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The HNRNPF/H RNA binding proteins and disease

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Abstract

The members of the HNRNPF/H family of heterogeneous nuclear RNA proteins – HNRNPF, HNRNPH1, HNRNPH2, HNRNPH3, and GRSF1, are critical regulators of RNA maturation. Documented functions of these proteins include regulating splicing, particularly alternative splicing, 5'capping and 3' polyadenylation of RNAs, and RNA export. The assignment of these proteins to the HNRNPF/H protein family members relates to differences in the amino acid composition of their RNA recognition motifs, which differ from those of other RNA binding proteins (RBPs). HNRNPF/H proteins typically bind RNA sequences enriched with guanine (G) residues, including sequences that, in the presence of a cation, have the potential to form higher-order G-quadruplex structures. The need to further investigate members of the HNRNPF/H family of RBPs has intensified with the recent descriptions of their involvement in several disease states, including the pediatric tumor Ewing sarcoma and the hematological malignancy mantle cell lymphoma; newly described groups of developmental syndromes; and neuronal-related disorders, including addictive behavior. Here, to foster the study of the HNRNPF/H family of RBPs, we discuss features of the genes encoding these proteins, their structures and functions, and emerging contributions to disease.

Graphical Abstract

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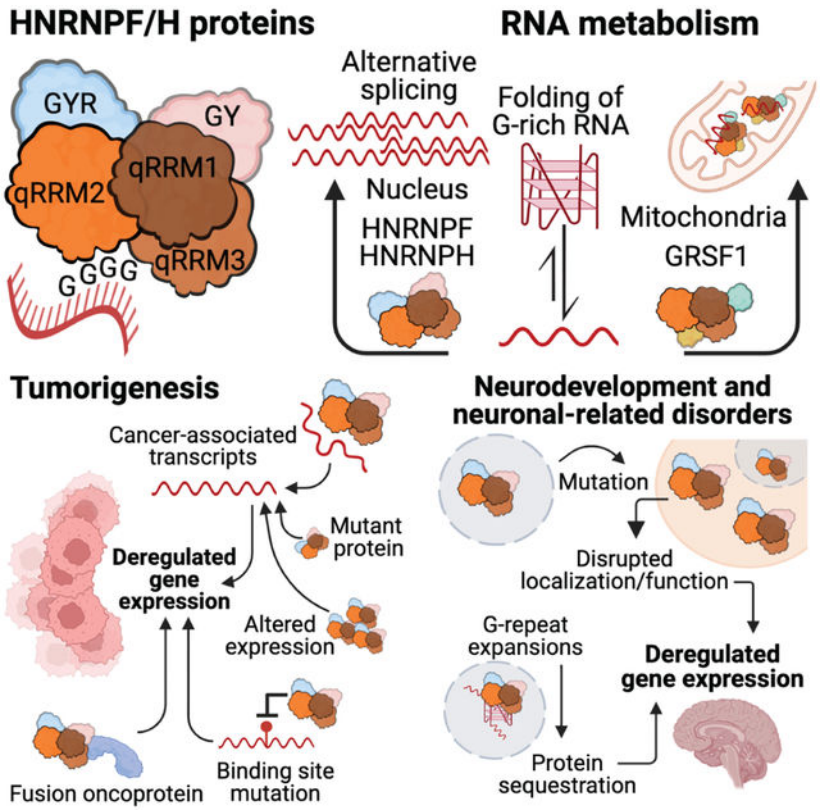
Author Contributions

Tayvia Brownmiller: Writing – original draft; writing – review and editing. **Natasha Caplen:** Conceptualization and funding (lead); writing – original draft (lead); writing – review and editing (lead); visualization (lead).

Conflict of Interest

The authors have declared no conflicts of interest for this article.



The HNRNPF/H RNA binding proteins are regulators of RNA maturation, particularly alternative splicing. Recent studies have highlighted the discovery of mutations in genes encoding members of the *HNRNPF/H* protein family in specific diseases, their function in regulating the expression of disease-specific transcripts, and the effect of sequence variants in their binding sites on the regulation of gene expression. Figure created with BioRender.com.

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The contributions of the HNRNPF/H family of RNA binding proteins to cancer and other diseases highlight their importance in regulating RNA maturation.

Keywords

HNRNPF/H RNA binding proteins; cancer; alternative splicing; neurodevelopment; HNRNPF; HNRNPH1; HNRNPH2; HNRNPH3; GRSF1

This article is categorized under:

RNA Processing > Splicing Regulation/Alternative Splicing; RNA in Disease; RNA interactions with proteins and other Molecules > Protein-RNA Interactions: Functional Implications

1 INTRODUCTION

The HNRNPF/H family of heterogeneous nuclear RNA proteins (hnRNPs) comprises five proteins, HNRNPF, HNRNPH1, HNRNPH2, HNRNPH3, and GRSF1. First identified and characterized in the late 1980s through the early to mid-1990s, these homologous RNA binding proteins (RBPs) lack the conserved aromatic amino acid residues in their RNA recognition motifs (RRMs) typical of most hnRNPs. This observation led to their designation as proteins containing quasi-RNA recognition motifs (qRRMs) (Honoré et al., 1995). Structural and functional studies have highlighted HNRNPF/H proteins binding of guanine (G)-rich sequences and how these interactions may alter RNA secondary structures. Studies of these RNA-protein interactions, through the generation of transcriptome-wide datasets and analysis of gene-specific events, have revealed the importance of the HNRNPF/H proteins' function in regulating RNA processing, particularly alternative splicing. This review begins with summaries of key features of the HNRNPF/H genes and proteins. We then discuss the contributions of this family of RBPs to specific cancer sub-types and neurodevelopmental syndromes, other disease states, and the aging process. We aim to stimulate further study of the functions of the HNRNPF/H proteins to inform our understanding of the mechanism underlying these conditions and aid the development of new treatments based on these findings.

2 THE HNRNPF/H RNA BINDING PROTEINS

Dreyfuss and colleagues first described HNRNPF and what is now known as HNRNPH1 (formally HNRNPH) in a series of studies detailing their amino acid sequence and their binding of poly-G RNA (Matunis et al., 1994; Piñol-Roma et al., 1988; Swanson & Dreyfuss, 1988). In parallel and subsequently, Wilusz and co-workers identified GRSF-1, currently referred to as GRSF1 (Qian & Wilusz, 1994), and described a protein highly homologous to HNRNPH1 initially named DSEF-1 or HNRNPH' and now designated as HNRNPH2 (Bagga et al., 1998; Bagga et al., 1995; Qian & Wilusz, 1991). HNRNPF, HNRNPH1, HNRNPH2, and GRSF1 have three qRRM domains but exhibit different cellular localizations. The HNRNPF, HNRNPH1, and HNRNPH2 proteins concentrate in the nucleoplasm, while the predominant isoform of GRSF1 (Isoform 1) localizes to mitochondria (Antonicka et al., 2013; Jourdain et al., 2013). The family member, HNRNPH3 (previously known as HNRNP2H9), has different N and C-termini; however, its two qRRM domains share significant sequence homology with the qRRM2 and qRRM3 domains of the other HNRNPF/H proteins (Mahé et al., 1997). Figure 1 summarizes the domain structure of the HNRNPF/H proteins (Figure 1A) and their sequence alignments (Figure 1B).

2.1 The *HNRNPF/H* genes and gene expression

The human *HNRNPF/H* genes map to four human chromosomes (Banga et al., 1996; Honoré et al., 1995). The *HNRNPF* and *HNRNPH3* genes map to chromosome 10, while *HNRNPH1* maps to chromosome 5, *HNRNPH2* to chromosome X, and *GRSF1* to chromosome 4 (Figure 2A). Bulk RNA sequencing of various human tissues (GTEx Portal: https://gtexportal.org/home/) demonstrates the ubiquitous expression of the human

HNRNPF/H gene family members with some variation in the expression levels detected in different tissues, particularly in brain-derived tissues (Figure 2B). The overall expression of *HNRNPF* and *HNRNPH1* is higher than that of other family members, with *GRSF1* RNA levels demonstrating the lowest abundance. Interestingly, the transcript variants expressed by the HNRNPF/H genes (Figure 3) are more distinctive and complex than the gene-level expression data, or the amino acid homology of these proteins (Figure 1B) may suggest. The *HNRNPF* gene (ENSG00000169813) expresses several protein-encoding transcript variants with different 5' untranslated regions (Figure 3A), each of which encodes the same 415 amino acid protein (UniProt – P52597; ~55 kDa) (Figure 1B). In contrast, the Ensembl genome database reports ~50 annotated transcript variants expressed by the human *HNRNPH1* gene (ENSG00000169045), including the subset of transcripts (coding and non-coding) detailed in Figure 3B. The dominant full-length *HNRNPH1* transcripts ENST00000393432/NM_001257293 and ENST00000442819/NM_005520 and another variant (ENST00000356731) encode the same 449 amino acid protein (UniProt – P31943; ~50–55 kDa) while additional transcripts have the potential to encode small peptides (<200 amino acids), as well as putative protein isoforms of 212 to 472 amino acids. Interestingly, a substantial proportion of transcription from the *HNRNPH1* locus results in the expression of transcripts that lack coding potential. The functional significance of the expression of non-dominant HNRNPH1 isoforms and *HNRNPH1* non-coding transcripts is unclear. However, as discussed below (3.2), a study of cancer-associated mutations suggests that the ratio of the dominant protein-coding transcripts and other transcripts may regulate the expression of full-length HNRNPH1 protein (Pararajalingam et al., 2020). Interestingly, though the HNRNPH1 and HNRNPH2 proteins are over 90% homologous, the organization of the genes encoding these proteins differs substantially. Specifically, unlike the multi-exon *HNRNPH1* gene, the *HNRNPH2* locus expresses a single two-exon transcript (Figure 3C) that encodes the HNRNPH2 449 amino acid protein (UniProt – P55795; ~50–55 kDa). *HNRNPH3* (Figure 3D) and *GRSF1* (Figure 3E) express several transcript variants. Two *HNRNPH3* variants (ENST00000265866 and ENST00000354695) encode proteins of 346 (UniProt – P31942; ~ 37 kDa) and 331 amino acids, respectively, while the longest *GRSF1* transcript variant encodes the mitochondrial-localized GRSF1 isoform 1 (480 amino acids, UniProt – Q12849; ~55 kDa).

2.2 HNRNPF/H protein stucture

The HNRNPF/H proteins' structural similarities include their qRRM domains and two other domains, a glycine-tyrosine-arginine-rich (GYR) domain and in the case of HNRNPH1, HNRNPH2, and HNRNPH3, a C-terminal glycine-rich (GY) domain (Figure 1A). HNRNPH1 and HNRNPH2 are over 90% homologous, differing by only nine residues, with most in the qRRM1 domains of each protein (five amino acid differences) and other differences in the other qRRM domains (one in the qRRM2 and two in the qRRM3 domains) and one in the GYR region (Figure 1B). The similarity of the HNRNPH1 and HNRNPH2 proteins means that in some cases, experimentation cannot definitively determine whether one protein or the other, or both, are responsible for a given result. Therefore, in this review, when discussing studies that could not report HNRNPH1 or HNRNPH2-specific results, we will use the designation HNRNPH. HNRNPF and HNRNPH1 or HNRNPH2 are about 75% homologous, with amino acid differences found

throughout the HNRNPF protein but particularly in the C-terminus region (Figure 1B). The family member, HNRNPH3, has different N and C-termini; however, its two qRRM domains share significant sequence homology with the qRRM 2 and 3 domains of the other HRNPF/H proteins (Figure 1B).

