schwann cell proliferation and apoptosis. We found that HuR was bound to and regulated several key mRNAs, co-ordinately regulating them at the post-transcriptional level (12). Subsequently, as immature Schwann cells differentiated, we found that they lost expression of HuR, and the production of HuR targets encoding proliferation and apoptosis proteins were downregulated. Notably, many of the HuR targets in immature Schwann cells become re-expressed in MPNSTs, and the encoded proteins play key roles in tumour growth, as shown for SOX9 (8) and BRD4 (13). HuR is frequently upregulated in different cancer types (14), leading us to hypothesize that HuR could become re-expressed in MPNSTs, where it would have a key role in driving the dysregulated transcriptomic programs. Here, we present evidence that HuR is potently tumorigenic in MPNSTs and that suppressing HuR expression reduces tumour growth and metastasis. We propose that the malignant influence of HuR is linked to enhancing multiple key oncogenic programs operating in MPNST cells.

RESULTS

HuR is upregulated in human MPNSTs

To explore the potential role of HuR in Schwann cell cancers, we searched a publicly available expression dataset (GEO: GSE41747)(7) and found that *HuR/ELAVL1* mRNA levels were significantly upregulated in MPNSTs, both in patients and in mouse samples (Figure 1A, B). Next, we analysed HuR protein abundance by immunohistochemistry in a human tissue microarray panel comprising normal nerves (n=7), benign neurofibromas (n=76) and MPNSTs (n=109)(15), and similarly, found a strong upregulation of HuR protein expression in MPNSTs (Figure 1C). Finally, we validated these results by examining HuR protein and mRNA expression in an independent cohort of frozen human normal nerves (n=5), neurofibromas (n=12) and MPNSTs (n=15), obtained from the Stanmore Musculoskeletal Biobank (UK). We confirmed that total HuR protein levels were significantly elevated in the MPNST samples (Figure 1D, E). We also examined cytoplasmic HuR levels, since HuR export from the nucleus to the cytoplasm is linked to its function as post-transcriptional regulator of target mRNAs (16). As shown (Figure 1D, F), cytoplasmic HuR levels were higher in MPNST samples. *HuR* mRNA levels were also higher in these samples (Figure 1G).

We did not find significant differences in the abundance of HuR mRNA or protein when comparing dermal neurofibromas and plexiform neurofibromas from our frozen cancer panel (Supplemental Figure 1A), nor were there differences in *HuR* mRNA levels in publicly available datasets (Supplemental Figure 1B, C). Notably, we also did not find significant differences in the levels of HuR mRNA or protein when comparing NF1-derived and sporadic MPNSTs (Supplemental Figure 1D, E).

The high abundance and cytoplasmic localisation of HuR point to a potentially important role in both sporadic and NF1-derived MPNSTs.

RIP-Chip identifies HuR mRNA targets associated with key cancer traits

To examine the biological significance of the high HuR levels in MPNSTs, we analysed HuR-associated mRNAs on a genome-wide scale by ribonucleoprotein (RNP) immunoprecipitation (RIP) under conditions that preserve endogenous RNA-protein interactions, followed by microarray detection of bound mRNAs (RIP-Chip) (17,18). Notably RIP-chip preferentially enriches for the stably–bound subsets of putative mRNA targets rather than transient targets that might be detected by RNP crosslinking. This favours the identification of targets forming part of functional complexes that lead to mRNA target stabilization and increased translation (19).

RIP analysis was performed using an anti-HuR antibody or control anti-IgG antibody and cytoplasmic extracts from human neurofibroma (n=8) and MPNST (n=12) tumour samples from the aforementioned frozen cancer panel, and the isolated RNA was identified by microarray analysis (Supplemental Figure 2A, B). Background mRNAs identified in side-by-side control IP reactions using mouse anti-IgG antibody were subtracted to identify *bona fide* HuR-interacting mRNAs. We found that HuR was

specifically bound to 71 and 276 mRNAs in neurofibroma and MPNST samples respectively (Figure 2A, Supplemental Table 1 and 2). The large majority of neurofibroma targets (60 out of 71; 85%) were also associated with HuR in the MPNST samples, whereas 216 HuR-bound transcripts were exclusively expressed in MPNST samples (Figure 2A), in line with the high HuR expression in MPNST samples, and supporting a role for HuR in malignant Schwann cell tumours.

To identify molecular pathways associated with HuR-bound transcripts in MPNST, we performed gene set enrichment analysis (GSEA) using Molecular Signature Database (MSigDB) hallmarks (20). GSEA revealed significant enrichment of signatures associated with oncogenic traits that include cell cycle progression, EMT and angiogenesis (21), as well as signatures for key oncogenic transcription factors MYC and E2F (22,23) (Figure 2B). In addition, key signalling pathways in MPNSTs that include the Wnt/β-Catenin, PI3K/AKT/mTOR and RAS pathways (1,2), were also enriched (Figure 2B–D; Supplemental Table 3).

Together, these data point to a potentially important biological function for HuR in MPNSTs, possibly regulating key signalling pathways that control oncogenic traits in these malignant tumours.

HuR promotes MPNST cell growth in vitro and in vivo

To characterize the functional importance of HuR, we first evaluated its abundance in established MPNST cell lines. Using a publicly available expression dataset (GEO: GSE14038) (8), we found that *HuR* mRNA levels were significantly upregulated in MPNST cell lines compared to neurofibroma-derived Schwann cells (Supplemental Figure 3A). Furthermore, higher *HuR* mRNA and protein levels were observed in 4 commonly used MPNST cell lines (ST88-14, 90-8, S462, STS-26T), compared to freshly isolated Schwann cells derived from human nerves (Supplemental Figure 3B, C).

We then silenced HuR in these 4 MPNST cell lines by lentiviral delivery of two distinct HuR-specific short hairpin (sh) RNAs and examined cellular growth. Three of the cell lines (ST-8814, 90–8 and S462) were derived from NF1 patients with confirmed loss of heterozygosity at the *NF1* locus for all 3 cell lines; the 90–8 cell line additionally showed a known microdeletion of *NF1*, whereas the sporadic MPNST line STS–26T had no mutations detected in the 60 exons of the *NF1* gene. These MPNST cell lines share a common gene expression profile distinct from that of normal Schwann cells, although they differ in proliferation rates and in expression of cell cycle proteins (24). Both constitutively expressed shRNAs reduced HuR levels efficiently, and suppressed cell growth, as shown by measuring ATP levels and by direct cell counts in all 4 cell lines (Supplemental Figure 3D–F). Furthermore, HuR silencing significantly reduced the clonogenic and anchorage-independent growth capacity of all 4 cell lines (Supplemental Figure 4A–C).

Since the in vitro consequences of HuR perturbation were shared by all MPNST cell lines, we selected as representative STS-26T for in vivo validation. STS-26T cells in

which HuR levels were normal or reduced by silencing were implanted subcutaneously in immunodeficient mice. Subsequent analysis revealed that whereas control cells efficiently formed tumours (5/5), tumour formation was completely abolished in HuR-silenced cells (0/5) (Figure 3A, B).

Next, we examined the effects of HuR depletion on the growth of already established tumours. To this end, we used a doxycycline-inducible lentiviral shRNA system targeting HuR. STS-26T cells expressing pTRIPZ-sh Control (sh *i*Ctrl) or pTRIPZ-sh HuR (sh *i*HuR) were injected subcutaneously in the left and right flanks of immunodeficient mice respectively, and tumours were allowed to form for about 20 days until they were palpable (~100 mm^3 average). Mice were randomly assigned to standard chow diet or doxycycline diet for a period of 10 days, whereupon mice were sacrificed and tumours extracted (Figure 3C). Remarkably, induction of *HuR* shRNA by doxycycline treatment severely blunted tumour growth, and even led to visible tumour regression (Figure 3D). Tumour regression was observed in all mice following HuR depletion, and on average tumours shrank by 40% (Figure 3E), and weighed significantly less than control tumours (Figure 3F). HuR-depleted cells formed smaller tumours, and the efficiency of HuR silencing was confirmed in those tumours by Western blotting analysis (Supplemental Figure 5A, B). Notably, tumours arising from sh *i*HuR cells without doxycycline treatment were indistinguishable in all parameters from tumours arising from sh *i*Ctrl cells with or without doxycycline treatment, supporting the efficiency and specificity of the inducible system used.

We also documented a marked reduction in the proliferation marker Ki67 and a rise in an apoptotic marker, active Caspase-3, in HuR-depleted tumours (Supplemental Figure 5C). We validated these results in the panel of cultured MPNST cell lines, where, similarly, genetic depletion of HuR in the 4 cell lines potently reduced cell proliferation as measured by (1) cell cycle analysis by flow cytometry, which showed a general increase in the percentage of cells in the G1 phase and a decrease in cells in the S and G2/M phases, consistent with a G1 cell cycle arrest, and (2) BrdU incorporation (Supplemental Figure 6A, B). We also found a marked increase in senescence after HuR silencing in all 4 cell lines (Supplemental Figure 6C). Detection of Annexin V by flow cytometry indicated a trend towards increased apoptosis in all 4 cell lines after HuR knockdown when cells were cultured under basal, growth-promoting conditions (10% serum). Exposure to cellular stress, such as culture of cells under growth-limiting conditions (2% serum) (13), exaggerated this effect and led to a marked increase in apoptotic and necrotic cell death after HuR depletion (Supplemental Figure 6D).

Together, these data indicate that HuR play an important functional role in MPNST cell biology by controlling key features such as survival, proliferation and replicative immortality.

HuR promotes MPNST metastasis in vivo

MPNSTs have a high metastatic potential, and up to 50% of patients develop metastatic disease, usually to the lung, further worsening 5-year survival rates of patients (1,25). Yet, metastasis is one of the least understood aspects of MPNSTs. Given the profound antitumoral effects of HuR in MPNST cells, and the identification of EMT signature as one of the top enriched categories of HuR targets from GSEA analysis (Figure 2B, C), we decided to examine whether HuR could be important for MPNST metastasis, using a surrogate model of lung metastasis. Briefly, this model measures the ability of cells injected in the lateral tail vein of immunodeficient mice to survive in circulation, arrest at a distant organ, extravasate, adapt to growth in the foreign microenvironments of a distant tissue, and grow into metastatic lesions, recapitulating many of the essential late steps in metastasis (26).

We silenced HuR constitutively in STS-26T cells, an MPNST cell line which exhibits metastatic tropism to the lung (27), injected them in the tail vein, and examined metastatic colonization of the lung 4 weeks later by histology (Figure 4A). Remarkably, very few metastatic lesions were observed in HuR-depleted cells in contrast to control cells, which formed numerous and large metastatic foci (Figure 4B, C). Next, we used our inducible lentiviral silencing system to examine the effect on HuR silencing on the colonization step of the metastatic process, i.e. the survival of the cells in these foreign microenvironments during the metastatic process, and the reactivation of their proliferation programs to form overt metastatic lesions. We injected STS-26T cells expressing pTRIPZ-sh Control (sh *i*Ctr) or pTRIPZ-sh HuR (sh *i*HuR) in the tail vein, and allowed them to begin to form metastases for 2 weeks. Mice were randomly assigned to standard chow or doxycycline-containing diet for 4 weeks, whereupon mice were sacrificed and lungs extracted for histological analysis (Figure 4D). Induction of HuR shRNA by doxycycline treatment blocked the conversion of these micrometastases into the macroscopic neoplastic growth seen in the control cells with or without doxycycline treatment and in sh *i*HuR cells without doxycycline treatment (Figure 4D–F).

Together, these data support a key role for HuR in MPNST metastasis, potentially controlling key aspects of the metastatic process, from the survival of the cells in the bloodstream to their colonization and proliferation within distant organs.

HuR overexpression in Schwann cells is not sufficient to trigger oncogenic transformation or dissemination

Since HuR is expressed at notably higher levels in MPNST compared to Schwann cells (Figure 1 and Supplemental Figure 3A–C), we next examined whether elevating HuR levels in human Schwann cells was sufficient elicit to cell transformation and dissemination.

