hnRNP H/F drive RNA G-quadruplex-mediated translation linked to genomic instability and therapy resistance in glioblastoma

Pauline Herviou, Morgane Le Bras, Leïla Dumas, Corinne Hieblot, Julia Gilhodes, Gianluca Cioci, Jean-Philippe Hugnot, Alfred Ameadan, Erik Dassi, Anne Cammas, et al.

To cite this version:

Pauline Herviou, Morgane Le Bras, Leïla Dumas, Corinne Hieblot, Julia Gilhodes, et al.. hnRNP H/F drive RNA G-quadruplex-mediated translation linked to genomic instability and therapy resistance in glioblastoma. Nature Communications, Nature Publishing Group, 2020, 11 (1), pp.1-17. 10.1038/s41467-020-16168-x . hal-02879110

HAL Id: hal-02879110
https://hal.inrae.fr/hal-02879110
Submitted on 23 Jun 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
hnRNP H/F drive RNA G-quadruplex-mediated translation linked to genomic instability and therapy resistance in glioblastoma

Pauline Herviou1,2,3,9, Morgane Le Bras1,2,3,9, Leïla Dumas1,2,3, Corinne Hieblot1,2,3, Julia Gilhodes4, Gianluca Cioci5, Jean-Philippe Hugnot6, Alfred Ameadan7, François Guillonneau7, Erik Dassi8, Anne Cammas1,2,3 & Stefania Millevoi1,2,3

RNA G-quadruplexes (RG4s) are four-stranded structures known to control mRNA translation of cancer relevant genes. RG4 formation is pervasive in vitro but not in cellulo, indicating the existence of poorly characterized molecular machinery that remodels RG4s and maintains them unfolded. Here, we performed a quantitative proteomic screen to identify cytosolic proteins that interact with a canonical RG4 in its folded and unfolded conformation. Our results identified hnRNP H/F as important components of the cytoplasmic machinery modulating the structural integrity of RG4s, revealed their function in RG4-mediated translation and uncovered the underlying molecular mechanism impacting the cellular stress response linked to the outcome of glioblastoma.
Most steps in the gene expression pathway are regulated by the ability of specific RNA regions to form duplexes and other types of RNA conformations involving both Watson-Crick and non-canonical interactions. RNA-binding proteins (RBPs) establish highly dynamic interactions with such RNA elements, creating functional ribonucleoprotein complexes that are essential for every step of post-transcriptional control, including mRNA processing, stability, transport and translation. Accumulating evidences showed that RBPs are dysregulated in cancers, with a major proportion altered in glioblastomas (GBM), one of the deadliest forms of brain cancer, and impact on the expression and function of oncogenic and tumor-suppressor proteins. A detailed knowledge of the interactions between RBPs and their cancer-related RNA targets is vital to better understand tumor biology and potentially unveil new targets for anti-cancer therapy.

Among the many unusual RNA conformations, RNA G-quadruplex (RG4) structures are intriguing not only because they possess unique properties and have been implicated in key cellular functions and gene expression mechanisms but also, and more importantly, their dysregulation has been proposed to have a tremendous impact on human diseases, including cancer. RG4s are extremely stable structures formed by stacking of two or more G-quartets, each composed of four guanines interacting via Hoogsteen bonding. RG4 motif hotspots include both 5’ and 3’ untranslated regions (5’UTR and 3’UTR, respectively), suggesting an important role in mRNA translation. Their formation is regulated by intrinsic properties (e.g., the nature of the coordinating ion, the loop sequence and length, the number of G-quartets) and extrinsic interacting factors, with RBPs being critical regulators of RG4 conformation and function in cancer cells. This notion is supported by studies demonstrating the role of RG4-protein interactions on the expression of cancer-relevant genes as well as by affinity proteomic approaches identifying RG4-binding proteins (or RG4-BPs) known to modulate multiple cancer traits. Important insights on the impact of RBPs on RG4 formation have been recently provided by high-throughput RG4 mapping studies which showed that RG4 formation is pervasive in vitro but not in cellulo. This led to propose that RBPs might be critical to maintain RG4s unfolded in eukaryotic cells. However, the notion of global in cellulo unfolding is in contrast with cellular imaging studies showing RG4 formation in cellulo as well as with functional in cellulo analysis of RG4-driven endogenous or reporter gene expression. This view has been recently revisited by in vitro transcription experiments and in cellulo RG4s capturing approaches which provided evidence of transient RG4 formation. These observations, together with other findings suggesting that the rate of protein-RNA complex assembly is faster than RG4 structuration, reinforced the view that RBPs play a major role in shifting RG4s toward an unfolded state, yet the RG4s dynamics and function remain poorly investigated. Recent unbiased affinity proteomic approaches identified several RG4 interactors, including RBPs (e.g., hnRNP H, hnRNP F, FMRP) and RNA helicases (e.g., DDX21, DDX3X, DHX36). However, given that the strategy used in these studies consisted in comparing RBP binding either to folded G4s or to their mutated version (haboring substitutions of the Gs), the question of which RBPs bind the unfolded RG4s and of whether, how and by what extent they impact on post-transcriptional gene expression in cancer cells have not been fully addressed. Answering these questions is essential to gain a better understanding of the role of RBP-RG4 interactions in translational control where RG4s function as strong repressors by different poorly elucidated mechanisms.

Here, we identify hnRNP H and hnRNP F as important components of the cytoplasmic molecular machinery that specifically bind RG4s in their unfolded state. Our findings establish a role for hnRNP H/F as translational regulators acting in synergy with the RNA helicase DHX36 and impacting the biology of GBM. This activity appears to be involved in the resistance mechanisms of GBM, possibly accounting for the failure of current treatments.