The qRRM domains of the HNRNPF/H proteins define their recognition and interactions with RNA (Honoré et al., 1995; Matunis et al., 1994) and their relative locations and sequences are detailed in Figure 1B. Honoré and colleagues first referred to the RNA recognition motifs of the HNRNPF/H family members as quasi-RRMs because their amino acid composition differed from those of the consensus RRM sequences, lacking positively charged and aromatic residues (Honoré et al., 1995). However, subsequently, studies showed that the qRRM domains adopt classical RRM folds. Specifically, analysis of the HNRNPF protein demonstrated that in the presence of RNA containing three consecutive guanines, each qRRM domain forms a β/α, β/β, α/β structural motif (Dominguez & Allain, 2006; Dominguez et al., 2010). These studies also defined the conserved amino acids within the qRRMs of the HNRNPF/H1/H2 proteins that contact RNA (Dominguez et al., 2010). Specifically, Dominguez and colleagues described a four amino acid motif (RGLP) followed by either W, F, or Y towards the beginning of the domain and an R or L in the middle as critical residues that contact RNA. Towards the end of the domain, conserved sequences in contact with RNA consist of an R or G residue followed by five amino acids and a motif consisting of A, T, I/V, E, V/L, and F (see Figure 1B for the location of each of these motifs). Structural studies of HNRNPH indicate a similar organization of their qRRM domains and that the first two qRRMs of HNRNPH1 can form compact and extended conformations (Penumutchu et al., 2018). Interestingly, a critical determinant of these conformational dynamics is a single amino acid between the qRRM1 and qRRM2 domains – an alanine in HNRNPF and a proline in the HNRNPH proteins (Penumutchu et al., 2018). The alanine residue present in HNRNPF favors the protein forming an extended state that has the potential to facilitate interactions with multiple G-tracts. In contrast, the proline present in HNRNPH confers a more compact state that facilitates interaction with a single G-tract (Penumutchu et al., 2018).

While the qRRM domains have defined structures, the GYR and GY portions of the HNRNPF/H proteins have the repetitive amino acid composition typical of low-complexity domains (LCDs). Many recent studies have LCDs facilitate the multivalent interactions regulating many dynamic processes, including gene expression (reviewed in Alberti & Hyman, 2021; Bhat et al., 2021; Sharp et al., 2022). One assayable property of proteins containing putative LCDs assesses their propensity to form a gel-like state. For example, recent *in vitro* studies demonstrated that the purified GYR domain region of HNRNPH1 can form a hydrogel detectable by confocal microscopy that transmission electron microscopy revealed consists of polymer-like fibers (Kim & Kwon, 2021). A subsequent analysis presented in the same study highlighted that the GYR domain could interact with other RBPs containing similar LCDs, including HNRNPF, and the importance of three tyrosine residues – Y236, Y240, and Y243 – within this domain as critical determinants of the interaction with other LCD containing proteins. Interestingly, Kim and Kwon observed that the GY domain of HNRNPH1 did not form hydrogel droplets. However, they did observe

using a transgene reporter assay system that this domain can activate transcription, the potential significance of which we will discuss in sections 3.3 and 4.1.

Close to and overlapping with the GYR domains, HNRNPF, HNRNPH1, HNRNPH2, and HNRNPH3 are motifs associated with nuclear localization. Specifically, a region that includes a conserved motif recognized by the karyopherin receptor complex and associated with the nuclear transport of HNRNPF - YSDPP (Lee et al., 2006; Siomi et al., 1997) (YDPP in HNRNPH1, HNRNPH2, and HNRNPH3) and a region adjacent to this motif (amino acids 205 – 213 of HNRNPF, HNRNPH1, and HNRNPH2) (Van Dusen et al., 2010) (Figure 1B). Results supporting the importance of this latter region in the nuclear localization of HNRNPF/H proteins include studies showing that deletion or single amino acid substitutions affecting the highly conserved QRPGPYYDRP sequence results in a shift from a nuclear-only localization to nuclear and cytoplasmic localizations. As discussed below (3.2.2 and 4.1), this observation has proven particularly critical in assessing the potential effect of recurrent mutations in several *HNRNPF/H* genes.

GRSF1 is the only member of the HNRNPF/H family to contain an alanine-rich (A-rich) domain, which is present in the N-terminus of the protein's longest isoform (480 amino acids), and it also has an acidic (glutamate and proline-rich) region between its qRRM2 and qRRM3 domains (Figure 1B). The mitochondrial location of GRSF1's longest isoform, which contains the A-rich domain, suggests that this may contribute to this cellular localization (Antonicka et al., 2013; Jourdain et al., 2013). These and a subsequent study that observes a GRSF1 isoform lacking the A-rich domain concentrates in the cytoplasm support this hypothesis (Sofi et al., 2018). The function of the acidic region is undefined; however, one study has suggested that it may function as a negative regulator of RNA binding (Sofi et al., 2018).

2.3 HNRNPF/H protein functions

The regulation of many facets of RNA metabolism, including splice site selection and polyadenylation, involves the interaction of RNPs with G-tracts (3 or more consecutive guanines); (Huppert et al., 2008; McCullough & Berget, 1997; Wang et al., 2004; Xiao et al., 2009; Yeo et al., 2004; Zarudnaya et al., 2003). Multiple studies, including transcriptome-wide analysis, have demonstrated the enriched presence of G-tracts within the binding sites of the HNRNPF/H proteins (for example, (Caputi & Zahler, 2001; Huelga et al., 2012; Uren et al., 2016) and ENCODE RNA Bind-n-seq datasets – HNRNPF: ENCSR376SUZ and HNRNPH2 ENCSR328PGZ). Research related to the functions of the HNRNPF/H proteins has thus probed two broad themes. One theme focuses on the HNRNPF/H proteins' regulation of RNA processing through their interaction with G-tracts adjacent to splice sites or polyadenylation signals. Complementing these efforts are studies focused on the interaction of the HNRNPF/H proteins with regions of RNA that can form complex G-quadruplex (G4) secondary structures. Here, we will first consider the functions of the nuclear-localized HNRNPF/H proteins, focusing on more general findings rather than reports of their regulation of gene-specific RNA processing, followed by a discussion of GRSF1's functions in the mitochondria.

2.3.1 Nuclear-localized *HNRNPF/H proteins*—Before discussing findings related to the function of the nuclear HNRNPF/H proteins, it is crucial to note that due to their homology, assessing their protein-specific functions is particularly challenging. For example, sequence-based reagents (e.g., siRNAs) may target the transcripts expressed by more than one HNRNPF/H gene unless carefully designed. Also, antibodies against these proteins may detect more than one family member, particularly HNRNPH1 and HNRNPH2. It is also likely that the HNRNPF/H proteins regulate the expression of each other, and perturbation of one may alter the expression of another family member. Furthermore, much of our understanding of HNRNPF/H protein function comes from *in vitro* biochemical or cell-based studies, which will not capture the complexity of their protein-specific functions *in vivo*. For example, transgenic mice harboring disruptions of *Hnrnph1* (Hnrnph1$^{tm1b(KOMP)Wts1}$) or *Hnrnph2* (Hnrnph2$^{em1(IMPC)J}$) loci exhibit quite different phenotypes. *Hnrnph2* hemizygous male and homozygous female mice are viable, though phenotypic analysis noted abnormal vocalization and coat/hair morphologies. In contrast, homozygous disruption of *Hnrnph1* on a C57BL/6N background results in preweaning lethality, whereas heterozygous mice exhibit disruption of erythropoiesis. Of note, a recent study by Shan and co-workers highlighted this latter observation as part of their analysis of the differential binding of RBPs, including HNRNPF/H proteins, to RNAs that exhibit changes in expression during red blood cell differentiation (Shan et al., 2021). Generation of another line of *Hnrnph1* knockout mice resulted in no post-13.5-week embryos, though its conditional deletion in germline cells produced viable offspring (Feng et al., 2022). However, assessment of the germline *Hnrnph1*-deleted mice at five months of age demonstrated both male and female infertility due to defects in spermatogenesis and a failure of oogenesis and folliculogenesis, respectively (Feng et al., 2022). Complete ablation of *Hnrnpf* (*Hnrnpf*−/− mice generated using B6.C-Tg(CMV-Cre)1Cgn/J x *Hnrnpf*-fl/fl mice) is embryonically lethal. However, the tissue-specific depletion of HNRNPF (principally in kidney tubules) achieved using a *Pax8-Cre*-derived mouse line did result in viable mice which exhibited hypertension and glycosuria (Lo et al., 2019). Notably, germline *Hnrnph1*-deleted mice and the *Pax8-Cre-HNRNPF* knockout mice exhibited evidence of altered maturation of specific transcripts, consistent with their functions as alternative splicing factors (Feng et al., 2022; Lo et al., 2019).

Multiple studies have described the functions of the HNRNPF/H proteins in alternative splicing, including studies of gene-specific splicing events (for example, (Buratti et al., 2004; Caputi & Zahler, 2001; Chen et al., 1999; Chou et al., 1999; Fisette et al., 2012; Garneau et al., 2005; Hastings et al., 2001; Královičová & Vořechovský, 2006; Marcucci et al., 2007; Mauger et al., 2008; McCullough & Berget, 1997; Min et al., 1995). Complementing these studies are more global analyses that have used increasingly sophisticated approaches for determining and quantifying alternative splicing events (for example, (Huelga et al., 2012; Katz et al., 2010; Venables et al., 2008; Wang et al., 2012; Xiao et al., 2009). G-tracts, such as those bound by HNRNPF/H proteins, are over-represented near splice sites, particularly within regions about 70 nts downstream of 5’ splice sites that contribute to defining exon-intron boundaries (McCullough & Berget, 1997; Xiao et al., 2007; Yeo et al., 2004), though G-tracts are also present within other intronic and exonic regions. Thus, at least one mechanism by which HNRNPF/H proteins regulate

alternative splicing involves their interaction with these sequences, which combined with 5' splice sites of varying strengths, determines the frequency of an exon inclusion or exclusion event (Xiao et al., 2009). Additionally, the length and organization of other G-tracts and their intronic or exonic location, may contribute to the regulation of splicing events by the HNRNPF/H proteins. For example, HNRNPF/H proteins may inhibit or promote the recruitment of the spliceosome complex to specific sites in a precursor mRNA. HNRNPF/H proteins may also compete with other RBPs for binding if other *cis*-regulatory sequences are in proximity to target sites of the HNRNPF/H proteins. Collectively, these observations mean that the output of HNRNPF/H protein function can vary, resulting in exon inclusion in one context and exon exclusion in another.