We ectopically expressed HA-tagged HuR using a doxycycline-inducible lentiviral system (28) in immortalized normal human Schwann cells (iHSCλ2) (Supplemental Figure 7A), and found that this led to a small but significant increase in cell growth, as

shown by measuring ATP levels and by direct cell counts, and increased the clonogenic and anchorage-independent growth capacity (Supplemental Figure 7B). However, no major effect was observed in tumour formation in vivo using mouse xenograft models (Supplemental Figure 7C, D). We also did not observe any major effect on tumour formation after ectopic expression of HuR in immortalized plexiform neurofibroma-derived human Schwann cells (ipNF SC). These data suggest that while HuR overexpression promotes proliferation in Schwann cells in culture, it is not sufficient to induce Schwann cell-derived tumors in mice.

Similarly, we examined metastatic properties using tail vein injections of control or HuR-overexpressing normal and plexiform neurofibroma-derived human Schwann cell lines. We did not find any metastatic nodules in the controls of either cell line. Unexpectedly, we found the presence of small micrometastases in HuR-overexpressing normal Schwann cell lines (1 out of 4) and plexiform human Schwann cell lines (3 out of 4) (Supplemental Figure 7E), although these were far fewer and smaller than after injection of STS-26T MPNST cell line (Figure 4).

Taken together, HuR overexpression in normal or plexiform neurofibroma Schwann cells was not sufficient to trigger oncogenic transformation but modestly increased their metastatic capacity.

Pharmacological inhibition of HuR reduces MPNST growth and metastasis

Our results demonstrate that elevated HuR levels in MPNST cells are required for cell growth and metastasis. We next ascertained whether the inhibition of HuR could be exploited as a therapeutic strategy using small molecule HuR inhibitors.

Pharmacological inhibition of HuR with MS-444, which inhibits HuR homodimerization to prevent the binding of 3'UTR AU-rich elements (29), pyrvinium pamoate (PP), an FDA-approved anthelminthic drug that blocks HuR nucleocytoplasmic translocation (30), and tanshinone mimic 6N (TM-6N), which inhibits HuR-RNA complex formation (31), each strongly reduced MPNST cell growth in culture, in ST88-14 and STS-26T cells (Figure 5A, B), similar to what we found through the genetic inhibition of HuR (Supplemental Figures 3 and 4).

Importantly, intra-peritoneal injection with MS-444 (25 mg/kg body weight; treatment every 48h for 10 days) of nude mice bearing already established tumours (formed 20 days after xenograft transplant of STS-26T cells in flanks) severely blunted tumour growth, and even led to tumour regression in some cases. In addition, MS-444-treated tumours weighed significantly less than control tumours (Figure 5C–E). Remarkably, MS-444 treatment of nude mice (intra-peritoneal injection every 48h for 2 weeks; 25 mg/kg body weight) with established micrometastases that had formed for 2 weeks after intravenous injection of STS-26T cells, also reduced the conversion of these micrometastases into the macroscopic neoplastic growth, and smaller metastatic areas were measured in MS-444-treated mice than in vehicle-treated mice (Figure 5F–H).

Our results show that pharmacological inhibition of HuR reduces MPNST growth and metastasis, thus highlighting the relevance of HuR as a potential therapeutical target for MPNST.

HuR regulates key oncogenic transcriptional programs

Next, we sought to define the mechanisms by which HuR exerts such profound effects on MPNST cells. We silenced HuR expression using lentiviral vectors expressing constitutive shRNA directed at HuR in the ST88-14 cell line, and performed RNA-sequencing profiling. We identified distinct transcriptomic profiles between the control and HuR-depleted cells (Figure 6A), and differential expression analysis revealed that a notable proportion of the transcriptome in MPNST cells changed significantly. There were similar numbers of significantly upregulated and downregulated transcripts exhibiting >2-fold changes (1563 and 1627 respectively) (Figure 6B, Supplemental Table 4).

GSEA analysis revealed an inhibition of several tumorigenic pathways in MPNST cells by HuR silencing, including the YAP/TAZ, Wnt/β-Catenin, PI3K/AKT/mTOR and RAS pathways, in addition to signatures for the key oncogenic transcription factors MYC and E2F (Figure 6C, Supplemental Table 5 and 6). There was also significant overlap between the signature sets we identified by this RNA-Seq analysis and the RIP-chip analysis for putative HuR mRNA targets (Figure 2B). These results strongly suggested that HuR could be affecting MPNST cells by directly regulating these pathways, several of which have already been established to play key roles in MPNST tumorigenesis (6,7,13,32-34). We thus set out to examine the importance of HuR for these pathways.

We first focused on the YAP/TAZ pathway. In an elegant study, it was recently shown that human MPNSTs exhibit elevated HIPPO-TAZ/YAP expression, and hyperactivation of YAP/TAZ in Schwann cells activated an oncogenic program with development of MPNSTs (6). GSEA analysis of RNA-Seq data showed that HuR silencing in MPNST cells led to a strong suppression of a YAP-conserved signature (35) as well as a functionally validated YAP-activated signature (36) (Figure 7A,B). Notably, GSEA also showed a strong negative correlation of genes regulated by YAP/TAZ hyperactivation in Schwann cells (6) and genes regulated by HuR silencing in ST88-14 MPNST cells (Supplemental Figure 8A), suggesting that HuR could be responsible for regulating expression of YAP/TAZ pathway components.

To test this hypothesis, we examined whether HuR associated with mRNAs encoding key protein components of the YAP/TAZ pathway by performing RIP followed by reverse transcription and quantitative PCR analysis (RIP-qPCR) in MPNSTs. To avoid possible confounding effects of cell type heterogeneity in tumour samples, we used MPNST cell lines instead. We found that there was a strong enrichment of *YAP1* mRNA, encoding YAP1, and *TEAD1* and *TEAD2* mRNAs, encoding TEAD1 and TEAD2, two transcriptional partners of YAP1, in HuR RIP samples (Figure 7C). Notably, HuR depletion led to a striking reduction in the levels of these proteins in both the NF1-derived ST88-

14 cell line (Figure 7D), and the sporadic cell line STS-26T (Supplemental Figure 8B). Suppressing the YAP/TAZ pathway by HuR silencing reduced the mRNA levels of downstream YAP/TAZ target genes in both cell lines (Figure 7E; Supplemental Figure 8C). Interestingly, although we did not find that *TAZ* or *TEAD4* mRNAs were direct targets of HuR, HuR depletion led to a strong reduction in their expression levels, likely due to an indirect effect on their transcription, mRNA stability or translation.

Our results indicate that HuR can regulate expression of key YAP/TAZ pathway components in MPNST cells, which could lead to the aberrant elevated HIPPO-TAZ/YAP expression seen in mouse and human tumour tissue samples and the hyperactivation of this essential oncogenic pathway for MPNST pathogenesis.

HuR regulates key cell cycle genes in MPNST via a RB-E2F axis

The RAS-MEK-ERK and PI3K-AKT-mTOR are two other major signalling pathways that are upregulated in MPNSTs and have important roles in MPNST pathogenesis (1,2). GSEA analysis revealed that many components of these pathways were significantly downregulated by HuR silencing, including activation of receptor tyrosine kinases such as PDGF (Supplemental Figure 9A, B). Along the same lines, GSEA also showed a strong positive correlation among genes regulated by the MEK inhibitor PD0325901 in the MPNST cell line 90-8TL (13) and genes regulated by HuR silencing in ST88-14 MPNST cells (Supplemental Figure 9C). In addition, several signatures of downstream effectors of these pathways, including the RB and E2F transcription factors, were inhibited by HuR silencing (Figure 8A, B). These effectors are frequently dysregulated in cancer and play an important role in cell cycle regulation (22).

To examine the specific function of HuR in controlling these tumorigenic pathways, we examined whether HuR was bound to and regulated expression of different components of these pathways (Figure 8C). By RIP-qPCR analysis, we found that HuR was bound to *CCND1, CCND2, CDK2, CDK6, p27, E2F1, E2F2* and *E2F3* mRNAs (Figure 8D). In both the ST88-14 and STS-26T cell lines, we found that silencing HuR strongly reduced the levels of several proteins encoded by these mRNAs, accordingly to the role of HuR in promoting mRNA stability and translation (Figure 8E, Supplemental Figure 9D). However, in specific cases, HuR can also inhibit protein translation (16), as described for *P27* (37). Here, similarly, we found that HuR depletion led to an increase in P27 protein level. P21 protein levels were also induced, not as a direct target of HuR, but possibly as one of the key proteins increasing in abundance during cell senescence.

Collectively, our results suggest that HuR may play a direct role in driving cell cycle progression via E2F transcription factors in MPNST cells by acting at several levels: (1) by enhancing the expression of specific cyclins and CDKs to promote phosphorylation of RB1 to release E2F transcription factors (TFs), (2) by repressing production of P27, an inhibitor of CDKs, and (3) by directly regulating the levels of E2F TFs.

HuR activates a key Wnt/β-Catenin oncogenic program in MPNST

Using the *Sleeping Beauty* forward genetic screen, signalling through the canonical Wnt/β-Catenin pathway was recently identified as a key genetic driver of MPNST tumorigenesis (33,34). Canonical Wnt pathway is activated after Wnt stimulation leading to an inhibition in degradation of β-Catenin, which then enters the nucleus and binds to a member of the lymphoid-enhancing factor 1/T-cell factor (LEF1/TCF) and other transcriptional cofactors including BCL9 and Pygopus to regulate the expression of target genes involved in diverse cellular processes (38). We found that HuR silencing led to a downregulation of *MYC*, *SOX9* and *AURKA/B* mRNAs (Supplemental Table 4), which have previously been identified as downstream targets of the Wnt/β-Catenin pathway in different cell systems (39-41). Here, we confirmed that these genes were also downstream target genes in MPNST cells by silencing β-Catenin using specific shRNAs in the ST88-14 and STS-26T cell lines (Supplemental Figure 10A, B).

In line with this set of targets, GSEA analysis showed that several signature data sets associated with this pathway were significantly downregulated by HuR silencing (Figure 9A, B; Supplemental Table 5 and 6). RIP analysis showed that HuR associated with *CTNNB1* mRNA, which encodes β-Catenin, as well as *BCL9* mRNA, which we also identified as a target of HuR from our RIP-Chip analyses (Supplemental Table 2) (Figure 9C). A functional role of β-Catenin in controlling MPNST cell growth and viability had already been shown using shRNA-mediated silencing of CTNNB1 (34).

HuR silencing led to a notable reduction in the levels of β-CATENIN and BCL9 in ST88-14 (Figure 9D) and STS-26T cells (Supplemental Figure 10C), showing that HuR controlled protein production from these mRNAs. HuR depletion also significantly lowered the levels of the key downstream targets, including c-MYC, SOX9, AURKA and AURKB, further supporting a role for HuR in regulating Wnt/β-Catenin-mediated gene transcription. To confirm these results, we examined the capacity of ectopically expressing a degradation-resistant form of β-Catenin to abrogate the effects of HuR silencing in MPNST cells. We found that constitutive expression of the β-Catenin mutant partially blocked the effects of HuR silencing on downregulation of the downstream Wnt pathway targets in ST88-14 MPNST cells (Figure 9E). In line with this, although silencing HuR still reduced ATP levels and cell number in cells overexpressing the β-Catenin mutant, there was a significant recovery in these parameters when compared to *HuR*-silenced cells expressing the empty vector plasmid (Figure 9F). Similar results were obtained in the sporadic MPNST STS-26T cell line (Supplemental Figure 10D, E).