Results
Identification of the protein machinery binding to folded or unfolded RG4s. Previous work demonstrated that the canonical RG4 sequence G3A2G3A2G3A2G3 (hereafter referred to as the G3A2) is highly prone to form a RG4 structure in vitro but remained largely unfolded when ectopically expressed in cells. This led to propose that RNA helicases and RBPs unfold RG4s and maintain them in an unfolded state. To identify the protein machinery that recognizes RG4 forming G-rich sequences and modulates their function in mRNA translation, we used an unbiased proteomic approach based on RNA affinity purification of cytoplasmic proteins (refer to Supplementary Fig. 1a for fractionation control) with immobilized biotinylated RNAs followed by mass spectrometry (RP-MS). Unlike other studies using RP-MS to identify proteins bound to wild-type RG4-forming or mutated G-less sequences, we compared affinity enrichment between the G3A2 RNA (G3A2 WT) folded into a RG4 (as described in the Methods section) and its modified version (hereafter referred to as G3A2 7dG) in which replacement of guanines by 7-deaza-guanines prevented Hoogsteen base-pairing and RG4 formation (Supplementary Fig. 1b), as revealed by circular dichroism spectra (Supplementary Fig. 1c). Gel electrophoresis followed by silver staining displayed different complex protein patterns between the native and 7-deaza modified G3A2 RNAs, whereas mock pull-downs with control beads were remarkably clean (Supplementary Fig. 1d). Proteins bound to the G3A2 WT and 7dG RNAs were subjected to tryptic digestion followed by HCD-MS/MS allowing quantitative label free proteomic analysis of RNA-protein interaction data. RG4-BPs (i.e., proteins binding to the G3A2 WT) and G-rich-BPs (i.e., proteins binding to the G3A2 7dG) were defined by the ratio WT/7dG and high confidence proteins (false discovery rate (FDR < 0.05)) were ranked according to an arbitrary 1.5-fold enrichment cutoff after subtraction of the background proteins resulting from non-specific protein binding to the bait RNA sequences (Supplementary Data 1). This quantitative analysis performed with four biological replicates revealed 370 significant G3A2 protein interactors (with 237 proteins found in all replicates), among which we experimentally characterized 328 RG4-BPs and 42 G-rich-BPs. The RP-MS screen (Fig. 1a, Supplementary Data 1) selectively enriched known RG4-BPs, revealed RBPs that have not previously been reported to interact with RG4s and, more importantly, underscored the RBPs that preferentially bind folded or unfolded RG4s. As expected, RNA helicases were found preferentially associated to structured RG4s (Fig. 1a). We compared these results with a recent qualitative RP-MS data set identifying cytoplasmic proteins associated to the RG4 inhibiting NRAS mRNA translation. Of the 370 high-confidence proteins identified in our screen, 27 overlapped with the 80 high-confidence proteins bound to the NRAS RG4, resulting in 343 additional cytoplasmic RG4 binders, of which 320 were assigned to specific functional pathways, including translation and RNA metabolism. In addition, the intersection of our RP-MS and the RNA-binding total proteome (using a compilation of recent RNA interactome capture methods), revealed that 260 out of the 370 identified proteins were annotated as RBPs (Fig. 1b and Supplementary Data 2). It is noteworthy that several recently identified m6A-responsive RBPs (based on ref. 20) were found
among the RG4-binders, highlighting a possible interplay between RG4s and epitranscriptomic modifications of RNAs.

To confirm the RP-MS results and further validate preferential binding to the RG4 sequence or structure, RNA pull-down experiments were repeated by incubating cytoplasmic extracts with RNAs containing the G3A2 WT, the G3A2 7dG or the mutated (G-tract-less or G-less) G3A2 sequence (Mut) (Supplementary Fig. 1b). The recovered proteins, for which the silver stained SDS-PAGE gel displayed distinct protein profiles (Supplementary Fig. 1d), were then probed for some RBPs and

![Diagram](https://example.com/diagram.jpg)

with RNAs containing the G3A2 WT, the G3A2 7dG or the mutated (G-tract-less or G-less) G3A2 sequence (Mut) (Supplementary Fig. 1b). The recovered proteins, for which the silver stained SDS-PAGE gel displayed distinct protein profiles (Supplementary Fig. 1d), were then probed for some RBPs and
RNA helicases found to bind preferentially either the native or the 7dG G3A2 RNAs (Fig. 1d, e). We focused on proteins known to bind RG4s, as for instance DHX34,25, DHX924, DDX3X6, and additional proteins, as KSRP or LARP1, whose function was not associated to RG4 binding. Similar bead-bound RNA elution profiles suggested that the differential interaction was not related to the amount of loaded RNA (Fig. 1d, f). As expected, RG4 helicases recently identified as translational regulators, specifically DHX34, DHX924, DDX3X6, were enriched by pull-down with the G3A2 WT and showed less interaction with the G3A2 7dG or the Mut sequence, corroborating the requirement of a RG4 for RNA binding. Similar results were obtained for the translational regulator LARP1 (Fig. 1d, e), who was also found at the NRAS RNA binding. Similar results were obtained for the translational machinery preferentially binding unfolded RG4s, as for instance DHX34,25, DHX924, DDX3X6, and which ones prefer to bind the G-rich sequence per se.

To bring further insights into the role and mechanism of action of the machinery preferentially binding unfolded RG4s, we focused on two closely (structurally and functionally) related RBPs30–31, hnRNPH and hnRNPF (or hnRNPH/ F), since these factors have been reported to regulate mRNA expression through binding RG4-forming sequences29,32,33 but their role in translation via these motifs or structures has not been investigated yet. As observed for the G3A2, hnRNPH/F binding to the NRAS RG417 depended on RG4 unfolding and the presence of G-stretches (Supplementary Fig. 2). It is interesting to note that the overall binding protein profile was similar between NRAS and G3A2 but differed between cytoplasmic and total extracts (Supplementary Fig. 2b, c). The RG4 structuring-dependency of hnRNPH/F binding was further analyzed by RNA-pull down with RNA baits pre-incubated with either the small-molecule ligand carboxypyridostatin (cPDS) or pyridostatin (PDS) known to stabilize cytoplasmic RG4s24 or RNA/DNA G4s35, respectively. We found that the binding of hnRNPH/F, but not that of the control polypyrimidine tract-binding protein hnRNPI (described in the Methods section), to both the G3A2 RG4 (Fig. 1f and Supplementary Fig. 3a) and NRAS RG4 (Supplementary Fig. 2d) was decreased upon cPDS or PDS treatment. Similar results were obtained by reversed pull-down (i.e., RBP/helicase immunoprecipitation of G3A2 RNAs (WT or 7dG), followed by RNA detection; Supplementary Fig. 3b, c) and surface plasmon resonance (Supplementary Fig. 3d, e), further validating that the binding of hnRNPH/F and helicases depend on RG4 structuration.