Related to the interaction of HNRNPF/H proteins with G-rich sequences is the unusual property of nucleic acids containing multiple G-tracts. Specifically, in the presence of a monovalent cation (e.g., K^+ or Na^+), G-rich sequences can, *via* Hoosgteen-hydrogen bonds, form planer G-tetrads that can stack to generate a G-quadruplex (G4) structure (Varshney et al., 2020). Experimental studies have estimated that the human transcriptome contains thousands of G-rich sequences with the potential to form RNA parallel G4s stacking two or three guanine-tetrads (consensus motif: $G_{\geq 2}N_{1-7}G_{\geq 2}N_{1-7}\ G_{\geq 2}N_{1-7}G_{\geq 2}$) (Guo & Bartel, 2016; Kwok et al., 2016). However, it is unclear to what extent rG4s occur within cells, as many RBPs can suppress or resolve such structures. The recognition of G-tracts by HRNPHF/H proteins has meant these are prime candidates for interacting with regions of G-rich RNA with the potential to form rG4s and that their regulation of splicing includes altering rG4 formation as a result of the inhibition of folding (Dominguez et al., 2010; Samatanga et al., 2013), or as our recent *in vitro* studies have indicated the potential for destabilization of the folded G4 state (Vo et al., 2022).

Another important facet of the RNA processing functions mediated by members of the HNRNPF/H protein family includes their interaction with G-tracts present in the GU rich region 3' of the AAUAAA polyadenylation (poly A) signal. In a series of studies employing viral or cellular RNAs, *in vitro* analysis demonstrated that HNRNPF/H proteins can bind G-rich sequences downstream of a poly A signal and as a consequence regulate the efficiency of 3' end processing (Alkan et al., 2006; Arhin et al., 2002; Bagga et al., 1998; Bagga et al., 1995; Qian & Wilusz, 1991). Initial experiments demonstrated the promotion of cleavage stimulation factor (CSTF) complex assembly by HNRNPF/H proteins and thus 3'-end processing (Arhin et al., 2002; Bagga et al., 1998; Bagga et al., 1995). Subsequent experimentation showed that in other contexts, for example, the processing of B-cell nascent RNAs, HNRNPF/H proteins can also inhibit CSTF binding and RNA cleavage (Veraldi et al., 2001). More recent studies examining the human *β-globin* locus demonstrated that HNRNPH interacts with the polypyrimidine tract-binding protein PTBP1 and that, in this case, PTBP1 can recruit HNRNPH to a G-rich sequence 5' of the poly A signal (Millevoi et al., 2009). In addition, analyses of the 3'-end processing of *TP53* transcripts have linked the function of the HNRNPF/H proteins to G-rich sequences that can form G-quadruplexes. Specifically, Decorsiere et al. and Newman et al. showed that to overcome the inhibition of 3' end processing following DNA damage, HNRNPF/H binds a G-rich sequence 3' of the *TP53* poly A signal that can form a quadruplex structure. This binding event together with the DHX36 resolution of the G4 structure maintains *TP53* RNA processing and thus protein

expression (Decorsiere et al., 2011; Newman et al., 2017). Finally, there is some evidence that the HNRNPF/H proteins may regulate the alternative splicing and polyadenylation of transcripts expressed by a single gene to mediate expression of different protein isoforms, for example isoforms of the acetylcholinesterase enzyme (Nazim et al., 2017).

2.3.2 GRSF1 and mitochondrial function—To date, the most detailed analyses of the cellular functions of GRSF1 have focused on the activity of its longest isoform – isoform 1 – in regulating the post-transcriptional processing of transcripts expressed in mitochondria (mt). As mentioned above (2.2), the 480 amino acid GRSF1-isoform 1 contains an N-terminus A-rich domain that functions as a mitochondrial localizing signal. In mitochondria, GRSF1 forms RNA-dependent foci (Antonicka et al., 2013; Jourdain et al., 2013). In the absence of GRSF1, studies have demonstrated significant disruption of the expression of several mitochondrial RNA (mtRNA) species. For example, Jourdain and co-workers and Antonicka and colleagues reported decreased levels of *COX1*, *COX2*, and *MT-ND5* protein-encoding mRNAs following the silencing of *GRSF1* (Antonicka et al., 2013; Jourdain et al., 2013). The same studies also noted alterations in the steady state levels of ribosomal RNAs, notably decreased detection of 16S ribosomal RNA (rRNA). Jourdain and co-workers also reported changes in the ratio of some mature and precursor tRNAs; for example, depletion of GRSF1 resulted in decreases in the levels of some mature transfer RNAs ($tRNA^{Phe}$ and $tRNA^{leu}$) but increases in their respective precursor RNAs (Jourdain et al., 2013). Additional experimentation by Jourdain et al. demonstrated that GRSF1 interacts with subunits of RNase P, an enzyme required for processing many mtRNAs, and the RNase P-independent processing of other transcripts (Jourdain et al., 2013). Complementary findings of Antonicka and co-workers demonstrated GRSF1's binding of RNAs encoded by the mitochondrial genome's light strand (L-strand) (Antonicka et al., 2013). A subsequent study by Pietras and colleagues confirmed GRSF1's binding of the mtRNA L-strand and showed GRSF1 functions as a critical component of a mtRNA degradation mechanism (Pietras et al., 2018). The nomenclature of the mtRNA L and heavy (H) strands refers to their asymmetric densities in a gradient, which reflect differences in their sequence distribution. Specifically, L-strand-transcripts are G-rich and thus predicted to have a greater likelihood of containing G-tracts that can form rG4s. In brief, Pietras et al. demonstrated that in mitochondria, GRSF1 could bind and disrupt G4 structures. A component of the mitochondrial degradosome – SUPVSL1 (previously SUV3) interacts with the GRSF1-bound G4 containing RNA to release GRSF1 and facilitate degradation of the RNA by the 3' – 5' ribonuclease PNPT1 (previously PNPase) (Pietras et al., 2018). This cooperative mechanism between GRSF1 and the mitochondrial degradosome is vital for the maintenance of the mitochondrial transcriptome. Interestingly, Pietras et al. speculate that the GC sequence bias of vertebrate mitochondrial genomes also required an adaption of a mechanism that limits the accumulation of transcripts prone to rG4 formation and that this may, at least in part, explain the late evolutionary emergence of *GRSF1* (Pietras et al., 2018).

Other functions ascribed to GRSF1 include a report by Ufer and co-workers describing its interaction with a G-rich sequence in the 5' untranslated region (UTR) of a transcript variant of glutathione peroxidase 4 (*GPX4*) that encodes a mitochondrial-specific isoform of the GPX4 protein (Ufer et al., 2008). Ufer and colleagues also showed that depletion of GRSF1

in embryos *ex vivo* by RNAi resulted in the impairment of mid and hind-brain development; effects rescued by the co-administration of a cDNA expressing the mitochondrial-specific isoform of GPX4 (Ufer et al., 2008). Noh, Kim, and colleagues noted the binding of GRSF1 to a similar sequence motif in a long non-coding RNA – *RMRP* (RNA component of mitochondrial RNA processing endoribonuclease) (Noh et al., 2016), as described for the interaction of GRSF1 and the transcript variant of GPX4. RMRP is a component of a complex required for the processing of mature 5.8S rRNAs, and subsequent experiments performed by Noh, Kim et al. showed that GRSF1 contributes to an accumulation of RMRP RNAs in the mitochondrial matrix (Noh et al., 2016).

3 THE HNRNPF/H RNA BINDING PROTEINS AND CANCER

Many proteins that regulate RNA maturation have emerged as contributing to the expression of cancer-specific oncogenic transcripts and alterations in the expression profiles of cancer cells, including core components of the spliceosome and hnRNPs. We discuss below recent studies that highlight the different mechanisms by which HNRNPF/H proteins can promote tumorigenesis (Figure 4A), including their function in regulating the expression of mRNAs encoding proteins that promote tumorigenesis or alter responses to treatment. We also discuss the effect of somatic mutations on the expression or function of members of this protein family or the effect of mutations that disrupt their binding sites and chromosomal rearrangements involving the *HNRNPF/H* genes.

3.1 Cancer-associated transcripts and the HNRNPF/H proteins

One mechanism by which members of the HNRNPF/H proteins contribute to tumorigenesis is their promotion of the expression of specific transcript variants encoding proteins that tumor cells depend on for cancer initiation or progression or could modulate treatment efficacy. Here, we describe our recent studies highlighting the function of HNRNPH1 in processing the oncogenic fusion transcript *EWSR1::FLI1*. We also discuss studies performed by others that illustrate further examples of how the HNRNPF/H proteins regulate the expression of cancer-specific transcripts or gene expression more broadly.

3.1.1 HNRNPH1 and EWSR1::FLI1—Ewing sarcoma (EWS) is a pediatric bone and soft tissue cancer. In about 85% of Ewing sarcomas, a chromosomal translocation t(11:22)(q24:q12) generates the fusion oncogene *EWSR1::FLI1* consisting of 5’ *EWSR1*-derived and 3’ *FLI1*-derived sequences. The resulting oncoprotein (EWSR1::FLI1) functions as an aberrant transcription factor that promotes malignant transformation and the proliferation and survival of EWS cells. Of the 85% of Ewing sarcomas that harbor an *EWSR1::FLI1* fusion gene, a subset (approximately 35% of cases, see (Vo et al., 2022)) harbor *EWSR1*-intron 8 breakpoints. To express the EWSR1::FLI1 fusion oncoprotein, tumor cells that harbor *EWSR1*-intron 8 breakpoints must exclude *EWSR1*-exon 8 during RNA processing to generate an in-frame *EWSR1::FLI1* mRNA that expresses the fusion oncoprotein. Failure to exclude *EWSR1*-exon 8 results in the expression of an *EWSR1::FLI1* mRNA that includes a premature stop codon. The results of a genome-wide RNAi screen performed in an EWS cell line dependent on excluding *EWSR1*-exon 8 to express EWSR1::FLI1 and our subsequent studies determined that HNRNPH1 functions as the critical regulator

of this splicing event (Grohar et al., 2016) (Figure 4B). In brief, silencing of *HNRNPH1* in EWS cell lines dependent on the exclusion of *EWSR1*-exon 8 to express in-frame *EWSR1::FLI1* fusion transcripts (e.g., the EWS cell lines TC-32 and SK-N-MC) results in the inclusion of *EWSR1*-exon 8, a decrease in the expression of the fusion oncoprotein, reversal of the well-characterized EWS gene expression signature, and loss of cell viability (Grohar et al., 2016). Interestingly, PCR analysis of *EWSR1::FLI1* transcripts (Neckles et al., 2019) and minigene assays (Vo et al., 2022) demonstrated that neither HNRNPH2 nor HNRNPF function as regulators of *EWSR1*-exon 8 exclusion, indicating that this is an HNRNPH1-specific splicing event. Building on these observations, we showed that HNRNPH1 binds at least one G-rich sequence present at the 3' end of *EWSR1*-exon 8 *in vitro* and that in the presence of cations, these RNAs can form G-quadruplex (G4) secondary structures (Neckles et al., 2019; Vo et al., 2022). Using this information, we assessed if TC-32 and SK-N-MC EWS cell lines are sensitive to the pan-G4-binder pyridostatin (PDS). We determined that these *EWSR1*-exon 8-positive EWS cell lines exhibit reduced cell viability at lower concentrations of PDS than other EWS cell lines or non-EWS cells. Furthermore, we observed that PDS generates a concentration-dependent increase in the inclusion of *EWSR1*-exon 8 and a concomitant decrease in the expression of the EWSR1::FLI1 protein and reverses the expression of established transcriptional targets of the fusion oncoprotein (activated and repressed genes) (Neckles et al., 2019). Collectively, these studies suggest a means for inhibiting EWSR1:FLI1 expression through disruption of the HNRNPH1-dependent processing of the pre-mRNA expressed in a subset of Ewing sarcomas.