Conversely, attempts to rescue by overexpressing BCL9 using a lentiviral vector in HuR-silenced cells failed to affect MPNST growth (data not shown), suggesting that regulation of BCL9 is not central to the biological consequences elicited by HuR in MPNST cells. Our results above also show that a number of key oncogenic TFs, including c-MYC, SOX9 or E2Fs, were downregulated by HuR silencing. Similarly, here, we found no major effect on MPNST growth when the expression of these TFs was restored using

lentiviral overexpression constructs after HuR silencing (Supplemental Figures 11 and 12).

These data suggest that there is hierarchical prioritization for HuR function in MPNSTs: HuR primarily regulates the expression of key master regulators (e.g., β-Catenin, YAP/TAZ), which in turn control the production of cell cycle proteins and TFs.

HuR regulates a core transcriptional circuitry in MPNST cells by controlling expression of BRD proteins

Deregulation of the epigenome has also emerged as an important component of the pathogenesis of MPNST. In particular, two recent studies pointed to a role of bromodomain (BRD) and extra-terminal domain (BET) proteins in the activation and maintenance of an aberrant transcriptional program (13,32). BET proteins (BRD2, BRD3 and BRD4) bind to hyperacetylated lysines in promoter/enhancer regions, and subsequently recruit cofactors to control transcription of oncogenic drivers, such as MYC and E2F (42).

In MPNST cells, a small-molecule inhibitor of the BET proteins, JQ1, blocks proliferation and can induce apoptosis in MPNST cells in vitro and in vivo, highlighting the importance of these proteins in MPNST pathogenesis(13,32). Using GSEA analysis, we found a striking enrichment of genes activated by JQ1 treatment in MPNST cells (13), and genes upregulated by HuR silencing in our dataset (Figure 10A). Furthermore, in line with our RIP-Chip analyses, where we found enrichment of *BRD2* mRNA in HuR-bound fractions (Supplemental Table 2), we found that mRNAs encoding all 3 BET family members, BRD2, BRD3 and BRD4, were highly enriched in HuR fractions in RIP-qPCR analyses of MPNST cell lines (Figure 10B). HuR silencing strongly reduced all three BET proteins in both the ST88-14 and STS-26T cell lines, suggesting a role for HuR in promoting the stability and/or translation of the corresponding mRNAs (Figure 10C and Supplemental Figure 13A). A functional role of BRD4 in controlling cell growth and viability in MPNST cells had already been shown using shRNA-mediated silencing (13,32). Here, we found that silencing BRD2 using two distinct *BRD2*-specific shRNAs also led to a reduction in cell growth and viability (Supplemental Figure 13B–D), underscoring the importance of this transcriptional regulator in MPNST.

Key recent studies have shown that JQ1 treatment in melanoma and multiple myeloma cells depletes BRD2 and BRD4 from promoter and enhancer regions of the genome and this was associated with the disruption of transcriptional programs in these cells (43,44). To gain mechanistic insight into the role of HuR in mediating the function of BET proteins in MPNST cells, we investigated changes in BET occupancy on a genome scale by ChIP-Seq in ST88-14 cells after HuR silencing. We found that HuR silencing significantly reduced genome-wide BRD2, BRD3 and BRD4 occupancy by ~60%, ~50% and ~45% respectively (Figure 10D).

Next, to examine the functional effects of the BETs occupancy on transcriptional regulation, we generated a chromatin landscape in ST88-14 cells using H3K4me3 to

identify promoters, H3K4me1 to identify enhancers, and H3K27ac to identify active promoter/enhancer regions. Using this information, we performed network analysis to generate a network of transcriptional regulators that are targets of BRD proteins and regulated by HuR expression. In brief, we identified BRD-bound epigenetically active (active promoters and enhancers) and differentially regulated TFs in control and HuR-silenced cells (Figure 10E). It is apparent from this analysis that there is a considerable number of transcriptional regulators in MPNST cells that are associated with BRD proteins and that control key processes, such as proliferation. Importantly, we found that a substantial proportion of these transcriptional regulators were especially sensitive to HuR inhibition, which depleted BRD proteins from their promoter/enhancer regions and downregulated their expression (red circles). Thus, GSEA analysis of BRD-bound transcriptional regulators demonstrated a significant enrichment of genes activated in control cells and repressed by HuR silencing (Figure 10F). Gene enrichment analysis by ToppGene suite (45) identified these repressed regulators as being overrepresented in different oncogenic pathways, including E2F and MYC TF networks, proliferation and TP53-regulated pathways, and for different cancers (Figure 10G, H).

Collectively, these data demonstrate that BRD proteins are enriched at promoter/enhancer regions of key transcriptional regulators in MPNST cells, and that a reduction in their levels and genome occupancy by HuR silencing suppresses this oncogenic transcriptional circuitry.

DISCUSSION

Cancer arises from multiple genetic lesions that lead to aberrant gene expression programs, which are increasingly being recognised as fundamental for the acquisition, development and maintenance of cancer phenotypes. Compelling recent evidence shows that cancer cells can develop absolute dependencies on discrete molecular regulators – out of the thousands of human proteins that contribute to control of gene expression – that drive these dysregulated transcriptional programs. There is nowadays an intense search for these key regulators through focussed mechanistic studies, since they represent attractive targets for effective and enduring therapies in cancer (9,10).

In this study, we present evidence that MPNST cells exhibit an exceptional reliance on the RNA-binding protein HuR for their abilities to survive, proliferate and disseminate. HuR inhibition prevented the formation of tumours in xenograft models, and even induced a striking regression of tumour volume in established tumours. Furthermore, HuR strongly promoted the metastatic capacity of MPNST cells, one of the worst prognostic features of this cancer. We propose that HuR-regulated transcriptomes promotes the survival and adaptation strategies that allow the disseminated cancer cells to survive in the circulation, migrate to, extravasate and thrive in incompatible foreign microenvironments of distant tissues. Consistent with these results, we found that HuR regulated essential biological capabilities of MPNST cells, including cell cycle progression and sustained proliferation, resisting cell death in stress conditions and enabling replicative immortality. Notably, the functional consequences of HuR inhibition was largely comparable in a representative panel of 4 MPNST cell lines, obtained from patients with different NF1 status, even though these exhibit heterogeneous cellular growth rates and alterations in expression of a number of cell cycle proteins (24). This lack of major differences, functionally or mechanistically, between NF1-derived and sporadic MPNSTs after HuR silencing support the view that relatively similar molecular mechanisms are involved in the pathogenesis of MPNST, and that HuR can be a broad target for MPNSTs, irrespective of NF1 status (1).

Mechanistically, our RIP-Chip and transcriptomics data showed a global dysregulation of several signalling pathways and molecular regulators in these cancer cells, including the HIPPO-YAP/TAZ, PI3K/AKT/mTOR, RAS/RAF-MEK-ERK and Wnt/β-Catenin pathways and bromodomain regulation of gene transcription, while our focused analyses revealed that regulation by HuR of specific components of these pathways likely led to the aberrant signalling. Several of these pathways, including WNT/β-Catenin, seem to be specific to MPNSTs, since we find that HuR cannot regulate them in normal Schwann cells (data not shown). We posit that this capacity of HuR to simultaneously control several essential molecular regulators that operate in these cancer cells explains in large part the striking effects of HuR inhibition in MPNST cells. All these pathways have been shown to contribute to MPNST pathogenesis in seminal studies from several laboratories using various genetically engineered mouse models or culture systems (1,2,6,13,32-34,46). However, genetic or pharmacological inhibition of

these targets individually have, in general, been modestly effective and largely cytostatic. Instead, concurrent targeting of different pathways has proved more potent. Thus, combined inhibition of YAP/TAZ and PDGFR signalling activity (6), BRD proteins and MEK activity (13), or the mTOR and Wnt/β-Catenin pathways (34) are strongly synergistic in blocking tumour growth, and can even induce apoptosis.

These observations strongly support the view that parallel and redundant pathways control the oncogenic traits in MPNST cells, a phenomenon that has been associated with the emergence of therapeutic resistance in cancer cells (10,21). This is particularly true for targeted therapies against kinase-mediated signalling cascades, which are organized in a linear and hierarchical manner, with different receptor tyrosine kinases at the top, activating a reduced number of kinase signalling cascades, including RAS-RAF-ERK, PI3K-AKT and JAK-STAT that operate in parallel but which can crosstalk with one another (10). This linear and redundant architecture facilitates bypassing of a signalling pathway after its inhibition for another, thus blocking the therapeutic effects of the drugs. This paradigm is also consistent with the disappointingly poor results obtained from Phase I and Phase II clinical trials from targeting individual components of kinase cascades in MPNST patients. Because of the superior results obtained from additive effects of cotargeting multiple pathways, current clinical trials are more focused on combination drug therapy (1,25). Our results strongly argue that targeting HuR could be as equally effective as the combined treatments, as we find that HuR inhibition potently suppresses several of the oncogenic signals in MPNSTs, leading to the profound cytostatic and cytotoxic effects on tumour growth, as well as on their metastatic capacity.

Strikingly, we find that targeting HuR can both prevent the formation of tumours and metastatic nodules, as well as lead to shrinkage of fully established tumours and metastases. Thus, therapeutic intervention could be equally effective in newly diagnosed or recurrent tumours, which has particularly appealing translational importance, since timing of intervention and stage of disease has been raised as a possibility for negative clinical trials in MPNSTs. However, even though our promising data on pharmacological inhibition of HuR could represent a viable therapy for patients with MPNST, it is still unclear whether the preclinical efficacy observed in this study using cell lines and mice can be translated to the clinic. Whether HuR is a valid therapeutic target in MPNST warrants direct investigation.

Our ChIP-Seq data reveal a large network of transcriptional regulators operating in MPNST cells that are associated with key biological functions. Notably, we find that this network is highly sensitive to levels of HuR that regulate expression of these regulators via regulation of BRD protein levels, and/or signalling pathways. An essential role for BRD proteins in regulating gene expression has also been shown in several malignancies, including MPNST, and interestingly, we find that HuR silencing in MPNST phenocopies to a large extent treatment with the BET inhibitor JQ1, at least in terms of gene expression profiles. In addition, the displacement of BRD proteins on

promoter/enhancer regions of active cancer-related genes by JQ1 in haematological malignancies and melanoma (42-44) is similar to the reduced promoter/enhancer occupancy on transcriptional regulators in MPNSTs we found after HuR silencing. Thus, our data suggest that the dysregulation of the transcriptional program in MPNST by HuR silencing, could be in large part mediated by the influence of HuR on the MPNST transcriptional network.

Remarkably, we found that HuR depletion led to strong suppression of MYC and E2Fs levels that was accompanied by a highly significant reduction in the abundance of MYC- and E2F-transcribed gene sets, pointing to a general suppression of transcription at E2F- and MYC-driven targets by HuR inhibition. These proto-oncogenic TFs are among the most important drivers of tumorigenesis, as they regulate cell growth, proliferation, apoptosis and metabolic pathways (22,23), and represent some of the important targets for cancer therapy, although efforts to directly target these TFs have proved unsuccessful so far (9). Our work could thus have far-reaching implications for cancer therapy, since it suggests an alternative strategy to effectively target these TFs. MYC regulation also represents one of the best examples of the "multifunctional" capacity of HuR in driving expression of particular oncoproteins. MYC levels are regulated by major growth-regulatory and oncogenic signalling pathways, including the Wnt/β-Catenin, JAK-STAT and Notch pathways that induce *MYC* transcription and mTOR pathway that increases the efficiency of *MYC* mRNA translation, together with function of the PI3K and RAS signalling, and AURKA/B in increasing the stability of MYC protein (22,47). The striking downregulation of MYC levels in MPNST cells by HuR inhibition could potentially be mediated by a combinatorial action at several of these nodes of regulation, that are themselves under strong HuR influence. Similarly, E2F levels/function in MPNST cells could be determined by HuR by affecting multiple regulatory mechanisms, including Cyclin-CDK expression, RB phosphorylation and *E2Fs* transcription/translation.