hnRNPH/F localization and association with translationally active fractions. The ability of cytoplasmic hnRNPH/F to bind to unfolded RG4s prompted us to study the function and mechanism of action of these interactions in regulating mRNA translation in cancer cells. We focused on high-grade glioma or GBM, highly aggressive, angiogenic and treatment-resistant brain tumors, for the following reasons. First, previous studies showed that RBPs are highly dysregulated in GBM1, with hnRNPH/F being over-expressed both at the protein and mRNA level28,36. Finally, it is well known that mRNA translation dysregulation contributes to GBM progression and response to current therapeutic treatments37,38, yet the molecular mechanisms and therapeutic targets remain to be fully elucidated. To address whether hnRNPH/F drive translational control of genes contributing to GBM progression and treatment, we first evaluated the expression level of hnRNPH/F in tumor and normal tissues from the TCGA database. We found that hnRNPH/F family members displayed higher expression levels in GBM compared to normal brain (Supplementary Fig. 4a), suggesting a potential role for both RBPs in GBM gene expression reprogramming. In addition, data from REMBRANDT (Repository for Molecular Brain Neoplasia Data), a publicly available dataset with information on tumor gene expression, treatment history, and survival, demonstrated that high hnRNPH/F expression is correlated with poor survival (Supplementary Fig. 4b), indicating that hnRNPH/F are likely clinically relevant molecular target in GBM. To gain insight into the role of hnRNPH/F in translation regulation in GBMs, we first addressed their specific localization by subcellular fractionation of three GBM cell lines (U251, LN18 and U87) that differ in their response to chemo- and radiotherapy treatments and in the mutational profiles (Supplementary Fig. 4c). In addition to being present in nuclear fractions, hnRNPH/F co-distributed with proteins associated with active translation (eIF4A) and was enriched in microsomal fractions, containing endoplasmic reticulum-associated proteins (Fig. 2a). This result is consistent with previous findings showing moderate to high cytoplasmic expression for both hnRNPH and hnRNPF, respectively.
Depending on the tissues and on the normal/tumoral status\(^3\). Then, we performed polysomes profiling combined with immunoblotting to monitor the distribution of hnRNP H/F between translational inactive (non-polysomes, NP) and active (polysomes, P) fractions, in the absence or presence of puromycin, a drug that causes ribosome dissociation. We found that hnRNP H/F and other RG4 helicases (e.g., DHX36, DHX9 and DDX3X) co-sedimented with translating polyribosomes and that their association depended on polysome integrity (Fig. 2b). The co-sedimentation profiles observed and their modifications after treatment with puromycin were similar to those of the initiation factor eIF4A. Specifically, the fraction of hnRNP H/F loaded on
Role for hnRNP H/F in translational regulation of DNA damage response genes. To demonstrate a functional role for hnRNP H/F in translational regulation, we transfected U87 or LN18 GBM cells with hnRNP H and/or hnRNP F specific or control siRNAs for 48 h, followed by quantification of global protein synthesis rates by pulse-labeling with puromycin and immunoblotting using an anti-puromycin antibody (i.e., SunSET assay). We found that hnRNP H/F silencing induced only minor reduction of global translation rates (Supplementary Fig. 6a–d). Consistent with this, the polysomal profile was slightly altered by hnRNP H/F depletion (Fig. 3a, Supplementary Fig. 6e), indicating that cells deficient in hnRNP H/F are not globally defective in protein synthesis. Neither apoptosis nor proliferation were affected under these treatment conditions (Supplementary Fig. 6f, g), suggesting that changes in translational efficiency after hnRNP H/F silencing were not directly related to these processes. Based on these results and our previous findings (Figs. 1 and 2), we reasoned that hnRNP H/F might selectively control translation of sub-groups of mRNAs containing RG4-forming sequences. To test this hypothesis, we first mapped RG4-forming sequences within hnRNP H/F-binding regions in 5'UTRs, 3'UTRs and CDSs by combining the bioinformatic prediction of RG4 formation (using QGRS Mapper40) and the reanalysis of previously published cellulo RNA-protein interactions using CLIP-seq (cross-linking immunoprecipitation (CLIP) combined with deep RNA sequencing) data41,42 (Supplementary Fig. 7a). Strikingly, hnRNP H/F bound an important fraction of RG4s over all the RG4s predicted in the transcriptome (11% of 5'UTR, 2.7% of CDS, and 11.4% of 3'UTR) (Fig. 3b). Similar results were obtained by intersecting experimentally validated RG4s (based on11) with hnRNP H/F CLIP-seq data (Supplementary Fig. 7b, c), although the magnitudes of the enrichment were different and reflected the shifted abundance of RG4s identified in the different regions of the mRNA by the rG4-seq method11 (Supplementary Fig. 7b). Overall, these results support the notion of widespread regulation of RG4-containing mRNAs by hnRNP H/F. In addition, RG4s were significantly enriched in the binding regions of hnRNP H/F relative to random control sequences (Fig. 3c, Supplementary Fig. 7d and Supplementary Data 3). Most hnRNP H/F sites in those regions contain a high-scoring RG4-forming sequence (Supplementary Fig. 7e), with hnRNP F sites being less dense but still highly enriched, especially in 5'UTRs (Fig. 3c). These results extend the notion of a physical link between hnRNP F and RG4s (recently investigated in ref. 32) to translational regulatory regions, but most notably underscore the extent of hnRNP H-RG4 interactions, which has not been reported so far. Gene Ontology enrichment analysis showed that hnRNP H and hnRNP F bind RG4-containing RNAs associated with genes regulating cell stress response, including those involved in the response to DNA damage (DDR) (Supplementary Fig. 7f). This result is particularly relevant to GBM since chemo- and radio-resistance of these tumors is largely influenced by the expression of DDR genes43. We then asked whether RG4-containing mRNAs bound by hnRNP H/F were candidates for direct translational control by these factors. To this end, we performed polysomal fractionation of hnRNP H/F-depleted cells followed by RNA isolation from non-polysome (NP), light (LP) and heavy (HP) polysome fractions and RT-qPCR analysis. Based on our bioinformatic analysis (Fig. 3b, c and Supplementary Fig. 7), we selected 5 mRNAs involved in the DDR and/or playing a function in GBM that contained an hnRNP H/F binding site overlapping RG4-forming sequences. Among them, the mRNA encoding VEGF (vascular endothelial growth factor) was chosen as positive control due to its pivotal role in regulating tumor angiogenesis in human gliomas44. Also, the VEGF mRNA is regulated at the translational level by a variety of mechanisms relying on different sequence/structure elements, including RG4s45. Furthermore, we previously demonstrated that RG4 stabilization strongly represses VEGF mRNA translation and protein expression in living cells45. The ability of these mRNAs to form RG4s was validated by performing RNA-immunoprecipitation (RIP) assays with cytoplasmic extracts and the BG4 antibody, known to recognize folded RG4s44. In agreement with the bioinformatic analysis of RG4 formation, we found that these mRNAs were prone to form RG4s in cellulo (Fig. 3d and Supplementary Fig. 8a). The translational efficiency of these mRNAs and the control HPRT mRNA, following hnRNP H/F silencing, was quantified either by analyzing the ratio HP/total RNA (Fig. 3e) or by measuring the distribution of each mRNA across the gradient (Supplementary Fig. 8b). We observed that hnRNP H/F depletion induced a significant modification in mRNAs association with translating polysomes, indicating a role of hnRNP H/F in both translational activation (MECP2 and PRR5) and repression (VEGF, USP1, CCNA2, BAF53A) (Fig. 3e and Supplementary Fig. 8b). Importantly, cPDS cellular treatments over short periods of time (1 h) also modified the translation efficiency of these targets (Fig. 3f), without affecting the mRNA amounts for all except the USP1 mRNA, for which the effect on transcripts levels was reversed compared to the translational effect (Supplementary Fig. 8c). For this target, and in agreement with previous findings obtained with the VEGF mRNA45, we further validated the direct effect of cPDS on RG4-dependent translation using USP1 RNA
reporters and in vitro translation assays (Supplementary Fig. 8d). It is important to note that hnRNP H/F depletion and cPDS treatment resulted in similar positive/negative effects in translation efficiency (Fig. 3e, f) that were consistent with the ability of cPDS to both diminish hnRNP H/F binding to RG4 forming sequences (Fig. 1 and Supplementary Figs. 1–3) and dissociate hnRNP H/F from translating ribosomes (Fig. 2c, d). To further explore the translational regulation mediated by hnRNP H/F bound to RG4 forming sequences, we transfected GBM U87 ((Fig. 3g) or U251, Supplementary Fig. 8e) cells with in vitro-transcribed USP1 RNA reporters containing the RG4 sequence WT (USP1 RG4 WT), 7dG-modified (USP1 RG4 7dG) or mutated (USP1 RG4 Mut). We observed that hnRNP H/F silencing significantly decreased the expression of the USP1 RG4 WT, but also, by a greater extent, that of the USP1 RG4 7dG, while leaving the USP1 RG4 Mut reporter unaffected (Fig. 3g, Supplementary Fig. 8e). Moreover, ligand-induced RG4-stabilization resulted in significant inhibition of the USP1 WT, not the USP1 RG4 7dG, expression (Supplementary Fig. 8f). These functional effects fully mirrored both the efficiency of hnRNP H/F binding to the RG4 RNAs (WT, 7dG, Mut) (Fig. 1d, e, Supplementary Figs. 2 and 3) and the effect of a RG4-stabilizing
ligand on these interactions (Fig. 1f, Supplementary Figs. 2 and 3). The observations that the translation of the USP1 mRNA driven by the unfolded RG4 (7dG-modified) was insensitive to stabilizing ligands (Supplementary Fig. 8f) but much more responsive to hnRNP H/F loss compared to the USP1 RG4 WT (Fig. 3g, Supplementary Fig. 8e) suggest that the dynamic equilibrium between RG4s and linear G-rich sequences in cellulo results in low binding of hnRNP H/F to RG4s but, when preventing RG4 from folding, hnRNP H/F strongly bind the G-rich RNA to potentiate translation. In agreement with the dual role of RG4s in translation, these results also demonstrate that, RG4 stabilization, resulting from either the absence of hnRNP H/F or from the addition of RG4 stabilizing ligands, can either activate or suppress mRNA translation.