3.1.2 HNRNPF/H and the regulation of cancer-associated transcripts—

Tumorigenesis requires the disruption of many biological processes, including the deregulation of specific signaling pathways. One HNRNPF/H-dependent splicing event that could contribute to the activation of multiple signaling pathways involves the expression of the Δ165 isoform of the receptor tyrosine kinase, MST1R (macrophage stimulating 1 receptor, also known as RON) (see (Cazes et al., 2022) for discussion of MST1R/RON). The Δ165 isoform of MST1R requires no ligand binding for activation, resulting in the stimulation of multiple pathways associated with cancer, including the TGFß and PI3K/AKT pathways. First detected in a gastric carcinoma cell line (Collesi et al., 1996), tumor types expressing the *MST1R-Δ165* transcript variant include colorectal and breast cancer (Ghigna et al., 2005; Zhou et al., 2003), glioblastoma multiforme (LeFave et al., 2011), and ovarian (Mayer et al., 2015) and pancreatic cancer (Chakedis et al., 2016). Expression of the *MST1R-Δ165* transcript variant involves the exclusion of *MST1R*-exon 11 from the full-length transcript. Analysis by LeFave and colleagues highlighted the presence of two G-tracts (3Gs) at the 5' end of *MST1R*-exon 11 (LeFave et al., 2011). Critically, depletion of HNRNPH1 in cells expressing the transcript encoding the MST1R-Δ165 isoform resulted in a significant shift from the exclusion of *MST1R*-exon 11 to the inclusion of this exon; findings confirmed using a minigene consisting of *MST1R*-exon 11 and flanking intronic sequences (LeFave et al., 2011). A subsequent study by Braun and co-workers confirmed and extended these findings. First, using a series of minigene constructs and random mutagenesis, this study reported the mapping of RBP sequence binding motifs to nucleotide (nt) changes that influence *MST1R*-exon 11 splicing (Braun et al., 2018). This analysis

highlighted several RBPs, particularly HNRNPH1 and HNRNPH2, as putative regulators of the splicing of *MST1R*-exon 11. Consistent with earlier findings, the silencing of *HNRNPH1* resulted in increased *MST1R*-exon 11 inclusion. Interestingly, further analysis indicated cooperativity of HNRNPH1/2's binding of multiple sites in *MST1R*-exon 11, suggesting that even minor changes in HNRNPH1/2 expression could affect the splicing of this exon significantly (Braun et al., 2018). Furthermore, the authors speculate that the *MST1R*-exon 11 G-tracts could form G4 structures and that multiple binding events may be critical to altering the local secondary structure to favor exon skipping (Braun et al., 2018). Considering the relevance of these observations to cancer, analysis of data extracted from The Cancer Genome Atlas (TCGA) indicated a correlation of mutations within *MST1R*-exon 11 G-tracts, including those that HNRNPH1 or HNRNPH2 could bind and exclude this exon, particularly in carcinomas of the head and neck (squamous cell) or thyroid (Braun et al., 2018). We will discuss additional cases of the potential effect of sequence changes in the putative binding sites of HNRNPF/H proteins in 3.2.

As the Braun et al. study suggests, slight differences in the levels of HNRNPF/H these proteins in a pre-malignant or transformed cell, compared with a healthy cell, could have a profound effect on the transcriptome. Several studies have thus examined correlations between HNRNPF/H expression and the expression of cancer-associated transcripts. For example, in prostate cancer, two independent studies have implicated HNRNPF/H proteins in regulating the expression of androgen receptor (AR), a transcription factor involved in the initiation and progression of castration-resistant prostate cancer. First, Yang and colleagues demonstrated using multiple independent cohorts that prostate tumor samples express elevated *HNRNPH1* transcript levels than matched healthy tissue (Yang et al., 2016). They also showed that prostate tumor samples from African American men exhibited further differential expression compared to similar tumor samples from Caucasian Americans (Yang et al., 2016). In addition, Yang and co-workers found a correlation between *AR* and *HNRNPH1* expression in prostate tumors. Finally, they demonstrated that depleting *HNRNPH1* expression *via* RNAi decreases the expression of full-length *AR* and the *AR-V7* transcript variants (Yang et al., 2016). The generation of *AR-V7* transcripts involves using a cryptic exon 3b present within *AR*-intron 3 and is associated with the resistance of prostate cancer cells to androgen deprivation therapy. Interestingly, a study by Fan and co-workers demonstrated that HNRNPF can bind a G-rich sequence within exon 3b of the *AR* gene and that HNRNPF promotes recruitment of U2AF65 to the *AR*-exon 3b 3' splice site, which aids in the recognition and inclusion of this exon and thus the expression of *AR-V7* (Fan et al., 2018).

Several reports have also noted deregulated expression of one or more HNRNPF/H proteins in the brain tumor glioblastoma multiforme (GBM). LeFave and colleagues first demonstrated elevated HNRNPH RNA and protein expression in GBM samples compared to healthy tissues using PCR-based and immunohistochemical analysis (LeFave et al., 2011). Critically, the authors speculated that HNRNPH's elevated expression might explain the altered splicing of *MST1R* their study revealed, along with changes in the splicing of transcripts expressed that encode the *MADD* (MAP kinase activating death domain) gene, which functions in TNF-α signaling. More recently, Herviou et al. utilized the TCGA database to demonstrate increased expression of the HNRNPF and HNRNPF RNAs in

tumors (n=154) compared with a small number of control samples (n=5) and confirmed elevated expression of the HNRNPH and HNRNPF proteins in three high-grade GBM samples versus low-grade glioma samples (Herviou et al., 2020). As part of the same study, Herviou and colleagues concluded that low levels of the HNRNPF/H proteins favor reduced translation of mRNAs encoding proteins involved in the DNA damage response. In contrast, higher levels of HNRNPF/H proteins promote the translation of these mRNAs. Evidence presented to support this conclusion included demonstrating the HNRNPF/H-DHX36 cooperative regulation of USP1, a protein that forms part of the Fanconi anemia DNA repair pathway (Herviou et al., 2020). Also, based on earlier studies that HNRNPH regulates the alternative splicing of transcripts encoding the serine/threonine kinase ARAF (Rauch et al., 2011; Rauch et al., 2010), Le Bras et al. recently confirmed that the depletion of HNRNPF/H increases the expression of an *ARAF* transcript variant that negatively regulates the MAPK pathway. (Le Bras et al., 2022). Additional analysis linked HNRNPF/H's indirect regulation of the MAPK pathway to alterations in the expression of MAPK protein targets, including modification of the translation initiation factor EIF4E (eukaryotic translation initiation factor 4E) (Le Bras et al., 2022). The authors of this study thus propose that elevated levels of HNRNPF/H in GBM enhance expression of the full-length ARAF protein, resulting in an indirect increase in translation by promoting EIF4E phosphorylation. These changes in protein translation increase tumor growth. It is unclear what mechanism or mechanisms result in the elevated expression of HNRNPF/H in some tumor types. However, one report has indicated that the transcription factor MYC may regulate *HNRNPH1* expression (Rauch et al., 2011).

3.2 Mutations in HNRNPF/H genes or the targets of HNRNPF/H proteins

Recurrent, loci-specific sequence changes are a feature of many cancer types, and their identification has proven critical to defining those genes that contribute to tumorigenesis. Here, we highlight recent studies reporting recurrent sequence changes in *HNRNPF/H* genes that may increase cancer risk or contribute directly to tumorigenesis. We will also feature one example of how sequence changes in sites bound by HNRNPF/H proteins can dramatically change the transcript variants expressed by a particular gene.

3.2.1 Ulcerative colitis, cancer risk, and HNRNPF—An increased risk of cancer is associated with the repetitive cycles of tissue damage and repair observed in clinical states involving chronic inflammation. One such inflammatory condition is ulcerative colitis, which affects the intestine and is associated with an increased risk of colorectal cancer. A recent study of sequence changes detected in epithelial cells isolated from the nondysplastic intestinal crypt structures of ulcerative colitis patients highlighted the selection for alterations in several genes, including *HNRNPF* (Kakiuchi et al., 2020). Observed in 18 samples, these sequence changes in *HNRNPF* are all predicted to affect the protein's putative nuclear localization signal (nls) (p.R206Q, p.R206W, p.P209S, p.P209L, pP209T, pY210C, pY210D, p207–213del, p.209–214del). It is unclear whether these mutations contribute to the clinical course of ulcerative colitis and/or increased cancer risk, but, as we will discuss below (4.1), this cluster of mutations occurs in the same region as several germline mutations in other *HNRNPF/H* genes, particularly *HNRNPH2*, reinforcing that this portion of the protein is critical to its function.

3.2.2 Mantle-cell lymphoma—The B-cell malignancy, mantle-cell lymphoma (MCL), constitutes about 5 to 7% of lymphoma diagnoses per year (Armitage & Longo, 2022). The most frequently observed genetic event in MCL involves a chromosomal translocation that results in the constitutive expression of cyclin D1, a regulator of the cell cycle (reviewed in Navarro et al., 2020). Additional recurrent somatic sequence changes observed in MCL include those affecting genes that function in the DNA damage response, NOTCH or NFκB-signaling pathways, epigenetic regulation (reviewed in Silkenstedt et al., 2021) or, most recently, RNA processing, specifically, *DAZAP1*, *EWSR1*, and *HNRNPH1* (Nadeu et al., 2020; Pararajalingam et al., 2020). Focusing on *HNRNPH1*, the sequence analysis of two independent cohorts of MCL samples (Nadeu et al., 2020; Pararajalingam et al., 2020) and retrospective analyses (Pararajalingam et al., 2020) of previous datasets (Agarwal et al., 2019; Beà et al., 2013; Khodadoust et al., 2017; Rule et al., 2018; Wu et al., 2016) revealed that as many as 10% of MCLs harbor sequence changes in *HNRNPH1* with most mapping to intronic regions adjacent to or within *HRNPNH1*-exon 5 (exon 4 of the coding sequence) (Figure 5A). Interestingly, analysis of other lymphoma subtypes suggests that *HNRNPH1* sequence changes represent an MCL-specific mutation (Pararajalingam et al., 2020).