RBPs play a central role in regulation of gene expression, and thus it is not surprising that their dysregulation has been linked to several human diseases, including neurological disorders and cancer (48,49). These dynamic regulators can bind to and regulate thousands of functionally related genes, regulating every hallmark of cancer cells. Recent detailed studies have shown that RBPs are rapidly emerging as key oncogenic drivers in a variety of malignancies (48,50-53). In this study, we show the RBP HuR has pleiotropic functions in MPNST, driving tumour growth and metastasis by, quite strikingly, influencing almost all key signalling pathways and regulators discovered in MPNSTs so far, including the PI3K/AKT/mTOR, RAS-RAF-MEK-ERK, WNT/β-Catenin and HIPPO-YAP/TAZ pathways, and transcriptional regulators that include SOX9, AURKA/B, BRD proteins, as well as the proto-oncogenic TFs MYC and E2Fs. Over 1500 RBPs have been described and several of them are dysregulated in MPNSTs (data not shown). It would be interesting to examine their biological and mechanistic functions in MPNSTs, and whether they have broad functions similar to HuR or regulate distinct pathways

This function of HuR in establishing the highly intricate regulatory networks operating in MPNSTs to coordinate multiple cancer hallmark traits supports a “master” regulatory function for HuR in MPNST. Thus, by elevating HuR levels, MPNST cells have elaborated an adaptive mechanism to amplify and regulate key oncogenic signals and modulate transcriptional programs to confer a competitive advantage to these cancer cells, promoting MPNST growth and metastatic spread (graphical abstract).

MATERIALS AND METHODS

Cell culture

Human MPNST cell lines S-462, STS-26T, ST88-14 and 90-8, and immortalized normal human Schwann cell line (iHSCλ2) were obtained from Nancy Ratner (Cincinnati Children's Hospital Medical Centre)(24,34). Immortalized plexiform neurofibroma-derived Schwann cells (ipNFSC) was purchased from ATCC (ATCC® CRL3390™). The cells were maintained in Dulbecco's modified Eagle Medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% (v/v) antibiotic/antimycotic (Thermo Fisher Scientific) under standard conditions of culture in 37 °C and 5% CO_2.

Human Schwann cells were isolated from the sural nerves of donors with informed consent (see below). Withdrawn nerves were stripped of their epineurium and fascicles were separated from the remaining interfascicular epineurium. Fascicles were cut into 2-mm-long pieces and incubated in humidified conditions and 10% CO_2 for 7-14 days in DMEM (Gibco) with 10% FBS (Sigma), 500 U/mL penicillin/streptomycin (Gibco) and 2.5 mg/mL Amphotericin (Gibco). Fascicles were then digested in DMEM with 10% FBS; 500 U/mL penicillin/streptomycin; 0.8 U/mL dispase grade I (Sigma) and collagenase type 1A 160 U/mL (Sigma). The resulting Schwann cells were then amplified on plates coated with 0.1 mg/mL Poly-L-lysine in DMEM with 20% FBS; 100 U/mL penicillin/streptomycin; 0.5 mM Forskolin (Sigma); 2.5 mg/mL Amphotericin 2.5 mg/mL; 2.5 mg/ml Insulin (Sigma); 10 nM β1-heregulin (Sigma) and 0.5 mM IBMX (Sigma). Amplified Schwann cells were plated in DMEM with 10% FBS in the absence of β1-heregulin for at least 24 hours before analysis.

Lentivirus preparation and infection of MPNST cells

Lentiviral particles (Supplemental Table 7) were produced in HEK293FT cells. For single-infection experiments, MPNST cells were incubated with different lentiviral particles together with 8 µg/mL polybrene (Merck) for enhancing infection efficiency. Selection of infected cells was started 48 h post-infection with puromycin (4 µg/mL) for 2 days in supplemented growth medium or with geneticin (G418 sulfate) (Thermo Fisher Scientific) at 50 µg/mL for 5 days in growth medium without antibiotic/antimycotic. For the inducible knockdown plasmids, after infection and selection, cell lines were treated with 0.5 µg/ml doxycycline every 24 h for 3 days. Infection efficacy was tested by western blot and RT-qPCR analyses. For rescue experiments, cells were first infected with control or β-Catenin overexpressing plasmids and selected with puromycin, and then further infected with shHuR#3 lentiviral particles followed by selection with geneticin, as above. After infection and selection, MPNST cells were replated at specific densities for the different functional assays. All assays were conducted in triplicate, with at least 3 independent experiments performed for each assay.

Small molecule HuR inhibitors

For culture studies, MPNST cell lines were treated with the following small molecule HuR inhibitors for 48h: (a) MS-444 (29), obtained from Philipp Krastel (Novartis Institutes for Biomedical Research), reconstituted in DMSO and used at a final concentration of 10 μM. (b) Pyrvinium pamoate (Sigma), reconstituted in DMSO and used at a final concentration of 2 μM. (c) Tanshinone mimic 6N (TM-6N) (31), obtained from Alessandro Provenzani (University of Trento), reconstituted in DMSO and used at a final concentration of 10 μM. 0.1% DMSO was used in control cultures.

For in vivo xenograft studies, 1 X 10^6 STS-26T cells mixed in 1:4 PBS:Matrigel were injected subcutaneously in the left and right back flanks of mice respectively under standard procedures and tumours allowed to grow up to 100 mm^3 average volume, as described above. The mice then received intraperitoneal (IP) injections of MS-444 (25 mg/kg) dissolved in PBS/5% N-Methyl Pyrrolidine (NMP) (Sigma-Aldrich) or vehicle control every 48 hr for a further 10 days.

For lung metastasis studies, 1 X 10^6 STS-26T cells were injected in the lateral tail vein of mice, and left for 2 weeks to allow basal formation of lung metastases, as described above. The mice then received IP injections of MS-444 (25 mg/kg) dissolved in PBS/5% N-Methyl Pyrrolidine (NMP) (Sigma-Aldrich) or vehicle control every 48 hr for a further 2 weeks.

Statistics

All analyses were done using Microsoft Excel or GraphPad Prism 6.00 (San Diego California, www.graphpad.com). Data are shown in dot plots or histograms as mean ± SEM, $p < 0.05$ is deemed statistically significant. Statistical analysis was performed by two-tailed unpaired Student's t tests, Mann Whitney U test, one-way ANOVA with post hoc analysis by Tukey's multiple comparison test, or Two-way ANOVA with Tukey's multiple comparisons test, or as indicated. Quantifications were performed from a minimum of three experimental groups.

Study Approval

Animal Studies: Xenograft or experimental lung metastasis experiments were carried out following the ethical guidelines established by the Biosafety and Welfare Committee (BAWC) at CIC bioGUNE following the recommendations from AAALAC.

Human Studies: Patient samples were obtained in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standard:

(1) the Stanmore Musculoskeletal Biobank, a satellite of the UCL/UCLH Biobank (HTA Licence Number 12055), which was approved by the National Research Ethics Committee (reference 15/YH/0311). This specific study was approved by the NREC-approved UCL/UCLH Biobank Ethical Review Committee (reference EC17.14).

(2) Peripheral nerves for Schwann cell culture were retrieved from donors after consent under NHS Blood and Transplant (NHSBT) research study entitled 'Collection of peripheral nerves for control cells for brain tumour treatment research', NHSBT study (61). Full research project title was 'Identifying and validating molecular targets in low grade brain tumours; REC number: 14/SW/0119; IRAS reference: 153351.

Accession Codes

All the microarray, RNA-seq and ChIP-Seq data are deposited in the NCBI Gene Expression Omnibus (GEO) with the accession number GSE120687.

AUTHOR CONTRIBUTIONS

In vitro & in vivo analyses: M.P.-I, E.P.-A, M.I-L, A.B.-M, M.T.-C, D.M., N.B. Sequencing & Bioinformatics analysis: A.M.Arans.., D.M.-A., J.J.L., J.L.L., A.M.A., D.G., M.J.A.-B.
Supervised network analysis: S.O., A.D.S.
Technical support and discussion: N.M.-M, Mon. G., J.D.S., D.F.-R., V.G.-d.J., N.M.-C., A.C., R.B., M.L.M.-C.
Human samples & Reagents: L.L., D.B.P., O.H., E.E., H.I., A.I., P.P., E.S., C.L., A.M.F, R.D.C., M.G., C.S., A.P., P.S., N.R.
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A.W. and M.V-R. directed the study and contributed to data acquisition and analysis.
A.W. wrote the manuscript.
Order of authorship for first co-authors and co-senior authors was determined by relative contribution to data acquisition and design of research study.

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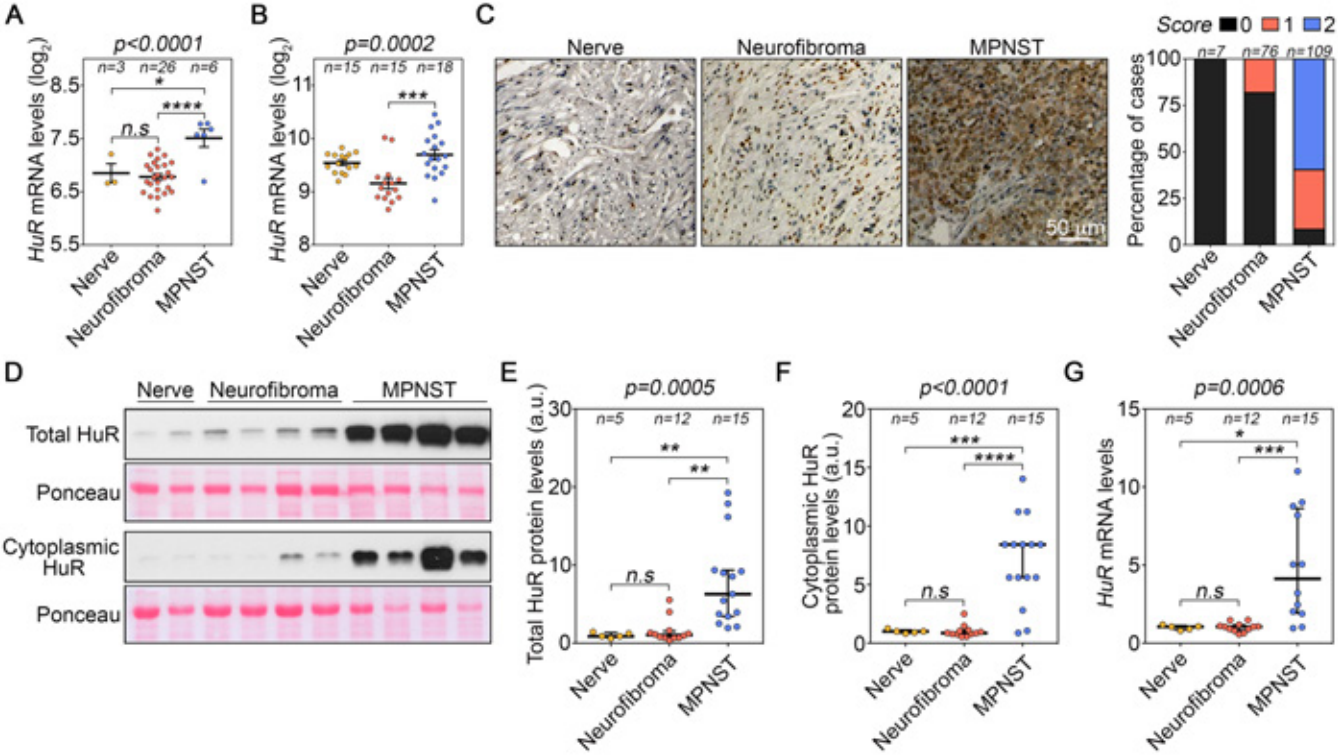


Figure 1: HuR is upregulated in human MPNSTs

(A, B) HuR mRNA levels in nerves, neurofibromas and MPNSTs from (A) patients and (B) mouse models from the Jessen cohort (GSE 41747)(7).