Cooperation between hnRNP H/F and DHX36 in translational regulation. We then sought to define the molecular mechanism underlying the function of hnRNP H/F in translation regulation involving RG4 motifs. Previous work speculated on the possibility that hnRNP H/F-RG4 interaction could be facilitated by helicases, specifically DHX36. This unwinding factor has been shown to bind RG4s both in vitro and in cellulo, and to be required for optimal translation of two mixed lineage leukemia proto-oncogenes in synergy with Aven. Furthermore, DHX36 is associated with translating polysomes (Fig. 2b) and regulates mRNA translation by specifically targeting RG4s. To investigate the possibility that hnRNP H/F and DHX36 cooperate to regulate RG4-dependent translation, we first performed co-immunoprecipitation assays using total (TE) or cytoplasmic (CE) extracts from U87 (Fig. 4a) or U251 (Supplementary Fig. 9a) GBM cells, in the presence of RNase and DNase to exclude nucleic acid-mediated interactions. In agreement with previous large-scale protein-protein interaction studies, we found that hnRNP H/F co-immunoprecipitated with DHX36 in both total and cytoplasmic extracts, irrespective of which protein was immunoprecipitated (Fig. 4a and Supplementary Fig. 9a). Unlike DHX36, DHX9 and DDXX were co-immunoprecipitated with hnRNP H/F in total extract, but weakly in the cytoplasmic extract, suggesting the formation of different RBP-helicase-RG4 complexes depending on their subcellular localization. However, neither hnRNP H/F nor DHX36 antibodies precipitated the translation initiation factor eIF4A (Fig. 4a and Supplementary Fig. 9a), recently proposed as an RG4 regulator. To analyze the formation of ribonucleoprotein complexes involving hnRNP H/F, DHX36 and RG4-containing mRNAs, we performed a series of RIP assays using cytoplasmic extracts from U87 cells. In agreement with CLIP-data, we found that the hnRNP H/F antibody immunoprecipitated endogenous mRNAs (Fig. 4b) previously identified as hnRNP H/F translational targets (Fig. 3). Since these mRNAs were also found in DHX36 RIP samples (Fig. 4b), we concluded that hnRNP H/F-DHX36 interactions might be involved in the translation regulation of RG4-containing mRNAs. However, as shown above, even if the two proteins shared similar distribution profiles in polysomes (Fig. 2b) and in microsomes (Fig. 2a and Supplementary Fig. 9b), they display opposite RNA-binding preferences, with hnRNP H/F preferentially associated to unfolded RG4s while DHX36 showing an improved association to structured RG4s (Fig. 1 and Supplementary Data 1). By combining RIP with the depletion of either of these factors, we tested the possibility of a sequential mechanism that would first unfold the RG4s and then keep them unfolded. As shown in Fig. 4c, d and Supplementary Fig. 9c, d, while DHX36 silencing reduced the binding of hnRNP H/F to RG4 targets, the recruitment of DHX36 was not affected by hnRNP H/F depletion, indicating that DHX36 is necessary for hnRNP H/F to bind to RG4s targets but not the opposite. Together, these results suggest that hnRNP H/F is recruited onto G-rich elements through direct interaction with DHX36 once the latter has bound and unfolded RG4s. To further test this model, we verified the in cellulo RG4 structuration after depletion of hnRNP H/F or DHX36 in LN18 (or U251), using the RG4 antibody and the treatment with cPDS as a positive control. For both cell lines, we observed that depletion of either of the two factors induced a similar increase in the BG4 signal, which was RNase-dependent and comparable in magnitude to that previously observed for DHX36 (Fig. 4e, f and Supplementary Fig. 9e). Therefore, hnRNP H/F and DHX36 might cooperate to maintain RG4s in an unfolded conformation, thus facilitating or repressing mRNA translation depending on whether the specific RG4 plays a negative or a positive role in this process, respectively.