Further observations made by Pararajalingam and colleagues included highlighting that the recurrent *HNRNPH1* intronic sequence changes predominately affect G-tracts (Figure 5A) and that at least one of these had the potential for binding by HNRNPH1. They also noted that the ratio of *HNRNPH1* transcripts that include or exclude the exon these sequences flank differed when they examined the *HNRNPH1* mRNAs expressed in mutated and unmutated tumor samples (Pararajalingam et al., 2020). These observations led Pararajalingam and co-workers to speculate that the non-coding mutations identified in the MCL samples disrupt the expression of one or more of its transcript variants (coding and/or non-coding), resulting in the increased expression of full-length *HNRNPH1* mRNAs and HNRNPH1 protein (Pararajalingam et al., 2020). Indeed, subsequent assessment of protein expression in MCL tissue samples demonstrated a correlation between mutation status and a shift in the splicing ratio that favors the generation of full-length transcripts with the intensity of anti-HNRNPH staining (Pararajalingam et al., 2020). Further studies are needed to determine how the altered expression of HNRNPH1 contributes to MCL. However, this study does highlight the need to consider how disruption of the autoregulation of HNRNPH1 expression may represent an important mechanism for deregulating its expression and potentially other protein family members and RNA binding proteins, a finding that warrants further investigation.

3.2.3 Burkitt's lymphoma—Cancer-specific sequence changes that alter the binding site for a member of the HNRNPF/H protein family can also influence the expression of a protein that contributes to tumorigenesis. One example is the regulation of two isoforms of the E-box helix-loop-helix transcription factor, TCF3. In a series of studies conducted by Yamazaki and colleagues, they demonstrated that HNRNPF/H proteins are critical regulators of the expression of two TCF3 isoforms, E12 and E47, by mediating an alternative splicing event involving *TCF3*-Exon 18a and *TCF3*-Exon 18b, respectively (Figure 5B) (Yamazaki et al., 2018; Yamazaki et al., 2019). HNRNPF/H proteins bind sites in *TCF3*-Exon 18b, suppressing the inclusion of this exon and favoring the expression of *TCF3*-Exon 18a

containing E12 isoform (Yamazaki et al., 2018). Regulation of the expression of the relative levels of each TCF3 isoform is critical for defining human embryonal stem cell fate as the TCF3-E47 isoform represses the transcription of *CDH1*, resulting in reduced E-cadherin expression and thus cell differentiation. In contrast, the expression of the E12 isoform favors *CDH1* expression and the maintenance of a pluripotent state (Yamazaki et al., 2018). A follow-up study identified a distal intronic conserved region (ICR) between the *TCF3* Exons 18a and 18b as an additional *cis*-regulatory element that regulates the usage of these exons (Yamazaki et al., 2019). Specifically, Yamazaki et al. determined that a second RBP, PTBP1, binds this ICR and functions cooperatively with HNRNPH1 bound to *TCF3*-Exon 18b to regulate the alternative splicing of *TCF3*. In brief, low levels of HNRNPH1 and PTBP1's binding of the ICR favors the inclusion of *TCF3*-exon 18b and *TCF3*-exon 18a exclusion and, thus, expression of the TCF3-E47 isoform. Contrastingly, elevated HNRNPH1 levels result in the expression of the TCF3-E12 isoform (Yamazaki et al., 2019). Interestingly, a study of recurrent mutations observed in the MYC-driven B-cell lymphoma, Burkitt's lymphoma, has shown significant enrichment of alterations that map to *TCF3*-Exon 18b (Schmitz et al., 2012). Furthermore, employing minigene reporters and gel shift assays, Yamazaki et al. demonstrated that two of these mutations – G1663C and G1681A – alter the ratio of E12 and E47 transcripts due to the loss of HNRNPH1 binding (Figure 5B) (Yamazaki et al., 2020). These results suggest that disruption of HNRNPF/H's regulation of *TCF3* splicing may contribute to the pathology of a subset of Burkitt's lymphoma.

3.3 Fusion oncogenes

Many tumors harbor genomic rearrangements that result in the fusion of sequences from two different genes, a proportion of which initiate or contribute to tumorigenesis. Fusion oncogenic events can include the juxtaposition of the regulatory regions from one gene such that these sequences deregulate the expression of another gene. In other cases, the fusion of the 5' regulatory region and a portion of the coding sequence from one gene adjacent to coding sequences derived from a second gene results in the expression of a protein with aberrant function. In recent years, the detection of fusion events in tumor samples involving the *HNRNPF/H* loci has increased primarily because of the availability of computational tools that enable the detection of fusion transcripts within RNA-seq data. The genomic rearrangement that underlies the expression of several of these *HNRNPF/H*-fusion transcripts remains to be determined. However, particularly in the case of two or more reports of a similar fusion event, these could indicate the generation of a *bona fide* fusion oncogene. If a *HNRNPF/H* family member is the 5' partner gene, the regulatory elements responsible for the ubiquitous constitutive expression of these genes could deregulate the expression of the 3' partner gene's protein product. Alternatively, or in addition, the HNRNPF/H protein domains could contribute directly to the function of a derived fusion oncoprotein by altering protein-nucleic acid or protein-protein interactions.

Cases of fusion oncogenes in which *HNRNPH1* is the 5' partner gene include t(5;10)(q35;p12) translocations found in pediatric leukemias, including T-cell acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) (Brady et al., 2022; Brandimarte et al., 2013; Chen et al., 2018; Kudo et al., 2022) (Figure 5C). Analysis of the fusion of 5' *HNRNPH1*-derived and *MLLT10*-derived 3' sequences in one case of early T-cell

precursor-ALL identified transcripts that joined either *HNRNPH1* nt 1324 (exon 11) or *HNRNPH1* nt 6701 (intron 10) to nt 2097 of *MLLT10* (exon 15) (Brandimarte et al., 2013). These transcripts are in-frame and predicted to express a fusion protein that retains most of the three qRRM domains of the RNA binding protein, along with the MLLT10-derived OM-LZ domain required for leukemogenesis. Sequencing of another *HNRNPH1-MLLT10* fusion expressed in a case of pediatric AML indicated fusion of *HNRNPH1*-intron 12 sequences to *MLLT10*-intron 14 sequences, which, following splicing, generates an in-frame transcript predicted to express a fusion protein including the three qRRM domains of HNRNPH1 (Kudo et al., 2022). Currently, it is unclear if the presence of the HNRNPH1 RNA binding domains contributes to the fusion oncoproteins function or whether it is only the utilization of *HNRNPH1*'s regulatory sequences to mediate the deregulated expression of the transcription factor MTTL10 that is critical to inducing tumorigenesis. The same applies to *HNRNPH1::ERG* fusions described in a small number of acute myeloid leukemia (AML) cases (Bolouri et al., 2018; Jiang et al., 2022; Kerbs et al., 2022; Liu et al., 2022; Smith et al., 2021) in which the chromosomal rearrangement results in the 5' *HNRNPH1*-derived regulatory sequences likely activating the expression of the ETS transcription factor ERG, which, deregulates gene expression to favor carcinogenesis. However, the study of other fusion proteins, such as EWSR1::FLI1, that include domains similar to those included within the HNRNPH1-fusions, suggest that the incorporation of protein regions, particularly those with low sequence complexity, could promote protein-protein interactions, inducing, for example, fusion protein multimerization, that enhances aspects of the aberrant function of these oncogenic proteins. (Boulay et al., 2017).

In addition to the rare but multiple reports of *HNRNPH1::MTTL10* and *HNRNPH1::ERG* fusions, several studies have reported single cases of *HNRNPF/H*-fusion transcripts, including an *HNRNPH3::ALK* rearrangement identified in a single case of a salivary duct carcinoma (Dogan et al., 2019) and an *HNRNPH3::TFE3* fusion detected in case of alveolar sort part sarcoma (Dickson et al., 2019). Also, as part of analyses of The Cancer Genome Atlas (TCGA) resource, several groups have used RNA-seq data to detect potential fusion transcripts and have noted cases involving *HNRNPF/H* genes (Gao et al., 2018; Hu et al., 2018; Yoshihara et al., 2015). *HNRNPF/H* fusion transcripts reported in TCGA datasets include *HNRNPF::ATAD1* and *HNRNPH3::ATAD1* fusions reported in breast cancer and lung adenocarcinoma, respectively. Other fusions include *HNRNPF::ZNF33B* and *HNRNPF::ZNF569* fusions in ovarian adenocarcinoma; an *HNRNPH1::RNF130* fusion in breast adenocarcinoma; an *HNRNPH2::CLTRN* fusion in a prostate adenocarcinoma; an *HNRNPH3::KLK1* fusion in a kidney adenocarcinoma; an *HNRNPH3::SLC29A3* in a stomach adenocarcinoma; and a *GRSF1::ELFN2* fusion in adenocarcinoma of the large intestine.

Interestingly, one recent study reported a fusion in which HNRNPH1 sequences form the C-terminus of the derived protein, specifically a MEF2D::HNRNPH1 fusion identified in a case of pediatric B-cell precursor acute lymphoblastic leukemia (ALL) (Ohki et al., 2019) (Figure 5C). In the report by Ohki and co-workers, they describe two in-frame transcripts that fused exons 4 or 5 of *MEF2D* to exon 5 of *HNRNPH1*. Modeling of fusion protein predicts it will contain part of the HNRNPH1 qRRM3 domain and its GY region. Other MEF2D-fusions (e.g., MEF2D::BCL9) function as aberrant transcription factors, which led

Kim and Kwon to assess if the qRRM3/GY region of HNRNPH1 can stimulate transcription (Kim & Kwon, 2021). Using a luciferase reporter system, Kim and Kwon demonstrated that the qRRM3/GY or the GY domain of HNRNPH1 can enhance transcriptional activity (Kim & Kwon, 2021); however, it remains to be determined how these domains contribute to the tumorigenic activity of the MEF2D::HNRNPH1 fusion protein.

4 THE HNRNPF/H RNA BINDING PROTEINS AND OTHER DISEASE STATES

4.1 Neurodevelopment

The functions of many RBPs are critical to ensuring the precise spatial and temporal regulation of gene expression required for normal neurodevelopment and neurological function. For example, the regulation of alternative splicing by specific RBPs during neuronal development can result in the expression of specific protein isoforms that exert different functional outcomes (Su et al., 2018). The HNRNPH-dependent splicing of *TERF2* (or *TRF2*) (telomeric repeat-binding factor 2) transcripts illustrates such a result. TRF2 forms part of the telomeric shelterin complex that also functions as a regulator of neuronal differentiation via its interaction with REST (repressor element-1 silencing transcription factor). In brief, studies conducted using rat cells demonstrated that HNRNPH promotes the expression of the *TRF2* full-length transcript and inhibits the expression of a *TRF2* transcript variant (TRF2-S) encoding an isoform lacking TRF2's DNA-binding domain and nls (Zhang et al., 2011). In the presence of HNRNPH, the nuclear expression of full-length TRF2 protein enhances the repression of neuronal genes by REST and the maintenance of a stem cell state (Grammatikakis et al., 2016). In contrast, lower levels of HNRNPH expression results in an accumulation of the TRF2-S isoform and sequestration of REST in the cytoplasm and the de-repression of neuronal genes silenced by REST and neuronal differentiation (Grammatikakis et al., 2016). Given these types of observations, it is unsurprising that deregulated RBP function is the underlying cause of several neurodevelopmental disorders (LaForce et al., 2022; Parra & Johnston, 2022; Prashad & Gopal, 2021), including Fragile X syndrome (FXS), which develops because of disrupted expression of the fragile X mental retardation protein (FMRP), a regulator of mRNA translation, RNA stability and subcellular localization (Protic et al., 2022). Added to this list in recent years are reports of neurodevelopmental disease-associated sequence changes in genes encoding hnRNPs, including the *HNRNPF/H* genes. Here, we discuss the genetic analyses that have identified multiple mutations in *HNRNPF/H* genes in individuals with clinical features characteristic of a neurodevelopmental disorder. Currently, there is limited research investigating the effect of these mutations on the organization of the HNRNPF/H proteins and their functions. However, by delineating these alterations in HNRNPF/H genes in this review, we aim to stimulate future research that will address these questions.