(C) Representative immunohistochemistry micrographs of endogenous HuR protein levels (brown) in a tissue microarray panel of human nerves (n=7), benign neurofibromas (n=76) and MPNSTs (n=109)(15). Score 0 represent low HuR staining, Score 1 intermediate staining and Score 2 represent high HuR staining.

(D–G) Western blot and RT-qPCR analysis of HuR levels in a panel of human nerves (n=5), benign neurofibromas (n=12) and MPNSTs (n=15) obtained from Stanmore Musculoskeletal Biobank. (D) Representative immunoblots showing total and cytoplasmic HuR levels in a selection of samples. (E–F) Graphs representing densitometry analysis of (E) total HuR protein levels, corrected for Ponceau red signals, (F) cytoplasmic HuR protein levels, corrected for Ponceau red signals. (G) HuR mRNA levels as measured by RT-qPCR analysis.

Data are presented as mean ± SEM (A, B) or median with interquartile range (E–G); one-way ANOVA with Tukey's multiple-comparisons test. n.s= not significant; *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001. The number of samples (n) per group is indicated.

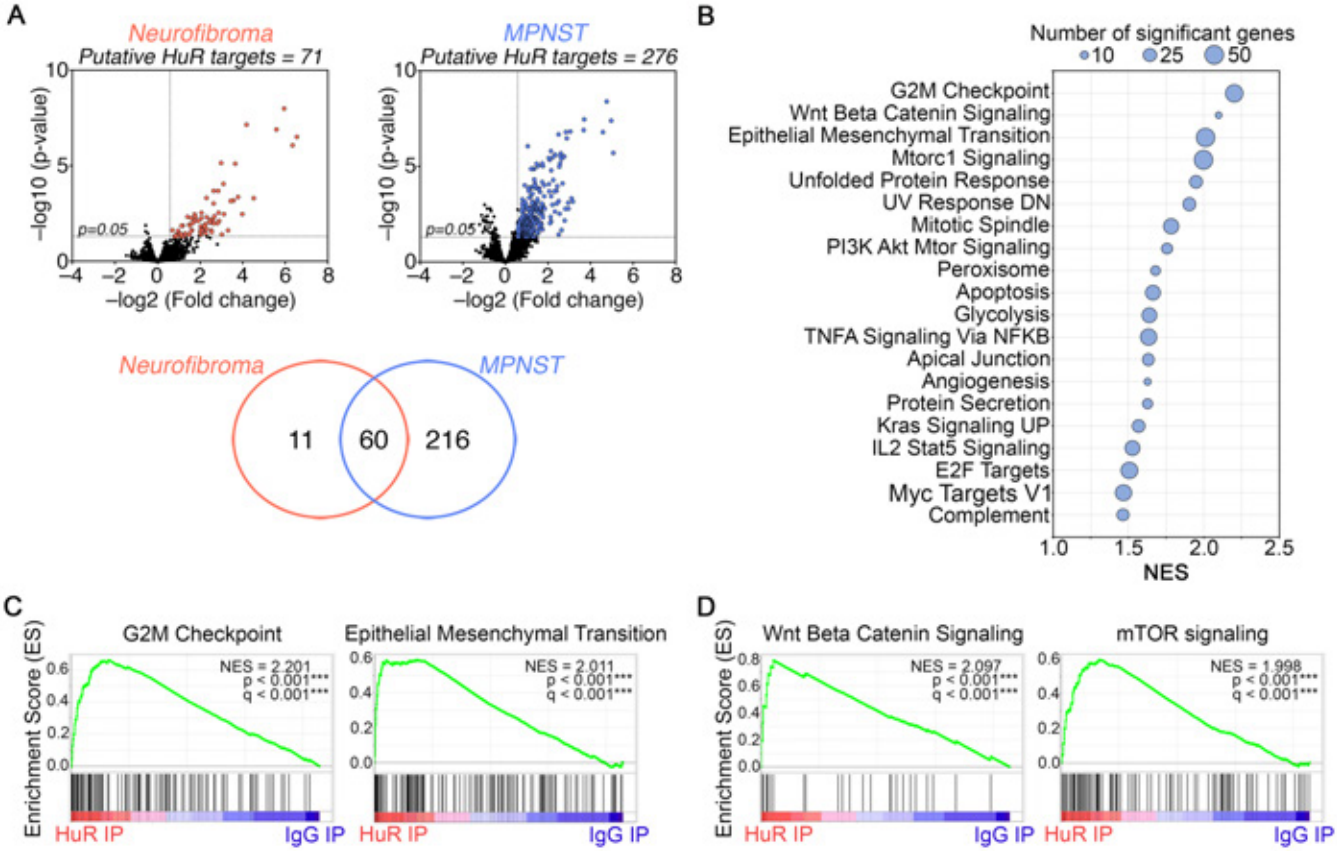


Figure 2: HuR is bound to key targets in human MPNSTs

(A) Volcano plots show enrichment of transcripts most significantly bound to HuR compared with control IgG in neurofibromas (left panel) (n=8) and MPNSTs (right panel) (n=12) obtained from Stanmore Musculoskeletal Biobank. (Red or blue coloured dots: fold-change >1.5; adjusted p-value<0.05). Venn diagram shows overlap between putative HuR mRNA targets from neurofibromas and MPNSTs.

(B) GSEA analysis of putative HuR mRNA targets in MPNSTs for MSigDB hallmarks. The top 20 gene sets (FDR q-values <0.1) are plotted relative to normalized enrichment score (NES). Circles denote the number of enriched genes in each category.

(C, D) GSEA plots of HuR IP and Control IgG IP for (C) key cancer traits and (D) oncogenic pathways in MPNSTs.

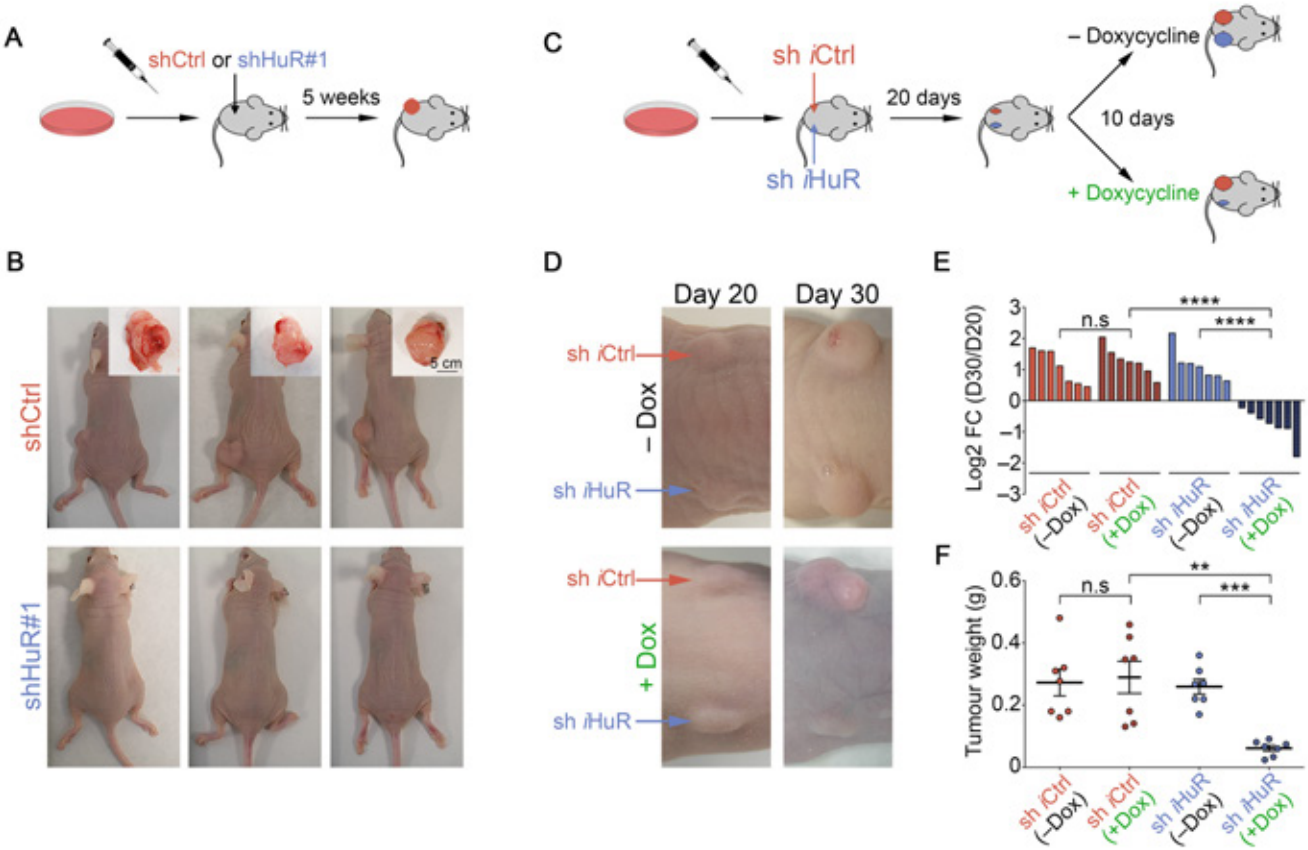


Figure 3: HuR promotes MPNST cell growth in vivo

(A, B) Constitutive HuR silencing prevents tumour formation in vivo. (A) Schematic representation of xenotransplantation experiments. (B) Representative pictures of tumours from nude mice injected with sh Ctrl or sh HuR#1 STS-26T MPNST cells, 5 weeks after transplant (n=5) for each condition. Scale bar= 5 mm. (C-F) Inducible HuR silencing promotes tumour regression in vivo. (C) Schematic representation of xenotransplantation experiments using inducible HuR silencing strategy. (D) Representative pictures of tumours from nude mice injected with sh iCtrl or sh iHuR STS-26T MPNST cells on left and right flank respectively at 20 days after injection (Day 20), and 10 days later (Day 30) with (+Dox) or without doxycycline diet (-–Dox). (E) Waterfall plot showing change in tumour volume (represented as log2 fold-change) of individual tumours formed at 20 days after transplant, and after 10 days with or without doxycycline treatment for each 4 groups of mice: sh iCtr (–Dox)(n=7), sh iCtr (+Dox)(n=7), sh iHuR (–Dox)(n=7) and sh iHuR (+Dox)(n=7). (F) Graph showing weight of excised tumours for the 4 groups of mice.

Each data point represents 1 mouse; one-way ANOVA with Tukey's multiple-comparisons test. n.s= not significant; **p<0.01, ***p<0.001; ****p<0.0001.