Impact of hnRNP H/F-RG4 mediated translational regulation on the DDR. Based on the observation that a sub-group of mRNAs containing RG4 and interacting with hnRNP H/F are associated with stress response (Supplementary Fig. 7d), we hypothesized that the RG4 formation induced by hnRNP H/F silencing or RG4 stabilization (Fig. 4e, f and Supplementary Fig. 9e) could interfere with the cells’ ability to synthesize proteins playing a cytoprotective role, resulting in cellular DNA damage stress. Combined analysis of two markers of genetic instability, γ-H2AX (i.e., phosphorylated H2AX) and 53BP1, by immunofluorescence microscopy revealed that hnRNP H/F removal from LN18 cells induced the appearance of nuclear foci of both factors (Fig. 5a). Consistent with this result, increased phosphorylation of H2AX was observed after treatment of LN18 cells with cPDS.
Ionizing radiation radiotherapy (IR) and temozolomide chemotherapy (TMZ) are part of the standard treatment for GBM patients. The ability of hnRNP H/F to induce DDR markers prompted us to determine whether hnRNP H/F silencing could enhance the cytotoxic effects of IR or TMZ. To this end, we depleted hnRNP H/F in the chemo- and radio-resistant GBM cell line LN18 and either analyzed DNA damage repair by monitoring H2AX phosphorylation or measured the cell survival fraction using clonogenic assays, after treatment with IR (Fig. 5d, e and Supplementary 10a–c) or TMZ (Supplementary Fig. 10d). Our results showed that hnRNP H and/or hnRNP F silencing induced a marked increase in γ-H2AX after exposure to IR (Fig. 5d and Supplementary Fig. 10a, b). Similar results were observed in the presence of the chemotherapeutic agent TMZ (Supplementary Fig. 10d). This reduced ability to cope with genotoxic stress correlated with reduced survival fraction upon IR (Fig. 5e and Supplementary Fig. 10c) or TMZ (Supplementary Fig. 10e). To define whether the LN18 cells’ ability to recover after genotoxic stress is dependent on translational regulation by hnRNP H/F, we analyzed the expression of γ-H2AX after treatment with TMZ, in the presence or absence of ectopically expressed hnRNP H/F and/or the translational inhibitor cycloheximide (CHX). As indicated in Fig. 5f, we observed that γ-H2AX was increased after CHX treatment, indicating that the recovery from TMZ-induced DNA...
damage was dependent on protein synthesis. Overexpression of hnRNP H/F markedly reduced H2AX phosphorylation, suggesting that these factors play a role in the recovery after TMZ-induced DNA damage. The observation that this effect is counteracted by CHX, led us to propose that hnRNP H/F control the cell response to a genotoxic insult by regulating the synthesis of proteins involved in the DDR.

**USP1 translational regulation by hnRNP H/F and DHX36 in GBM cells and tumors.** To strengthen the notion that hnRNP H/F and DHX36 cooperate to regulate the translation of RG4-containing DDR genes, we decided to focus on the mRNA encoding USP1, a ubiquitin peptidase with important functions in DNA repair.\(^{33,34}\) USP1 was chosen for further investigation also because its increased expression in GBM has been associated to resistance to treatments\(^{35}\) by providing a rationale for USP1 inhibition as a potential therapeutic approach against GBM. Furthermore, the USP1 mRNA translational regulation has been recently identified as a major mechanism of cisplatin resistance in non-small-cell lung cancer, yet the molecular mechanisms remain to be investigated.\(^{36}\) We first validated that, as shown for hnRNP H/F (Fig. 3), USP1 is a DHX36 translational target by performing polysomal analysis combined RT-qPCR analysis of the USP1 mRNA. We found that the polysome profile of U87 was only slightly altered by DHX36 depletion (Fig. 6a), in agreement with previous findings reporting a mRNA specific role of this helicase in translational regulation.\(^{34}\) In agreement with polysomal analysis of USP1 mRNA translation regulation by hnRNP H/F (Fig. 3e and Supplementary Fig. 8b) or DHX36 (Fig. 6b and Supplementary Fig. 11a), silencing of either of these factors or treatment with CPDS reduced USP1 protein expression (Fig. 6c–e and Supplementary Fig. 11b), providing further support for a RG4-dependent translational mechanism in which both hnRNP H/F and DHX36 cooperate to activate USP1 protein synthesis. In addition, loss of DHX36 or hnRNP H/F induced an increase in protein ubiquitination, in agreement with USP1 deubiquitinating function (Fig. 6c).

Finally, to investigate the potential clinical importance of our findings, we analyzed the expression of hnRNP H/F, DHX36 and USP1 in human glioma patient tissues. Gliomas are classified into low-grade (LGG) types with slow growth, and high-grade types (HGG) (or GBM), with fast growth and spread into normal brain tissue.\(^{57}\) Analysis of the protein expression of the three factors in four LGG and three GBM human tumor samples revealed that the human RNPs H/F, DHX36 and USP1 were markedly more expressed in GBM compared to LGG. In HGG, the fluctuation in the protein expression of USP1 appeared to correspond to that of hnRNP H/F and DHX36 (Fig. 6f). These results, together with the observation that hnRNP H/F and DHX36 interacted in the cytoplasm (Fig. 4a, Supplementary Fig. 9a), that both factors bound the USP1 mRNA and controlled its protein expression (Figs. 4b–d and 6b–d and Supplementary Fig. 11), strongly support a role for hnRNP H/F and DHX36 in coordinating USP1 expression in GBM.

**Discussion**

Recent data proposed that RG4s tend to massively form in vitro\(^{10,11}\) in accordance with their great stability, but their in cellulo formation was proposed to be highly dynamic due to the presence of a protein machinery that drive them to an unfolded state.\(^{10,13}\) In contrast to previous RP-MS data sets,\(^{5–9}\) we were able to capture and identify proteins binding to folded and unfolded RG4s by comparing native and 7dG-substituted G3A2 RNAs. Of note, incorporation of 7dG was instrumental in the identification of functionally relevant G4s in long RNAs.\(^{58}\)