4.1.1 Neurodevelopmental disease-associated sequence changes in HNRNPH2—

The first evidence of sequence changes in *HNRNPH2* emerged through an assessment of six unrelated females (2 to 34 years) who presented with developmental delay and/or intellectual disability (Bain et al., 2016). The report by Bain and colleagues described *de novo* heterozygous sequence changes at nucleotides 616 (c.616C>T, four cases), 617

(c.617G>A, one case), and 626 (c.626C>T, one case) of *HNRNPH2*. The first of these nucleotide variants changes the HNRNPH2 amino acid 206 from arginine to tryptophan – p.R206W, and the second, the same amino acid to glutamine – p.(R206Q). The sixth case alters the HNRNPH2 amino acid 209 from proline to leucine – p.P209L. Since the publication of this study, multiple reports have documented additional cases of *de novo* pathogenic *HNRNPH2* variants present in individuals with similar clinical phenotypes (Bain et al., 2021; Gillentine et al., 2021; Harmsen et al., 2019; Jepsen et al., 2019; Kreienkamp et al., 2022; Peron et al., 2020; Somashekar et al., 2020; White-Brown et al., 2021). Termed Bain-type X-linked Intellectual Development Disorder (MRXSB; OMIM #300986), clinical observations associated with heterozygous or hemizygous sequence variants in *HNRNPH2* include developmental delay, intellectual disability, deficits in speech and motor function, musculoskeletal abnormalities, and an increased diagnosis of autism spectrum disorders (Bain et al., 2021; Madhok & Bain, 2022; Salazar et al., 2021).

The distribution of changes in the *HNRNPH2* sequence is striking, with most altered nucleotides within the conserved region encoding the putative nls (Van Dusen et al., 2010) and the beginning of the GYR region between qRRM2 and qRRM3 (Figure 6A). Specifically, multiple reports have confirmed and extended the initial study by Bain and co-workers to show the presence of *de novo* heterozygous mutations in females and hemizygous mutations in males affecting nucleotides 616, 617, 626, 629, 634, 635, or 638 of *HNRNPH2* (c.616C>T, p.R206W or c616C>G, p.R206G; c.617G>A, p.R206Q or c617G>T, p.R206L; c626C>T, p.P209L; c629A>G p.Y210C; c634A>G, p.R212G; c635G>C p.R212T; c638C>T p.P213L (Bain et al., 2021; Gillentine et al., 2021; Harmsen et al., 2019; Jepsen et al., 2019; Peron et al., 2020; Somashekar et al., 2020; White-Brown et al., 2021). Though rarer, reports of *de novo* mutations predicted to alter the HNRNPH2 qRRM domains include the following: qRRM1 – c85C>T, p.R29C, qRRM2 – c340C>T, p.R114W, and c422T>A, p.M141K, or qRRM3 c1019A>T, p.D340V (Bain et al., 2021; Jepsen et al., 2019; Kreienkamp et al., 2022). Interestingly, an individual harboring a c85C>T mutation (p.R29C), which maps to the region that encodes the first qRRM domain, exhibited milder symptoms (Kreienkamp et al., 2022) in comparison with those that map to a region encoding the qRRM2 domain (Bain et al., 2021; Jepsen et al., 2019; Kreienkamp et al., 2022; Somashekar et al., 2020). This observation suggests that the HNRNPH2 qRRM2 domain may contribute more significantly to the protein's function in mediating splicing events needed for neurodevelopment, though this concept will require experimental confirmation.

A recent study by Kreienkamp and colleagues also described several male individuals harboring hemizygous sequence variants predicted to result in premature termination of translation. Specifically, monozygotic twins harboring a *de novo* c.562C>T, p.(R188*) variant; one case of a c.595C>T, p.R199* variant; and two sequence changes that result in frameshifts – c918_919dupTA, p.N307I*fs**10, and c1110dupT, p.A371C*fs**24 (Kreienkamp et al., 2022). In interpreting the effect of the predicted premature termination events, it is critical to consider HNRNPH2's exonic organization, which consists of only two exons (Figure 3C). This organization suggests that the mutant transcripts are less likely to be the subject of nonsense-mediated mRNA decay (Kurosaki & Maquat, 2016). Instead, it is probable that the c918_919dupTA mutation results in a transcript that expresses a truncated protein lacking part of the qRRM3 domain and the whole of the GY C-terminus, while

the c1110dupT mutation will result in a protein lacking most of the GY C-terminus. Interestingly, individuals harboring sequence changes predicted to express a truncated protein exhibited less severe developmental abnormalities, though several subsequently developed symptoms of a neuropsychiatric disorder (Kreienkamp et al., 2022). It is currently unclear if these milder symptoms are due to the truncated protein retaining its sequence-specific RNA binding and functions in RNA processing while disrupting other aspects of its functionality. For example, as discussed above (2.2), the inclusion of the HNRNPH1-GY domain in a reporter construct enhances gene expression (Kim & Kwon, 2021), which suggests that a truncated protein without this domain may lack this activity.

While most of the identified *HNRNPH2* sequence changes are *de novo* mutations, it is also crucial to consider that the location of the *HNRNPH2* locus on the X-chromosome means the potential for maternal inheritance of a disrupted allele. For example, the individual expressing the p.(N307I*fs**10) truncated proteins inherited this allele from a mosaic, unaffected mother (Kreienkamp et al., 2022). An earlier study also suggested that maternal germline mosaicism explains the inheritance of the c.616C>T p.R206W mutation by siblings (one male, one female) (Somashekar et al., 2020), though the authors did not confir this assertion experimentally. However, a study by White-Brown and colleagues showed inheritance of the same mutation c616C>T, p.R206W from an affected individual's biological mother, though she exhibited no symptoms because of skewed X-chromosome inactivation (by a ratio of 1:99) of the disease-causing allele. (White-Brown et al., 2021).

4.1.2 Neurodevelopmental disease-associated sequence changes in HNRNPH1 and other family members—

The identification of *HNRNPH2* mutations prompted the investigation of other family members, and in 2018 Pilch et al. described the first *HNRNPH1* sequence variant in a child who exhibited similarities in the clinical presentation of the first cases of Bain-type X-linked Intellectual Development Disorder. Specifically, Pilch and colleagues detected a *de novo HNRNPH1* c.616C>T, p.R206W mutation in a 13-year-old boy diagnosed with significant intellectual disability, dysmorphic features, and other developmental abnormalities (Pilch et al., 2018). A subsequent study by Reichert and co-workers described an additional seven individuals (five male, two female) harboring *HNRNPH1* mutations who exhibited similar clinical characteristics to the first case (Reichert et al., 2020). The *HNRNPH1* sequence changes observed in these seven cases included single nucleotide alterations at residues that are part of the GYR region associated with nuclear localization (c.616C>T, p.R206W) – two cases and c.617G>A (p.R206Q) one case), and a duplication that is predicted to generate a frameshift (c.618dupG, p.P207*fs**5) resulting in either expression of a truncated protein or reduced protein expression as a result of nonsense-mediated decay of the aberrant transcript. Another duplication (c1240_1243dup) also generates a frameshift (p.Q415*fs**30); however, as the mutation affects a residue in the C-terminus of the protein (within the GY region), this may result in truncated protein expression. Of note, the individual harboring this mutation had a less severe phenotype than the individuals reported in this study with mutations that affect the GYR region. Interestingly, an individual with an in-frame deletion (c.1116–1175del152, p.E373-W392del) that removes 19 amino acids within the HNRNPH1 GY domain also had a less severe phenotype. The study by Reichert and colleagues also described a large

duplication encompassing most of the *HNRNPH1* locus and an adjacent gene, *RUFY1* (Chr 5:178977572–179050134 GRCh37; Chr 5: 179550571–170623133 GRCh38). The functional effect of this duplication event is unknown; however, it is probable that disruption of HNRNPH1 function contributes to the clinical features of the individual harboring this mutation. A recent study also described two additional *de novo HNRNPH1* mutations, c.856A>G (p.(T286A; C-terminus end of GYR region) and c.1094C>T (p.(A365V; GY region), in individuals with a neurodevelopmental disorder (Gillentine et al., 2021). The study by Gillentine and colleagues also highlighted an earlier cohort study of individuals with a severe pediatric epilepsy disorder (Epi et al., 2013) that noted a c.1305A>G synonymous mutation, which cell-based studies showed results in the expression of a transcript missing *HNRNPH1*-exon 12. This *HNRNPH1*-exon 12 exclusion event has the potential to express a variant *HNRNPH1* transcript that expresses a protein lacking part of the GY domain, though to establish if this is the case, there is a need for further studies. See Figure 6B.

The study by Gillentine and co-workers also reported cases of mutations in *HNRNPF* (c.142A>G; c.634C>T; c.703G>A) in individuals with a neurodevelopmental disorder. The sequence changes in HNRNPF are located within the coding sequence for the first qRRM of the RBP (p.T48A), the second, the GYR region associated with nuclear localization (p.R212W)), and the third, the GYR region (p.A235T) (Figure 6C). Finally, the same study described alterations in the coding sequence of HNRNPH3 (c.293G>A, p.G98Q; c.946G>A, p.D316N; c.983G>A, p.s328N), which will alter a site between the first qRRM and the GYR region of HNRNPH3 and the other sites in its C-terminus region (Figure 6D).