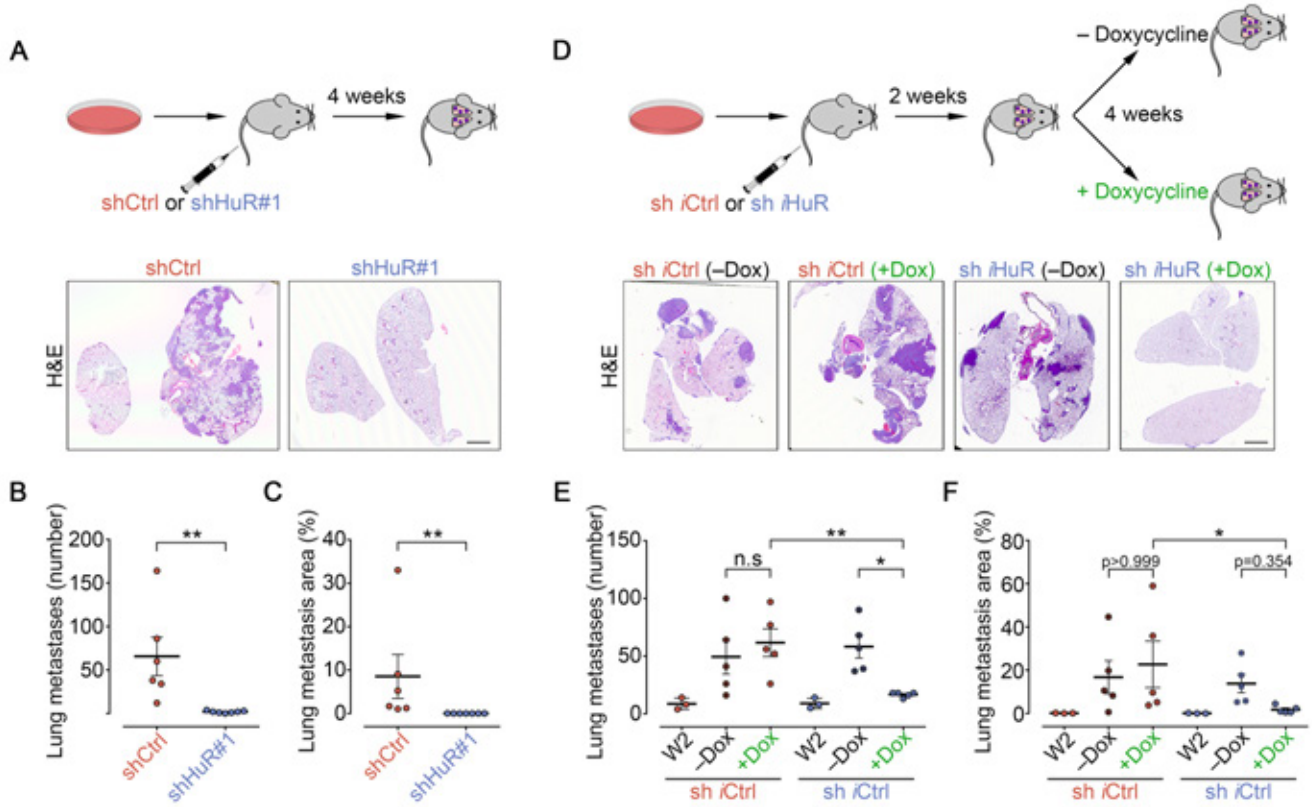


Figure 4: HuR promotes MPNST metastasis in vivo

(**A–C**) Constitutive HuR silencing prevents lung metastasis of STS-26T MPNST cells. (**A**) Schematic representation of lung metastasis experiments. Sh Control (n=6) or sh HuR#1-expressing (n=7) STS-26T MPNST cells were injected in the tail vein of nude mice, and mice sacrificed 4 weeks later and lung architecture analysed by haemaoxylin & eosin (H&E) staining. Representative pictures of lung histology for each group are shown. (**B, C**) Number of lung metastases (**B**) and lung metastatic area, expressed as a percentage of total lung area (**C**), was quantified by H&E histology. Scale bar, 2 mm.

Each data point represents 1 mouse; Data are presented as mean ± SEM, two-tailed unpaired Mann-Whitney U test. n.s= not significant; *p<0.05; **p<0.01.

(**D–F**) Inducible HuR silencing prevents growth of established lung metastatic nodules in vivo. (**D**) Schematic representation of experiments. Sh iControl or sh iHuR-expressing STS–26T MPNST cells were injected in the tail vein of nude mice. A group of mice (n=3, for each condition) was sacrificed at 2 weeks (W2) to analyse basal formation of lung metastases, and the rest of mice fed with normal diet (¬–Dox) or doxycycline diet to induce expression of shRNAs (+Dox), for a further 4 weeks, before sacrifice and analysis of lung histology by H&E staining. Representative pictures of lung histology for the following groups are shown: sh iCtrl with normal diet (sh iCtrl; –Dox)(n=5), sh iCtrl with doxycycline diet (sh iCtrl; +Dox)(n=5), sh iHuR with normal diet (sh iHuR; –Dox)(n=5), sh iHuR with doxycycline diet (sh iHuR; +Dox)(n=5), Scale bar, 2 mm. (**E, F**) Number of lung metastases (**E**) and lung metastatic area, expressed as a percentage of total lung area (**F**), was quantified for each of 6 groups by histology.

Each data point represents 1 mouse; Data are presented as mean ± SEM, one-way ANOVA with Sidak's multiple-comparisons test. n.s= not significant; *p<0.05; **p<0.01.

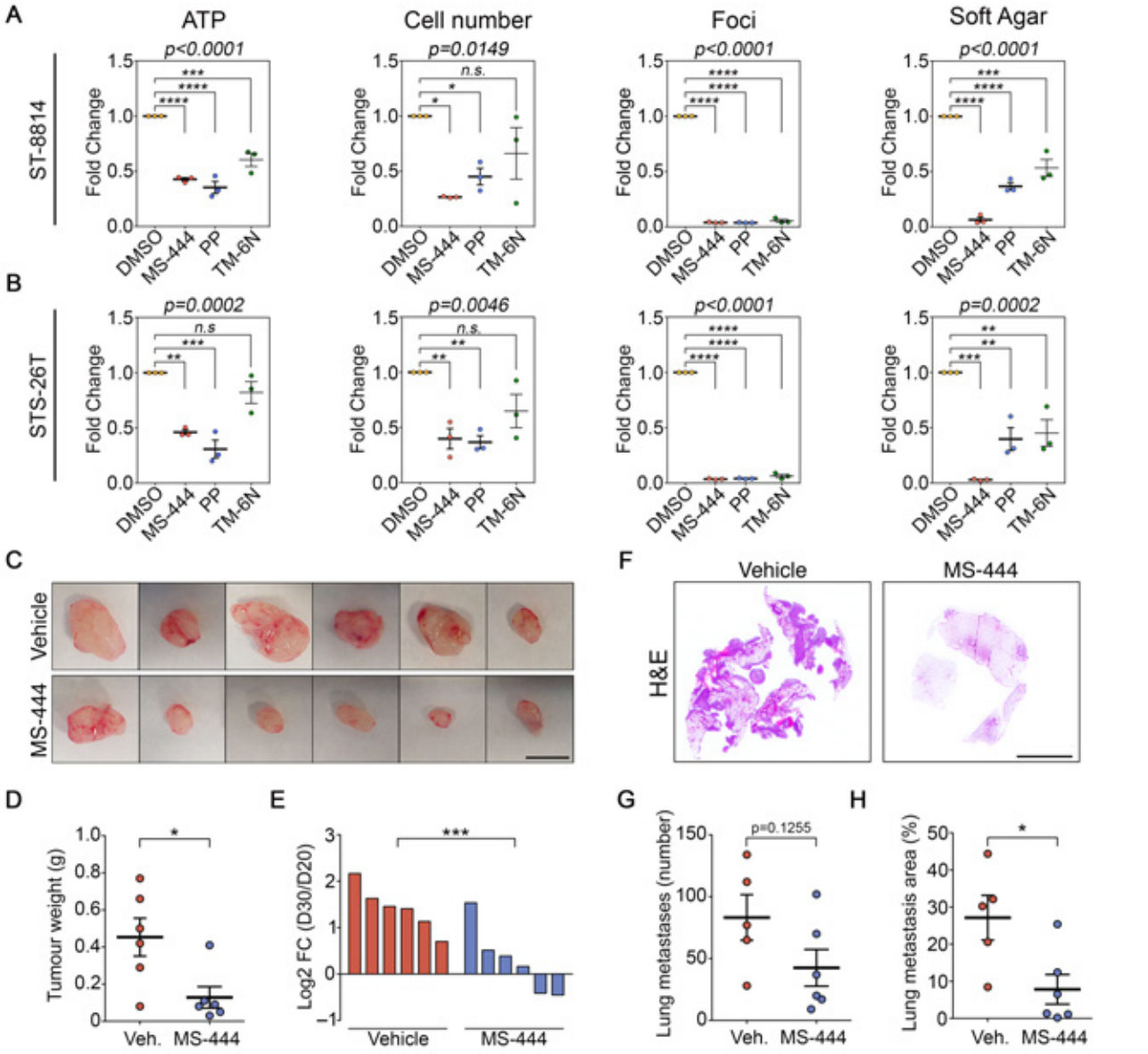


Figure 5: Pharmacological inhibition of HuR blocks MPNST cell growth and metastasis in vitro and in vivo

(A, B) Pharmacological inhibition of HuR activity leads to a reduction in cell growth in MPNST cell lines ST-8814 and STS-26T, as determined by ATP luminescence, counts of cell numbers, clonogenic assays (foci), and anchorage-independent growth using soft agar assays. Graphs represent absorbance of crystal-violet stained colonies for clonogenic assays, and number of colonies in soft agar assays.
Data are normalized to DMSO-treated and are presented as mean ± SEM; Each data point represents 1 independent experiment; one-way ANOVA with Tukey's multiple-comparisons test.

(C-E) Pharmacological inhibition of HuR activity by MS-444 promotes tumour regression in vivo. (C) Pictures of tumours from nude mice injected with STS-26T MPNST cells after vehicle or MS-444 treatment. Scale bar= 10 mm. (D) Graph showing weight of excised tumours for both groups of mice. (E) Waterfall plot showing change in tumour volume (represented as log2 fold-change) of individual tumours formed at 20 days after transplant, and after 10 days with pharmacological inhibition.
Each data point represents 1 mouse; Data are presented as mean ± SEM, two-tailed unpaired Mann-Whitney U test.

(F–H) Pharmacological inhibition of HuR activity by MS-444 prevents growth of established lung metastatic nodules in vivo. (F) Representative pictures of lung histology from nude mice injected with STS-26T MPNST cells after vehicle or MS-444 treatment. Number of lung metastases (G) and lung metastatic area, expressed as a percentage of total lung area (H), was quantified. Scale bar, 5 mm.
Each data point represents 1 mouse; Data are presented as mean ± SEM, two-tailed unpaired Mann-Whitney U test.
*p<0.05; **p<0.01, ***p<0.001; ****p<0.0001.

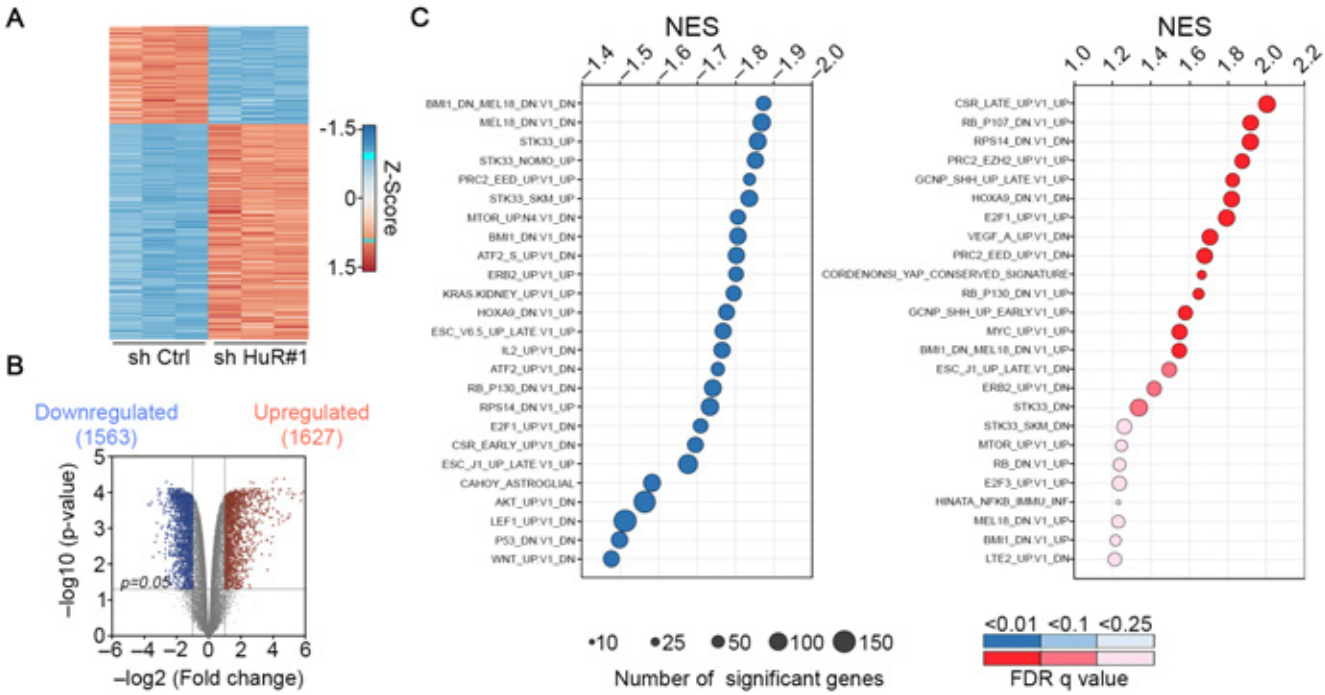


Figure 6: RNA-Seq reveals that HuR controls key oncogenic pathways in MPNST

(A) Heatmap representation of differentially expressed genes between sh Control (n=3) or sh HuR#1-expressing (n=3) ST88-14 MPNST cells (fold change >2 and adjusted p-value<0.05).