Our RP-MS screen (Supplementary Data 1) selectively enriched several RNA helicases (e.g., DHX36, DHX9, DDXX, DDX5, DDX17) (Fig. 1), reinforcing the concept of a dynamic equilibrium between the formation and resolution of RG4 structures. Surprisingly, while elf4A, who was previously found to be required for translation of two-quartet RG4-forming (CGG)\(_n\) motifs,\(^{31}\) did not associate with RG4s (as in ref. 6), its cofactor, elf4H, selectively bound the 7dG G3A2 RNA. In agreement with previous reports, elf4H could help to destabilize the RG4 by binding to the newly formed single-stranded region after partial strand structure unfolding by elf4A. However, the observation that hnRNP H/F did not interact with elf4A (Fig. 4a and Supplementary Fig. 9a) and bind G triplets,\(^{69}\) susceptible to structure in three-quartet RG4s, suggests the intriguing possibility that the requirement of a specific helicase-RBP pairs (elf4A-elf4H or DHX36-hnRNP H/F) depends on the number of quartets stacked to form RG4s. The intersection of our RP-MS data with the NRAS RG4-binding cytoplasmic proteome and the RNA-binding total proteome\(^{18–22}\) (Supplementary Data 2) revealed cytoplasmic RBPs whose function was not associated to RG4-binding, including known translation factors, such as LARP1.\(^{26}\) We also identified additional RG4-binding proteins, including known RNA-interactors but also proteins that have not been annotated as RBPs, thus extending the number of proteins binding the RG4-forming G-rich sequences (Fig. 1, Supplementary Data 1 and 2). Future studies will be needed to fully characterize the RG4/G-rich binding proteome in terms of specificity, selectivity, RG4/G-rich topology and mode of binding (direct or indirect).
More importantly, our work underscored the RBPs that preferentially bind unfolded RG4s, which included all hnRNP H/F family members (Supplementary Data 1). This result is consistent with RNA-protein interaction studies using either purified recombinant proteins\(^1\),\(^6\) or extracts from GBM (U87) cells\(^3\) showing that hnRNP H\(^3\) and hnRNP F\(^14\),\(^6\) preferentially binds linear G-tracts. The Drosophila hnRNP H/F homolog, Glorund, also recognizes G-tracts RNA in a single-stranded conformation\(^6\). In contrast, other sets of studies demonstrated that hnRNP H and/or hnRNP F\(^2\),\(^3\) bind RG4s, but not the mutated version, and that the small molecule TMPYP4 modulates this interaction\(^2\),\(^3\). To reconcile this whole set of results, and based on the observation that hnRNP H/F binding is modulated by DHX36 silencing but not the opposite (Fig. 4), we propose a two-step mechanism of binding in which RNA helicases first resolve RG4s and then recruit hnRNP H/F driving their binding to the linear G-rich regions. Thus, our findings refine the model of RBP recruitment by RNA helicases recently proposed\(^4\) by defining the RG4 folding status in the regulatory mechanism. A key question regarding the mechanistic of translational regulation was whether hnRNP H/F simply bind unfolded RG4s or had a function once bound to the linear G-rich regions. The last hypothesis is
supported by our results showing that unfolded RG4s (7dG) still require the presence of hnRNP H/F for their function in translational regulation (Fig. 3g and Supplementary Fig. 8e). While our results suggest that hnRNP H and hnRNP F behave similarly in their interactions (RNA-protein (Fig. 1) or protein-protein (Fig. 4)) and function (Fig. 3) (as previously reported\[^{29,30}\]), recent data showing that the two factors do not fully share the same set of protein interactors\[^{30}\], raise important questions about the possibility of differential translational effects discernable at the level of individual mRNAs or in specific translational compartments (cytosol versus microsomes). Finally, DHX36 and DHX9 were shown to stimulate mRNA translation by unfolding RG4s at upstream open reading frames (uORFs)\[^{24}\]. These results together with our findings support interesting future investigations to determine whether hnRNP H/F are involved in this regulatory mechanism.

In addition to highlighting the possibility that this mechanism may be important for splicing\[^{32}\] or polyadenylation\[^{29,46}\], our study extends the functions of hnRNP H/F to translational regulation and assigns to this mechanism a key role in the regulation of genes involved in resistance to treatments in GBM (Fig. 6). Although further work is needed to understand and characterize the full hnRNP H/F translome, we found that RG4s are over-represented in hnRNP H/F-binding sites at translational regulatory regions of mRNAs involved in pathways associated to genome instability and DNA damage and that hnRNP H/F bind an important fraction of predicted (Fig. 3b) or experimentally validated RG4s (based on ref. \[^{11}\]) (Supplementary Fig. 7c). Therefore, we predict that hnRNP H/F drive a substantial part of the RG4-dependent translational regulation and impact on the maintenance of genome integrity. In line with this view, RG4 stabilization by hnRNP H/F silencing or treatment with cPDS, induced the expression of markers of genome instability (Fig. 5). Although it could not be excluded that these effects are associated with the nuclear functions of hnRNP H/F\[^{29,46}\], we provided evidence that the link between hnRNP H/F and genome stability depends in part on mRNA translational regulation (Fig. 5). Moreover, hnRNP H/F inhibition not only induced but also enhanced chemo- and radio-therapy-induced DNA damage correlated with reduced cell survival (Fig. 5, Supplementary Fig. 10), indicating that targeting the RG4-dependent and hnRNP H/F-sensitive regulatory mechanism sensitizes cancer cells to treatments currently used to treat GBM patients (Fig. 7). Mining GBM TCGA and REMBRANDT data sets (Supplementary Fig. 4) as well as analyzing the protein expression in human glioma protein samples (Fig. 6), we found that hnRNP H/F is increased in GBM and correlates with poor survival, extending the notion of a key role of hnRNP H/F family members in cancer development and progression\[^{63}\]. Our results support a model (Fig. 7) in which hnRNP H/F overexpression in GBM coordinately regulate the translation of RG4-containing mRNAs encoding proteins involved in maintaining genome stability and in the response to genotoxic damage. The observation that 74 mRNAs coding for stress response factors are targeted by both hnRNP H/F and DHX36 (Supplementary Fig. 12) opens up new avenues for future research to investigate whether and how these regulations induce adaptive changes crucial for tumor cell survival during treatment and the development of resistance. Our results not only extend the notion of a link between G4 and genomic instability\[^{64}\] to mRNA translational regulation but also associates it with a role in resistance to treatments in GBM. Given that 1) our results were similar regardless of the GBM cell line (e.g., Fig. 2a, c, d and Supplementary Fig. 5b; Fig. 3g and Supplementary Fig. 8e, Fig. 5d and Supplementary Fig. 10b) or the cancer cell-type (Supplementary Fig. 13), 2) hnRNP H/F were found to be deregulated in many tumors (Supplementary Fig. 14), and 3) hnRNP H/F RG4-containing mRNA targets significantly enriched genes associated to GBM (adjusted \(P_{\text{value}} = 0.03284\) and 0.001729 for H and F targets, respectively) but also to other cancers, including breast (adjusted \(P_{\text{value}} = 0.033\) and 1.2E-06) and ovarian cancers (adjusted \(P_{\text{value}} = 0.013\) and 1.8E-05), we propose that the link between hnRNP H/F and cancer mediated by RG4-dependent translational regulation could apply to other cancer cells and tumors, thus making hnRNP H/F a potential target for therapeutic intervention.

Overall, our results support the notion that hnRNP H/F are an essential regulatory hub in GBM networks that drives translational control of RG4-containing genes contributing to GBM progression and response to treatments. Moreover, our RP-MS screen raises interesting future investigations to determine how modulation of RG4 structural integrity impacts cellular functions related to cancer hallmarks.

Methods

\section*{Cell culture and treatment.} Glioblastoma cells (LN18, ATCC CRL-2610; U251-MG ECACC #09063001 U87, SIGMA, #89081-402-1VL) were grown in DMEM media (4.5 g/l glucose) supplemented with 10% FBS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were tested for mycoplasma contamination by PCR. Cells were incubated/exposed at 37 °C with: 20 µM PDS (SelecTech S7444) or 20 µM cPDS (Sigma-Aldrich SML1176); or 10 µM PhenDC3 (Polysciences, #26000-1) for the indicated time, 100 µg/ml Puromycin (Sigma P8933) for 1 h, 500 µM or dose scale of TMZ for 24 h, 4 Gy or dose scale of γ-irradiation (Gammacell 40 Exactor).