4.1.3 Experimental analysis of HNRNPH2 mutants—As mentioned above, the interrogation of the functional effect of changes in the HNRNPH2 sequence is in its infancy, but one study has reported some initial findings (Kreienkamp et al., 2022). In the study by Kreienkamp and colleagues, the authors report the analysis of ectopically expressed wild-type HNRNPH2 protein and a panel of protein variants, HNRNPH2-R114W, HNRNPH2-R206Q, and HNRNPH2-P209L. First, they examined the cellular localization of these proteins, finding that the HNRNPH2 wild-type and the HNRNPH2-R114W protein that has an altered qRRM2 domain localized to the nucleoplasm only, while the HNRNPH2-R206Q and HNRNPH2-P209L proteins exhibited localization to both the nucleus and cytoplasm. These observations are consistent with mutations that cluster in a region around amino acids 206 – 212 of HNRNPH2, altering protein localization. Kreienkamp and colleagues next examined the interaction of the wild-type and mutant HNRNPH2 proteins with other RBPs with which it interacts. For example, the authors report reduced interaction of the HNRNPH2-R114W protein with DDX5 compared with the interaction of the wild-type protein and DDX5. This finding suggests that changes in the sequence of a qRRM domain could alter the protein-protein interactions needed to execute HNRNPH2-regulated splicing events. Critically, this study also examined the expression profiles of fibroblasts derived from an individual harboring a mutation that generates an HNRNPH2-R114W protein compared with cells derived from four unaffected males. Differential gene expression noted that the fibroblasts expressing the mutant protein exhibited deregulation of ~200 genes, while differential alternative splicing analysis indicated an alteration in the processing of

RNA transcripts, particularly a substantial change in the number of skipped exon events. These studies suggest that defects in splicing are a critical outcome of *de novo* or inherited mutation of HNRNPF/H genes. Hopefully, future studies, including the generation of mouse models, will address how such splicing changes result in the clinical phenotypes observed in individuals harboring mutations in these genes.

4.2 HNRNPF/H genes and other neuro-related disease states

4.2.1 Neurodegenerative disorders—The genetic basis of a substantial proportion of the neurodegenerative disorders amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) is the expansion of a GGGGCC (G_4C_2) repeat located in intron 1 of *C9ORF72* from an average of two repeats in healthy cells to several hundred or even over a thousand repeats in affected cells (Zampatti et al., 2022). The mechanisms by which expanded G_4C_2 repeats contribute to disease development remain unclear, but one focuses on the secondary conformations of transcripts containing expanded G_4C_2 repeats could form, including stable rG4s and the recruitment of RNA-binding proteins to these G-rich RNA molecules (reviewed in Malik et al., 2021). Collectively, the intramolecular RNA-RNA and intermolecular RNA-protein interactions associated with expanded G_4C_2 repeats appear to promote the appearance of nuclear-retained RNA foci. For example, fluorescent *in situ* hybridization (FISH) analysis showed, on average, the presence of six RNA foci per nucleus following the expression of transcripts containing 38 G_4C_2 repeats and twelve RNA foci per nucleus when the expressed transcripts contained 72 G_4C_2 repeats (Lee et al., 2013). Importantly, analysis of the RNA foci formed by transcripts expressed from constructs containing 38 or 72 G_4C_2 repeats demonstrated colocalization with HNRNPH, and subsequent RNA-immunoprecipitation studies confirmed HNRNPH's binding of RNA containing the expanded G_4C_2 repeats (Lee et al., 2013). Furthermore, the analysis of an HNRNPH-dependent splicing event – inclusion of *TARBP2*-exon 7 – demonstrated slightly reduced retention of this exon in the presence of transcripts containing 72 G_4C_2 repeats (Lee et al., 2013). Critically, the analysis of ALS and FTD brain tissues also indicated the colocalization of G_4C_2-RNA foci and HNRNPH in the cerebellum (~70% of foci) (Lee et al., 2013). A complementary study of *C9ORF72*-G_4C_2-ALS-derived brain tissues confirmed the localization of HRNPF/H proteins (immunofluorescence) and *C9ORF72*-RNA foci (*in situ* hybridization) in cerebellar granule cells and motor neuron cells (Cooper-Knock et al., 2014).

The results reported by Lee and colleagues prompted them to speculate that expanded G_4C_2 repeats sequester HNRNPH and that disruption of HNRNPH function could contribute to the pathology of ALS and FTD. A subsequent study by Conlon et al. built upon this concept (Conlon et al., 2016). First, they confirmed HNRNPH's interaction with RNAs containing *C9ORF72*-G_4C_2 repeats and that G_4C_2 repeats could form stable rG4 structures. To address the clinical relevance of their initial *in vitro* and cell line-based studies, Conlon and colleagues next examined the nuclear localization of HNRNPH proteins and rG4s in cells and tissue obtained from individuals with *C9ORF72*-G_4C_2-ALS compared with control samples. Among their reported findings, they detected many more HNRNPH-positive RNA foci in spinal cord sections from ALS patients than in non-ALS control samples. To assess the potential functional consequences of the observed altered localization of HNRNPH,

Conlon et al. next compared the outcome of putative and established HNRNPH-dependent alternative splicing events using RNA purified from postmortem-cerebellum. PCR analysis demonstrated differences in the percent inclusion of specific exons when compared to control and *C9ORF72*-G_4C_2-ALS-derived samples, a finding they confirmed and extended through analysis of RNA-seq data. Critically, this study determined that the motor cortex of *C9ORF72*-G_4C_2-ALS-derived tissue contained more insoluble HNRNPH protein (1.9 x) than control samples. Supporting data suggested that this increase in insoluble HNRNPH is related to the presence of rG4s formed by the expanded G_4C_2 repeats. However, it is still unclear to what extent alterations in the function of HNRNPF/H proteins contribute to the pathology of *C9ORF72*-G_4C_2-ALS and FTD; a more recent study offers evidence that sequestration of HNRNPH proteins does have transcriptome-wide effects (Wang et al., 2020). Specifically, Wang et al. demonstrated that elevated levels of the HNRNPH proteins in the insoluble fraction of *C9ORF72*-G_4C_2-ALS-derived samples are associated with increased intron retention events compared to control samples (healthy individuals and ALS-derived samples with less HNRNPH protein in the insoluble fraction). The function of the genes encoding the transcripts that exhibited enhanced intron retention indicated enrichment for those that function in many pathways implicated in ALS, including those coding for proteasome subunits. This study also reported enrichment for the presence of at least one HNRNPH binding site close to the 5' splice site of introns retained in *C9ORF72*-G_4C_2-ALS-derived samples (Wang et al., 2020).

4.2.2 Congenital myasthenic syndromes and other neuromuscular disorders

—Congenital myasthenic syndromes (CMS) encompasses a heterogeneous group of disorders characterized by muscle weakness that arise because of mutations in genes encoding proteins involved in neuromuscular transmission, including subunits of the acetylcholine receptor (AChR) and COLQ, a protein that anchors acetylcholinesterase on the surface of muscle fibers (Finsterer, 2019). The *CHRNA1* gene expresses a transcript variant ENST00000348749, consisting of nine exons, which encodes the full-length alpha subunit of AChR. Another variant, ENST00000261007, includes an additional 75 nt in-frame exon between *CHRNA1*-exons 3 and 4. Frequently referred to as *CHRNA1*-exon P3A, the inclusion of this exon results in a transcript that encodes a nonfunctional AChR protein that does not localize to the cell surface. Early studies (Beeson et al., 1990) and more recent RNA-seq analysis show that skeletal muscle expresses both variants (e.g., GTEx resources: ENST00000348749 – 28.8 TPM and ENST00000261007 – 12.1 TPM). In 2008, Masuda and colleagues demonstrated that an individual with severe myasthenic symptoms harbored two heterozygous *CHRNA1* mutations, one of which they identified as a G >A mutation within the intron preceding *CHRNA1*-exon P3A (Masuda et al., 2008). Subsequent analysis demonstrated that this mutation disrupts an intron splicing silencer (ISS) that HNRNPH can bind. Furthermore, using minigenes to model the relevant region of *CHRNA1*, Masuda and co-workers demonstrated that depletion of HNRNPH results in an increased inclusion of the *CHRNA1*-exon P3A (Masuda et al., 2008). In their discussion, the authors speculate that the HNRNPH-dependent exclusion of *CHRNA1*-exon P3A contributes to the synapse-specific expression of AChR, though this hypothesis still needs evaluation. Mutations in COLQ, which result in acetylcholinesterase deficiency at neuromuscular junctions, are also associated with CMS. Interestingly, one *COLQ* mutation involves the

HNRNPH1/HNRNPH2's regulation of splicing. Specifically, a missense mutation in *COLQ*, (c.1244A>G (p.(E415G) (Kimbell et al., 2004), results in a *COLQ*-exon 16 exclusion event (Rahman et al., 2015) that likely generates a transcript encoding a truncated protein lacking its C-terminal domain. Further analysis of the consequence of the c.1244A>G mutation focused on its alteration of a G-rich motif from GGAGG to GGGGG and a shift from the binding of SRSF1, which favors exon inclusion, to the binding of HNRNPH, which in this context, promotes exon exclusion (Rahman et al., 2015).

4.2.3 Addictive behavior—Addictive behaviors and addiction develop because of the complex interaction of many genetic and environmental risk factors, including the changes in gene expression promoted by a particular stimulus. The isolation of regulators of gene expression that may contribute to addictive behaviors and addiction has proven challenging; however, an increasing number of reports have described the critical function of RNA binding proteins in regulating the expression of proteins that influence an addiction-related phenotype, including members of the HNRNPF/H protein family (Bryant & Yazdani, 2016). Insights into the potential function of HNRNPH1 in altering the response to addictive stimuli, such as methamphetamine, emerged from quantitative trait locus (QTL) mapping in mice (Bryant et al., 2009; Parker et al., 2012). Subsequent fine mapping of one mouse chromosome 11 QTL highlighted a genomic region containing the *Hnrnph1* and *Rufy1* genes (Yazdani et al., 2015). Employing TALEN-mediated gene editing to introduce frameshift deletions into each gene, Yazdani et al. next generated *Hnrnph1*$^{+/-}$ and *Rufy1*$^{+/-}$ mice lines, assessed the expression of the targeted gene, and the sensitivity of the mice to methamphetamine compared to wild-type mice. Molecular analysis of *Hnrnph1*$^{+/-}$ mice confirmed the disrupted expression of *Hnrnph1*, and compared to their wild-type litter mates, a reduced increase in locomotor activity following the administration of methamphetamine. Disruption of the *Rufy1* locus generated no phenotypic differences.

In a follow-up study that further characterized the phenotype of *Hnrnph1*$^{+/-}$ mice, Ruan et al. demonstrated that the disrupted allele (a frameshift 16 bp deletion of the first coding exon of *Hnrnph1*) expresses an mRNA containing a premature stop codon (Ruan, Yazdani, Blum, et al., 2020). Using complementary assays of behavior, assessment of brain metabolism, and protein expression, Ruan and colleagues confirmed that *Hnrnph1*$^{+/-}$ mice exhibit reduced behavioral responses to methamphetamine and a decrease in the methamphetamine-induced release of dopamine in the nucleus accumbens region of the brain (Ruan, Yazdani, Blum, et al., 2020). Interestingly, the authors also linked alterations in the synaptic localization of the Hnrnph protein to a difference in mitochondrial function in the brain of *Hnrnph1*$^{+/-}$mice following exposure to methamphetamine (Ruan, Yazdani, Blum, et al., 2020). Further analysis of the murine *Hnrnph1* locus (specifically, its 5' UTR) determined that alterations in the levels of Hnrnph protein expression are sufficient to result in quantifiable differences in the behavioral response of mice to methamphetamine (Ruan, Yazdani, Reed, et al., 2020). Of note, an assessment of the sensitivity of *Hnrnph1*$^{+/-}$mice to other stimuli associated with addictive behavior showed that male mice consumed less alcohol than wild-type controls or female heterozygous mice (Fultz et al., 2021). Furthermore, Bryant et al. observed sex-independent and sex-specific effects when they examined the response of *Hnrnph1*$^{+/-}$ mice to the opioid fentanyl, nevertheless some effects, e.g., alterations in locomotor activity,

were less pronounced than that observed following exposure to methamphetamine (Bryant et al., 2021). The mechanistic basis for these observations remains to be determined, but one clue may come from a study of the *OPRM1 gene. OPRM1* codes for the mu-opioid receptor. Two studies have implicated the HNRNPF/H proteins in regulating the expression of *OPRM1* transcripts (Song et al., 2012), including the sequence-specific binding of an intron splice enhancer sequence by HNRNPH1 that increases the likelihood of a specific exon exclusion event and differences in opioid dependency (Xu et al., 2014).