(B) Volcano plot of transcriptome profiles between sh Control (n=3) and sh HuR#1-expressing (n=3) ST88-14 MPNST cells. Red and blue dots represent genes significantly upregulated and downregulated in sh HuR#1-expressing cells respectively (Red or blue dots: fold change >2 and adjusted p-value<0.05).

(C) GSEA analysis of sh Control and sh HuR#1-expressing ST88-14 MPNST cells for MSigDB Oncogenic signatures. Gene sets with FDR q values <0.25 are plotted relative to normalized enrichment score (NES). Categories with negative (left) and positive (right) NES are down- or upregulated, respectively, in sh Control cells. Circles denotes the number of enriched genes in each category and colour codes represent FDR q values as indicated.

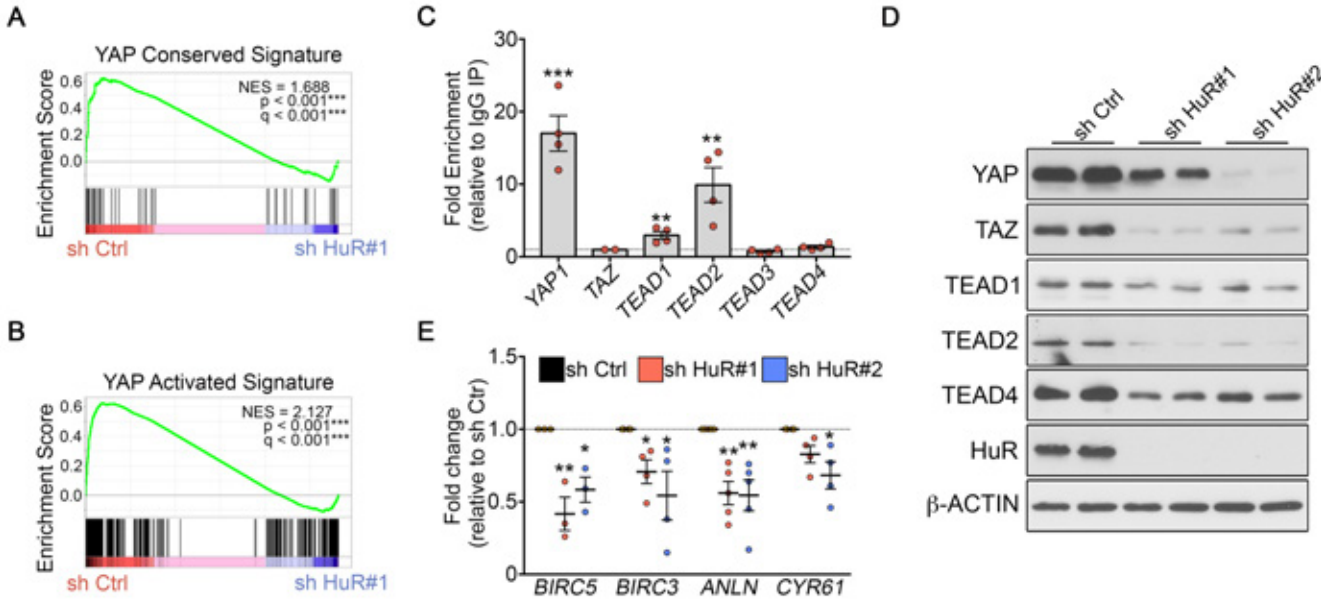


Figure 7: HuR regulates the YAP/TAZ pathway in MPNST

(A, B) GSEA plots showing enrichment of (A) Yap conserved signature from Figure 6C and (B) Yap activated signature set (36), in sh Control compared to sh HuR#1-infected ST88-14 MPNST cells.

(C) RIP-qPCR analysis showing binding of HuR to YAP1, TEAD1 and TEAD2 in 4 MPNST cell lines (ST88-14, 90-8, S462, STS-26T).

Data are normalised to control IgG IPs and are presented as mean ± SEM, two-tailed unpaired Student's t-test. n= 4 MPNST cell lines.

(D) Representative Western blot showing a general downregulation of key YAP/TAZ pathway components after HuR silencing in ST88-14 MPNST cells. Technical duplicates are shown, and similar results were obtained in at least 3 independent experiments.

(E) RT-qPCR analysis showing downregulation of YAP/TAZ pathway effector genes (6) after HuR silencing in ST88-14 MPNST cells.

Data are normalized to sh Ctrl cells and are presented as mean ± SEM; Each data point represents 1 independent experiment; one-way ANOVA with Tukey's multiple-comparisons test. *p<0.05; **p<0.01, ***p<0.001

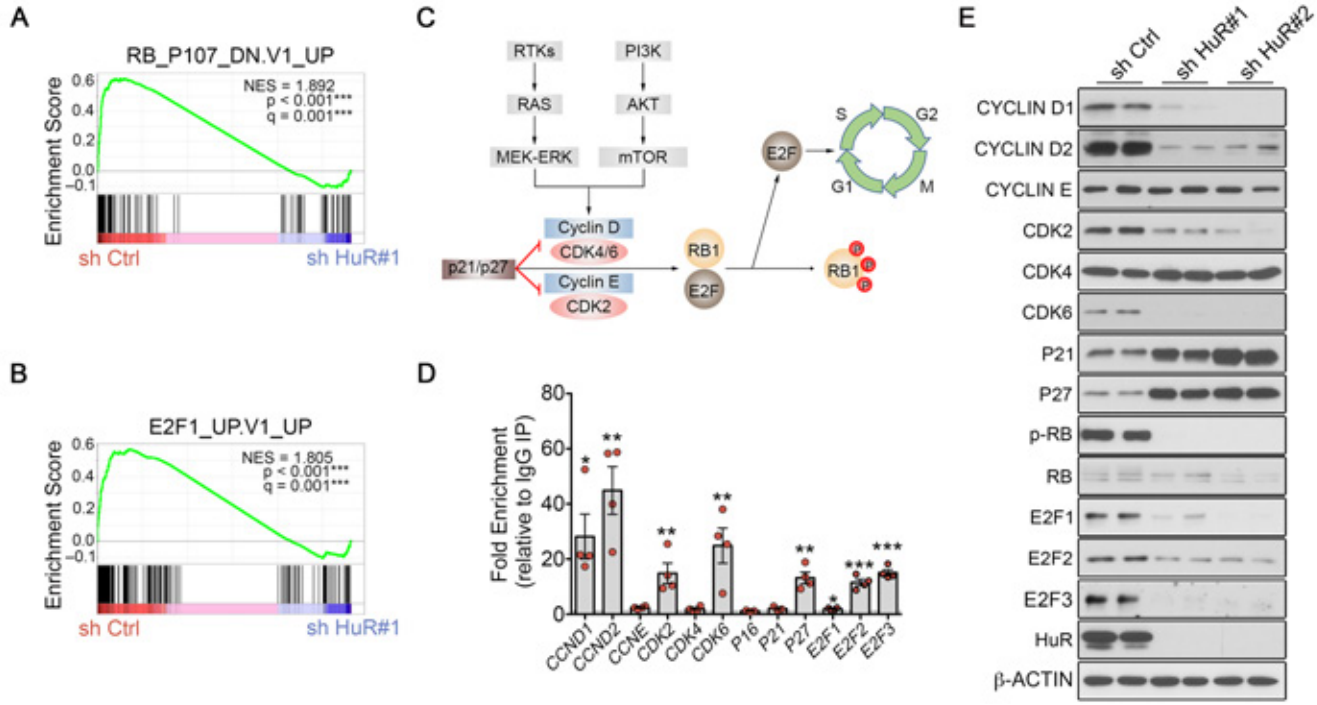


Figure 8: HuR regulates the RB-E2F pathway in MPNST

(A, B) GSEA plots showing enrichment of functionally defined (A) RB and (B) E2F1 signature set in sh Control-infected compared to sh HuR#1-infected ST88-14 MPNST cells from Figure 6C.

(C) Cartoon depicting regulatory components of the RB-E2F pathway in cell cycle regulation. By interacting with CDKs, cyclins form complexes (cyclin D with CDK4/6 and cyclin E with CDK2) that phosphorylate RB1 (phosphorylated RB1 is inactive). When RB1 is phosphorylated, E2F is released and can transcriptionally activate its target genes, enabling the G1/S transition of cell cycle. Cyclins can be regulated at the transcription level by the RAS-MEK-ERK pathway and at the translation level by mTOR via S6K and 4EBP1. P21 and p27 can bind to and inhibit Cyclin-CDK complexes.

(D) RIP-qPCR analysis showing binding of HuR to multiple genes in the RB-E2F pathway in 4 MPNST cell lines (ST88-14, 90-8, S462, STS-26T).

Data are normalised to control IgG IPs and are presented as mean ± SEM, two-tailed unpaired Student's t-test.

(E) Representative Western blot showing a downregulation of several key RB-E2F pathway components after HuR silencing in ST88-14 MPNST cells, in general concordance with RIP-qPCR data. Technical duplicates are shown, and similar results were obtained in 3 independent experiments. *p<0.05; **p<0.01, ***p<0.001.