\section*{Cell transfection.} siRNAs were transfected using the Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer’s instructions. In brief, cells were reverse-transfected with 2.5 nM siRNA for 48 h. siRNA oligonucleotides Control (5′-GGUGUCCCATCCCAAGUG-dTdT-3′), against hnRNP H (5′-GGUAUUUGGUCAUUUCAUG dTdT-3′), hnRNP F (5′-GG-GUGUCCGAAUUCAUG dTdT-3′) and DHX36 (5′-GGUGUGUGGAAUAUGUAA dTdT-3′)
2000 reagent according to the manufacturer. 250 ng of reporter mRNA was transfected in 48-well plates using lipofectamine hypotonic lysis buffer (10 mM Tris pH 8.0, 1.5 mM MgCl2, 10 mM NaCl, 1 mM DTT). Cells were subsequently incubated at 37 °C for 48 h or 16 h following DNA plasmid or mRNA reporter transfections respectively, before harvesting and analysis.

**Cell fractionation.** For cell fractionation, cells were gently resuspended in 500 µl of hypotonic lysis buffer (10 mM Tris pH 8.0, 1.5 mM MgCl2, 10 mM NaCl, 1 mM DTT) and vortexed for 4 s. After centrifugation at 1000 g (4 °C) for 5 min, supernatant (cytosolic fraction) was recovered. Pellet fraction (washed twice with buffer A) was resuspended in 500 µl of lysis buffer A (10 mM Tris pH 8.0, 140 mM NaCl, 1.5 mM MgCl2, 0.5% NP40, 1 mM DTT). The supernatant (nuclear fraction) was transferred into a fresh tube. Pellet-nuclear fraction (washed twice and resuspended in 500 µl of lysis buffer A) was transferred to a 5-ml round-bottom tube and 50 µl of detergent mix (3.3% (w/v) sodium deoxycholate, 6.6% (v/v) Tween 40) were added. After incubation on ice for 5 min, the supernatant-detergent mix (S-TRAP) was incubated at 37 °C for 30 min, supernatant (perinuclear fraction). The pellet-nuclear fraction (washed with buffer A) was resuspended in 500 µl of lysis buffer A supplemented with 0.1% SDS and sonicated. After centrifugation at 1000 g (4 °C) for 5 min, supernatant (nuclear fraction) was transferred into a fresh tube.

**Mass spectrometry.** Proteins were lysed and denatured in Tris 50 mM pH 8.5 and SDS 2% while disulfide bridges were reduced using TCEP 10 mM and subsequent free thiols groups were protected using chloroacetamide 50 mM for 5 min at 95 °C. Proteins were trypsin-digested overnight using the suspension trapping (S-TRAP) method to collect peptides as described in66. Eluted peptides were vacuum-dried while centrifuged in a Speed Vac (Eppendorf). C18 liquid nanochromatography and Mass Spectrometry (MS) nanoflowHPLC and MS analyses were performed at
the 3P5 proteomics facility (University de Paris) using an U3000 RSLC system hyphenated to an Orbitrap fusion MS (all from Thermo Fisher Scientific). All mobile phases are made with milliQ-grade H2O produced with a milliQ integral-3 polarimeter equipped with a temperature controller. CD spectra ranging from 190 to 250 nm were collected at room temperature.

**In vitro transcription.** RNAs used in RNA chromatography experiments were transcribed using the MEGAscript Kit (Invitrogen AM1333) as per manufacturer’s instructions. 7.5 mM ATP/UTP, 6.75 mM GTP/UTP (Biotin-14-UTP, Lucigen BU1053H) and either 7.5 mM GTP or 6.75 mM 7-deazaguanine (TriLink N-1044) plus 0.75 mM GTP was used. For luciferase reporter RNAs, m7G-cap was added using the Vaccinia capping system (M208S NEB) kit according to the manufacturer’s instructions. To generate the DNA templates to synthetize the luciferase reporter RNAs, oligonucleotides G3A2 WT, G3A2 Mut, G3A2 Mem were annealed and cloned in the pScoB-ampK plasmid from the Stratagene Blunt PCR cloning kit, then digested by NheI and purified. All oligonucleotide sequences are available in the Supplementary Table 1. RNA concentration was determined using the Claristart BM and software v.5.21 R4, Labtech and MARS Claristor Analysis Software v.3.20 R2.

**CD spectroscopy.** For the spectroscopy measurements, RNAs were prepared in buffers containing 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA in the presence of 100 mM KCl and annealed by heating to 95 °C and then cooling slowly to room temperature. CD of RNAs was determined at 20 °C by a Jasco J-815 spectropolarimeter equipped with a temperature controller. CD spectra ranging from 190 to 350 nm were recorded in a 1-mm path length cuvette, in triplicates, averaged and buffer subtracted.

**Silver staining.** Proteins co-purified by RNA chromatography or present in whole-cell lysates (inputs) were separated by SDS-PAGE and subjected to silver staining using Pierce Silver Stain Kit (Thermo Scientific, 24612) according to manufacturer’s instructions.

**Reversal affinity chromatography.** For RG4 formation, in vitro-transcribed biotinylated RNAs were heated to 95 °C for 5 min in one volume of 1 x phosphate-buffered saline supplemented with 2 M KCl and cooled down at room temperature in presence or absence of cPDS 10 µM. hrhNP H/F or DHX36 were immunoprecipitated overnight as described in "immunoprecipitation" of the Methods section. Beads were then washed three times in wash buffer (20 mM Tris HCl pH 8, 100 mM KCl, 0.5% NP-40, 0.4 mM EDTA, 1 mM DTT) and incubated with the in vitro-transcribed biotinylated RNAs in 300 µl of binding buffer (20 mM Tris pH 8, 0.5% NP-40, 1 mM DTT, 100 mM KCl, 0.2 mM EDTA) for 1 h or 2 h when RNAs were incubated with cPDS. After five washes in binding buffer, beads were resuspended in 60 µl of elution buffer (50 mM Tris pH8, 1% SDS), and boiled for 10 min. Immunoprecipitated proteins were analyzed by western blot and biotinylated RNAs were analyzed by urea PAGE followed by biotin detection (as described in "Biotinylated RNA detection section").

**Surface plasmon resonance.** All binding studies based on surface plasmon resonance technology were performed on BIACore T200 optical biosensor instrument (GE Healthcare) at 4 °C. Capture of the different biotinylated RNA (WT or 7dG) was performed on a Streptavidin (SA) sensorchip in HBS-EP + buffer
lysates were injected (Biotech) according to the manufacturer (Thermo Scientific H8901) or with 0.1 mg/ml cycloheximide (CHX) in PBS/CHX. Cells were then incubated for approximately 10 days until colonies were formed. For the detection of Grs, cells were seeded in 96-well plates coated with poly-D-lysine solution. 48 h post seeding cells were fixed with 100 µg/ml over the immobilized surface for 2 min at a flow rate of 30 ml/min. Thereafter, the hNRNP II/F antibody was injected at a concentration of 200 ng/ml and with 72-4 + nuclease setting (Amersham Biosciences, RPN203B). After cross-link under UV light (UV Stratalinker 1800), signals were probed using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific, 89880) according to the manufacturer’s instructions.