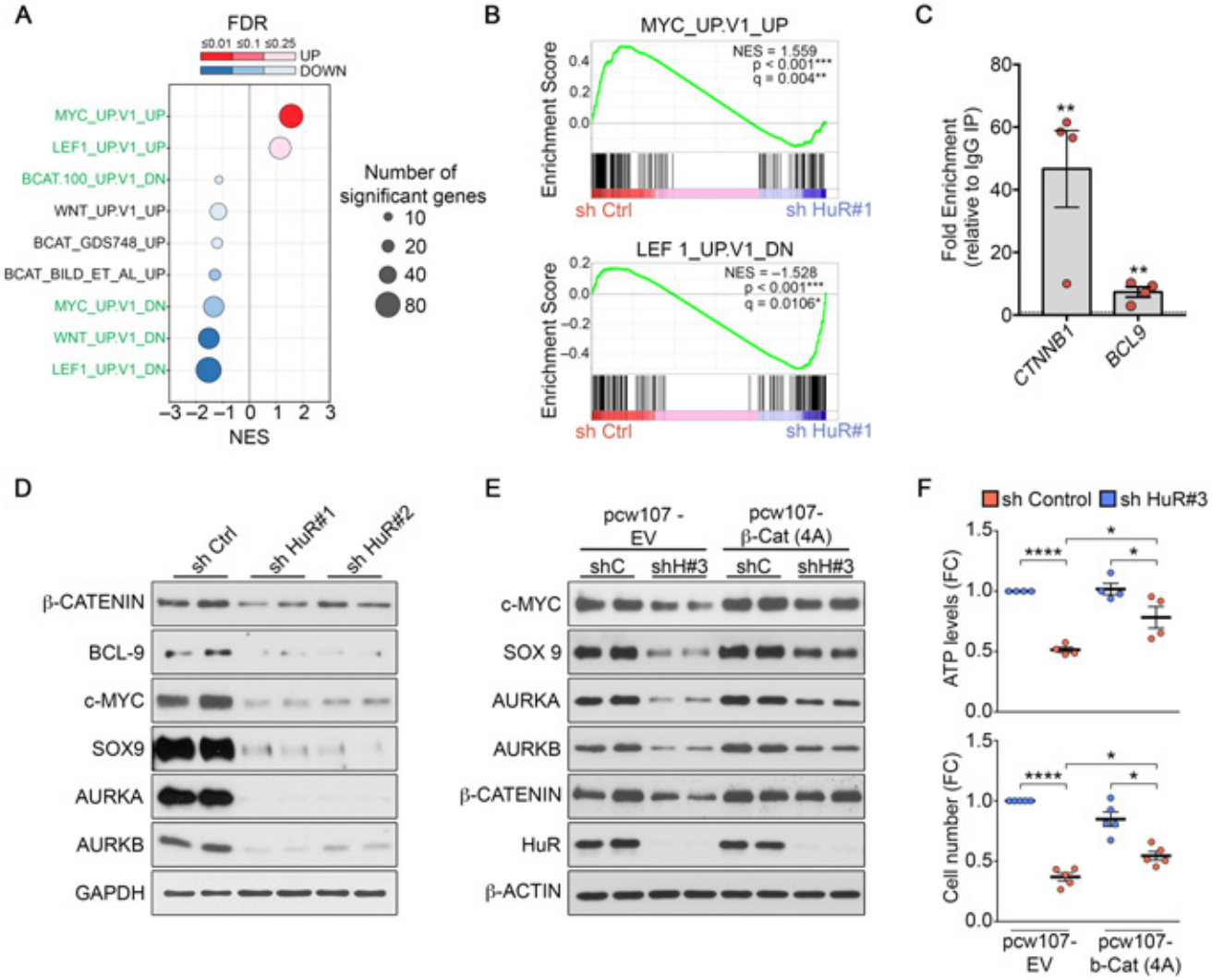


Figure 9: HuR activates key oncogenic programs by regulating the Wnt/β-Catenin pathway.

(A) Compendium of normalized enrichment scores (NES) of target gene sets associated with Wnt/β–Catenin pathway after GSEA analysis of HuR–silenced ST88-14 MPNST cells (Supplemental Tables 5 and 6). Notably, there is general positive correlation in the activation of the pathways (highlighted in green) in sh Control-infected cells. Circles denotes the number of enriched genes in each category and colour codes represent FDR q values.

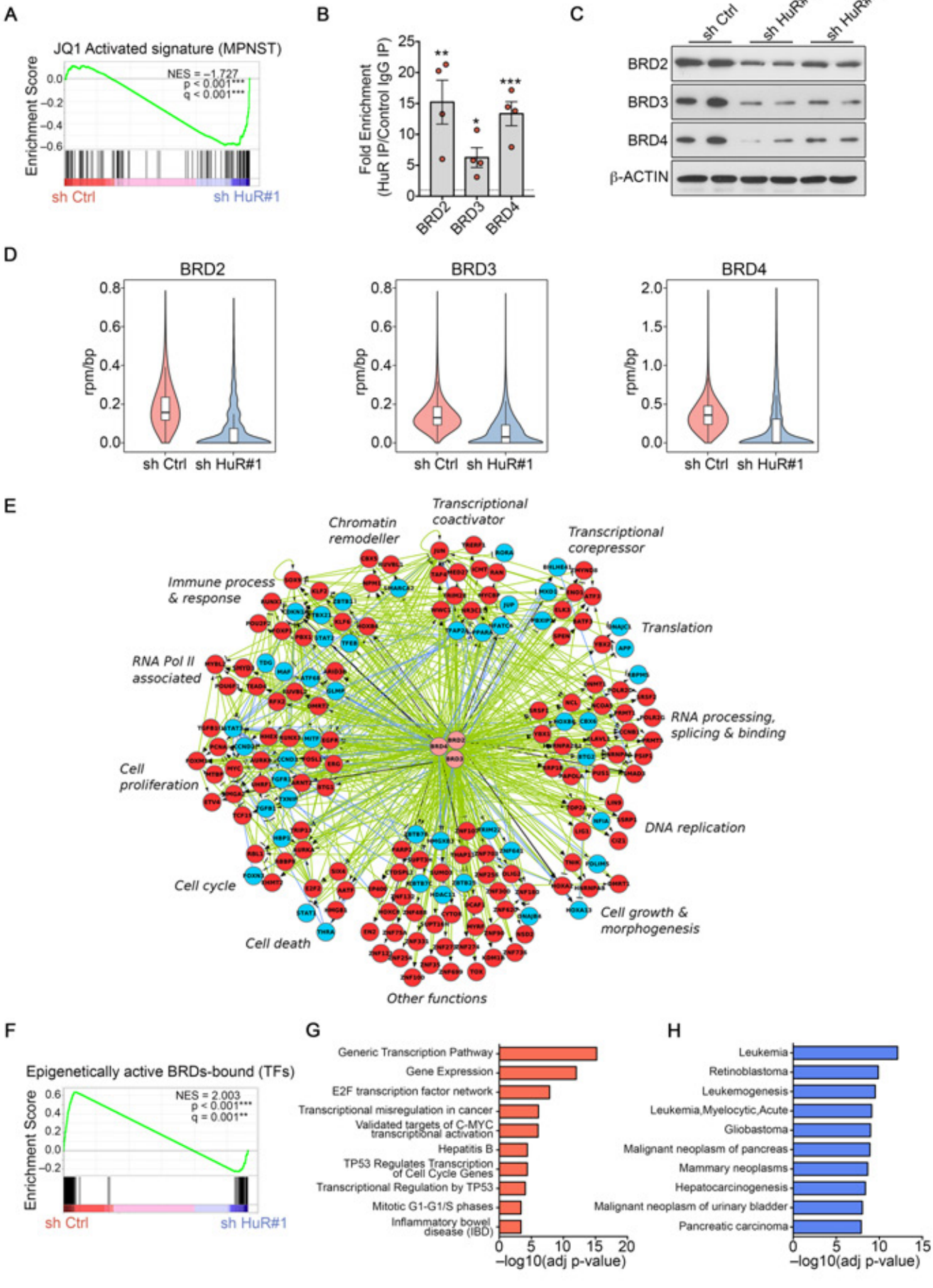
(B) GSEA plots showing enrichment of a MYC-induced target gene set in sh Control- cells, and a LEF1-repressed target gene set in sh HuR#1-infected cells.

(C) RIP-qPCR analysis showing binding of HuR to CTNNB1 and BCL9 in 4 MPNST cell lines (ST88-14, 90-8, S462, STS-26T). Data are normalised to control IgG IPs and are presented as mean ± SEM, two-tailed unpaired Student's t-test.

(D) Representative Western blot showing a general downregulation of Wnt/β-catenin pathway components, including key oncogenic downstream regulators, after HuR silencing in ST88-14 MPNST cells. Technical duplicates are shown, and similar results were obtained in 3 independent experiments.

(E) Representative Western blot showing that lentivirus-based expression of constitutively active β-catenin 4A mutant (harbours alanine substitutions at S33, S37, T41, and S45, preventing its degradation) [pcw107-β-Cat (4A)], partially blocks the downregulation of the key downstream regulators c-MYC, SOX9, AURKA and AURKB by HuR silencing (shH#3) in ST88-14 MPNST cells. Technical duplicates are shown, and similar results were obtained in 3 independent experiments.

(F) Ectopic expression of constitutively active β-catenin 4A mutant partially blocks the effects of HuR silencing on cell numbers and ATP levels in ST88-14 MPNST cells. Data are normalized to shC + pcw107-EV cells and are presented as mean ± SEM; Each data point represents 1 independent experiment; one-way ANOVA with Tukey's multiple-comparisons test. *p<0.05; **p<0.01, ***p<0.001, ****p<0.001.


A
JQ1 Activated signature (MPNST)
Enrichment Score
NES = −1.727
p < 0.001***
q < 0.001***
sh Ctrl
sh HuR#1
B
Fold Enrichment
(HuR IP/Control IgG IP)
BRD2
BRD3
BRD4
C
sh Ctrl
sh HuR#1
sh HuR#2
BRD2
BRD3
BRD4
β-ACTIN
D
BRD2
BRD3
BRD4
rpm/bp
sh Ctrl
sh HuR#1
E
Transcriptional coactivator
Chromatin remodeller
Transcriptional corepressor
Immune process & response
Translation
RNA Pol II associated
RNA processing, splicing & binding
Cell proliferation
DNA replication
Cell cycle
Cell growth & morphogenesis
Cell death
Other functions
F
Epigenetically active BRDs-bound (TFs)
Enrichment Score
NES = 2.003
p < 0.001***
q = 0.001**
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G
Generic Transcription Pathway
Gene Expression
E2F transcription factor network
Transcriptional misregulation in cancer
Validated targets of C-MYC transcriptional activation
Hepatitis B
TP53 Regulates Transcription of Cell Cycle Genes
Transcriptional Regulation by TP53
Mitotic G1-G1/S phases
Inflammatory bowel disease (IBD)
−log10(adj p-value)
H
Leukemia
Retinoblastoma
Leukemogenesis
Leukemia,Myelocytic,Acute
Gliobastoma
Malignant neoplasm of pancreas
Mammary neoplasms
Hepatocarcinogenesis
Malignant neoplasm of urinary bladder
Pancreatic carcinoma
−log10(adj p-value)

Figure 10: HuR regulates a core transcriptional circuitry in MPNST cells by controlling expression of BRD proteins

(A) GSEA plots showing an enrichment of genes upregulated in MPNST cells by JQ1 treatment (FC >1.5 and adjusted p-value<0.05)(13) and sh HuR#1-infected ST88-14 MPNST cells.

(B) RIP-qPCR analysis showing binding of HuR to BRD2, BRD3 and BRD4 in 4 MPNST cell lines (ST88-14, 90-8, S462, STS-26T).

Data are normalised to control IgG IPs and are presented as mean ± SEM, two-tailed unpaired Student's t-test.

(C) Representative Western blot showing downregulation of BRD proteins after HuR silencing in ST88-14 MPNST cells. Technical duplicates are shown, and similar results were obtained in 3 independent experiments.

(D) Violin plot showing the distributions of BRD2, BRD3 and BRD4 ChIP-seq signal at enriched regions in sh Control-infected and sh HuR#1-infected ST88-14 MPNST cells. The y axis shows BRD ChIP-seq signal in units of reads per million (rpm)/bp. The loss of BRD occupancy at BRD-enriched regions after HuR silencing is highly significant (BRD2: P = 4.44 x 10–16, BRD3: P = 1.332 x 10–15, BRD4: P = 1.332 x 10–15, Welch's t-test).

(E) Gene regulatory networks among differentially expressed, epigenetically active TFs that are either direct or indirect targets of BRD proteins. Red and blue circles represent differentially up-regulated TFs in sh Control-infected cells and down-regulated TFs in sh Control-infected cells respectively. Light green edges indicate regulatory interactions unique to sh Control cells, light blue edges indicate those unique to sh HuR#1-infected cells, and black edges indicate those present in both phenotypes. Pointed arrows indicate activation and blunted arrows indicate inhibition. Functional categories are based on Gene Ontology Biological Processes.

(F) GSEA plot showing strong enrichment of epigenetically active TFs that are either direct or indirect targets of BRD proteins in sh Control-infected compared to sh HuR#1-infected ST88-14 MPNST cells

(G, H) ToppGene analysis of HuR-regulated TF network in MPNST (Epigenetically active TFs that are either direct or indirect targets of BRD proteins in sh Control-infected cells and are downregulated in sh HuR#1-infected cells) (red circles from Figure 10E), as classified according to pathway categories (G) or disease (H).