**RT-qPCR**. Reverse transcription (RT) was performed on 1 µg total RNA (quantified with the SybrGreen (KAPA KK4605) using the StepOne software v2.2.2 (Applied Biosystems)). Expression of MEC2, PRR5, VEGF, USP1, BABAM1, CCNA2 was determined by RT-qPCR according to the manufacturer’s protocol. 12.5 ng of cDNA was analyzed by qPCR for 1 h at 4 °C. The coverslips were then incubated with primary antibodies in 1% normal goat serum/PBS at room temperature for 1 h using antibodies against γ-H2AX (BD301 Millipore 05-636; 1:500) and 53BP1 (Cell Signaling 2675; 1:200). The coverslips were washed twice for 10 min and incubated with goat anti-mouse IgG secondary antibody coupled to fluorescein isothiocyanate in 1% normal goat serum/PBS at room temperature for 1 h. The samples were then washed three times for 10 min each and mounted. For the detection of Grs, cells were seeded in 96-well plates coated with poly-D-lysine solution. 48 h post seeding cells were fixed with 0.1% DMEM and 50% methanol/acetic acid (3:1) at RT for 5 min. After a brief wash with methanol/acetic acid (3:1), cells were fixed with methanol/acetic acid (3:1) at RT for 10 min. Cells were then permeabilized with 0.1% Triton X-100 in PBS at RT for 3 min. For RNase treatment, coverslips were incubated with 100 µg/ml RNase A in PBS for 1 h at 37 °C. Cells were incubated with blocking solution (2% milk in PBS, pH 7.4) for 1 h at RT and then with 1 µg per slide of BG4 in blocking solution (2 h at RT). Cell nuclei were then incubated with 1:800 of a rabbit antibody against the Flag epitope (Cell Signaling ref# 2368) in blocking solution for 1 h. Next, cells were incubated with 1:800 Alexa Fluor 488 goat anti-rabbit IgG (Life technologies A-11001) in blocking solution in 1 h and with DAPI for 10 min. After each step, cells were washed three times for 10 min with 0.1% Tween-20 in PBS under gentle rocking. Cells were visualized at room temperature by using a confocal microscope (Zeiss, LSM780) or using the high-content Operetta High-Content Imaging System (Harmony Imaging 4.8; PerkinElmer). For the high-content analysis of cytoplasmic foci detection and subsequent analyses were performed with Columbus 2.8.2 software (PerkinElmer).

**Plating efficiency**, clonogenic assay. LN18 glioblastoma cells were transfected with siRNA (siCtrl or sifl), after twenty-four hours, cells were harvested and plated in 6-well plates at different concentration (500, 750, 1000 cells/well for siCtrl and 1500, 2500, 5000 for sifl) in duplicate. Twenty-four hours later cells were irradiated with an ionizing radiation dose (from 0 to 4 Gy) using the GammaCell 40 Exactor irradiator (Nordion, Ottawa, Canada) or with TMZ dose scale (from 100 to 500 µM). Cells were then incubated for approximately 10 days until colonies were visible with the naked eye without any joining between colonies. Then, plates were washed and cells were fixed with 10% formalin for 10 min, the formalin was removed and cells were covered with 10% crystal violet oxalate (Rl Diagnostics, Montreal, Canada) for 10 min. Then, the fixed target reaches a fixed threshold) between 10 and 100.

**GBM tumour sample**. Total protein lysates in RIPA Buffer (Sigma) (30 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.5 mM sodium fluoride, 0.5 mM sodium vanadate, and 1x protease inhibitor cocktail (Roche)) were extracted from 3 GBM (grade IV), and 4 Diffuse Low Grade Gliomas (Grade II: 2 astrocytomas and oligodendrogliomas 2). Tumors samples were obtained from the Montpellier hospital ("biological resource centre", (Collection NEUROLOGIE, DC-2013-2027/DC-2010-1185 /Authorization sher)) or Hybond-N RT (1:1000, Santa Cruz sc-233233), H2AZ (1:1000, Millipore E1014), FAM162-2MG (1:1000, ProteinTech), Ubiquitin (1:1000, Cell Signaling Technology 3936), Puromycin (1:1000, Millipore, MABE343), PARP (1:1000, Cell signaling 9542), Caspase-3 (1:1000, Cell signaling Technology 3192), Histone H3 (1:1000, Cell Signaling Technology 4499), EEA1 (1:500, Santa Cruz sc-35399), RP56 (1:1000, Santa Cruz sc-74495), RPL22 (1:1000, Novus Bio NBPI-06069), GAPDH (1:1000, Santa Cruz sc-23233), H2A.Z (1:1000, Millipore sc-53399), FAM162-2MG (1:1000, ProteinTech), Ubiquitin (1:1000, Cell Signaling Technology 3936), Puromycin (1:1000, Millipore, MABE343), PARP (1:1000, Cell signaling 9542), Caspase-3 (1:1000, Cell signaling 8G10), Anti-Rabbit IgG (1:5000, Ozyme 7074S), Anti-Mouse IgG (1:5000, Ozyme 7076S). The blots were developed using the ECL system (Amersham Pharmacia Biotech) according to the manufacturer’s directions.

**Polysomes.** Around 3.107 cells were treated with 0.1 mg/ml cycloheximide (CHX) for 15 min at 37 °C, washed twice with ice-cold phosphate-buffered saline supplemented with 0.1 mg/ml CHX (PBS/CHX), and scraped on ice in PBS/CHX. After centrifugation for 5 min at 200 g, the cell pellet was gently resuspended in 450 µl of hypotonic lysis buffer (5 mM Tris pH 7.5, 1.5 mM KCl, 1.5 mM MgCl2, 20 mM HEPES pH 7.6, 1 M KCl, 50 mM MgCl2). After 20 OD260 nm was layered on a 11.3 ml continuous sucrose gradient (5-50% sucrose density gradient fractionation system (Foxy Jr fraction collector coupled to UA-6UV detector, Lincoln, NE). The settings were as follows: fraction time, 62 s/ fraction; speed, 60 cm/h; sensitivity of the OD254 recorder, 0.5. The absorbance at 254 nm was measured continuously as a function of gradient depth; 16 fractions of approximately 0.8 ml were collected. The fractions recovered from the gradient were then incubated with 80 µg/ml cycloheximide (Sigma P8833) for 10 min at 37 °C. Cells were washed twice in ice-cold PBS, scraped on ice in PBS and collected by centrifugation at 200 g for 5 min. Cell lysates were made in 50 µM HEPES pH 7.6, 150 mM NaCl, 10% Glycerol, 1% Triton, 100 mM Na2PO4, 100 mM NaF, 1 µM EDTA, 1 mM MgCl2, and with 0.02% Protease Inhibitor Cocktail (Sigma, P8340) and protein concentration was determined by the Lowry method.