5 GRSF1, mitochondrial homeostasis, and cellular senescence

Cellular senescence, a state in which cells remain metabolically active but exhibit permanent cell cycle arrest, is a critical feature of aging, age-related disease, and other disease states, including cancer (Hernandez-Segura et al., 2018; McHugh & Gil, 2018; Ou et al., 2021). One feature of senescent cells is alterations in mitochondrial function and the accumulation of dysfunctional mitochondria (Ghosh-Choudhary et al., 2021). As discussed in sections 2.2 and 2.3, GRSF1 is the only HNRNPF/H protein that localizes to the cytoplasm, specifically mitochondria, where it is a component of the RNA granules that process precursor polycistronic mitochondrial RNAs and facilitates the loading of mature mRNA onto mitochondrial ribosomes. These observations have prompted several studies focused on exploring the function of GRSF1 in the maintenance of mitochondrial function and cellular senescence. As a starting point, Noh, Kim, and colleagues demonstrated that GRSF1 was one of only two RBPs that exhibited reduced expression in senescent fibroblasts and confirmed using a cell-based model of replicative senescence that GRSF1 protein levels decrease following the induction of senescence (Noh et al., 2018). A complementary report by Kim and colleagues confirmed the decrease in GRSF1 expression following the induction of senescence in a cell-based model and extended this observation to the study of liver, muscle, and heart tissues harvested from mice at 4, 18, and 32 months (Kim et al., 2019). PCR-based analysis of *GRSF1* mRNA levels in murine muscle and liver showed substantial decreases at the later time-points, and analysis of GRSF1 protein levels in muscle and heart showed similar declines, though the results for liver proved variable. It is unclear which mechanism or mechanisms result in the senescence-associated decrease in GRSF1 expression. However, the study by Noh, Kim, and co-workers reported evidence that in senescent fibroblasts, GRSF1 protein exhibits reduced stability (Noh et al., 2018), while Kim and colleagues describe an increase in the methylation of the *GRSF1* promoter in senescent cells compared to control cells (Kim et al., 2019).

Decreased expression of *GRSF1*, mediated using RNAi or CRISPR-Cas9 gene editing of HEK-293T cells, induces DNA damage, suppresses cell proliferation, and accelerates senescence following its induction (Noh et al., 2018; Noh et al., 2019). Associated with the acceleration of senescence was the enhanced secretion of interleukin 6 (IL6), a component of the senescence-associated secretory phenotype (SASP) (Noh et al., 2018; Noh et al., 2019). Analysis of the *GRSF1*-HEK-293T knockout cell lines also demonstrated reduced levels of mitochondrial protein complex components, increases in glycolysis, and changes in the redox balance of cells (Noh et al., 2019). Collectively, these results suggest that GRSF1 function is critical for maintaining mitochondrial activity. Furthermore, an examination of the mechanism of IL6 induction following GRSF1 loss revealed a signaling cascade that

results in the activation of mTOR and NFκB and the presence of a feedback mechanism that promotes the secretion of pro-inflammatory factors and SASP (Noh et al., 2019). These findings have implications for GRSF1 as a regulator of inflammation and warrant further studies to determine if its decreased expression contributes to the elevated levels of IL6 observed in chronic inflammation and cancer. However, one part of such efforts will need to consider the *in vivo* consequences of altered GRSF1 expression. As a first step in assessing GRSF1's function in a whole organism, Driscoll, Krasniewski, and colleagues generated tissue-specific knockout mice in which they ablated expression of *Grsf1* in skeletal muscle (Grsf1cKO) (Driscoll et al., 2021). Interestingly, the Grsf1cKO mice exhibited no overt phenotype until 16 to 18 months of age, when their endurance in a treadmill exercise test reduced significantly. However, this observation was not associated with any histological changes in skeletal muscle or detection of oxidative stress. It is unclear at this time if differences between results obtained using cell lines and those observed *in vivo* reflect the presence of compensatory mechanisms or if other tissue types may prove more sensitive to loss of GRSF1. For example, investigation of hematopoiesis may prove informative as homozygous *Grsf1* knockout mice ($Grsf1^{tm1b(EUCOMM)Wts1}$) exhibit decreased leukocyte cell numbers compared to control animals.

6 Conclusion

The need to understand both the shared and distinguishing functions of members of the HNRNPF/H family of RBPs in the regulation of gene expression has increased with the recent identification of several protein-specific disease-related phenotypes. Critical to addressing this need is the generation of resources that will aid the selective detection or perturbation of each family member. For example, the homology of these proteins has limited the utility of antibodies to detect family members, particularly HNRNPH1 and HNRNPH2, selectively. However, at least in cell-based systems, the specific tagging of these proteins through the employment of gene editing strategies should enhance our ability to distinguish, for example, the transcripts they bind and the analysis of their functions. Accompanying such efforts is the need for additional whole organism models, particularly mouse models that assess the effect of the recurrent mutations identified in the disease states discussed in this review. Critical questions that the generation of novel resources should focus on addressing include an enhanced understanding of the effect of the mutations that cluster between the qRRM2 and the GYR regions of the HNRNPF/H proteins. Specifically, do these mutations alter the cellular localization of the HNRNPF/H, as some early studies may suggest, or could they also alter the RNA binding functions of these proteins or have other consequences? To inform our understanding of how this protein family contributes to transcript diversity, we also need further analysis of the molecular, biophysical, and structural characteristics of the interaction of each protein with G-tracts of varied sizes and sequence configurations (e.g., single G-tracts, interspersed G-tracts of diverse sizes), RNA conformations (e.g., G4 or non-G-states), and context (e.g., within exons or introns). Such efforts will be critical to further delineating how members of the HNRNPF/H protein family contribute to pathology and, in time, improve the diagnosis and treatment of diseases involving these proteins.

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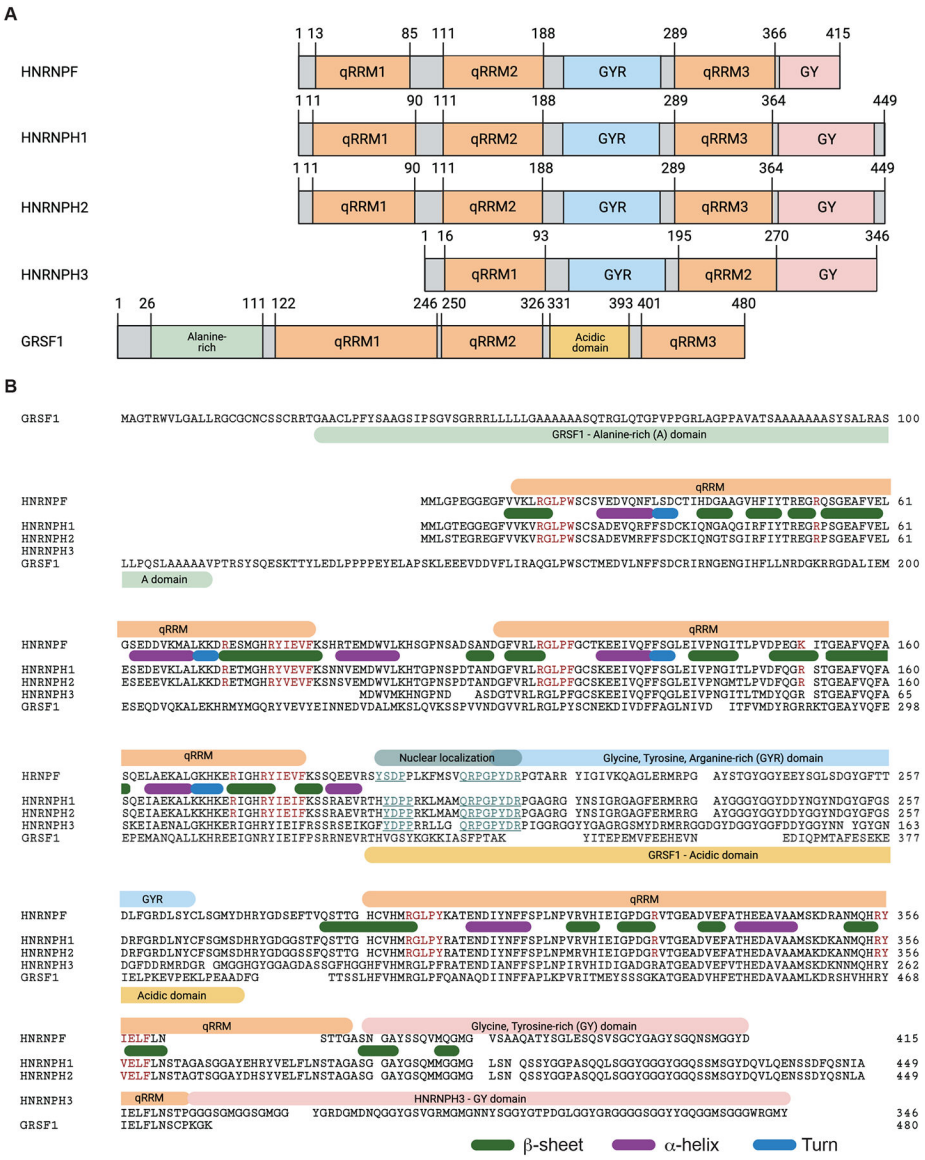

Figure 1. An overview of the HNRNPF/H proteins.

(**A**) Schematics of the HNRNPF/H protein family. The indicated domains are based on the UniProt reference human proteins as follows: HNRNPF - P52597, HNRNPH1 – P31943, HNRNPH2 – P55795, HNRNPH3 – P31942, GRSF1 – Q12849. (**B**) The amino acid sequence alignment of the reference HNRNPF/H proteins were generated using R-packages msa (Bodenhofer et al., 2015) and ggmsa (Zhou et al., 2022). The indicated qRRMs, related structural elements (β-sheet, α-helix, turns), and low complexity domains are based on those reported for HNRNPF (P52597), except for the C-terminus of HNRNPH3 (P31942) and the additional GRSF1 domains (Q12849). The amino acids in red indicate residues that contact RNA, and those in blue, indicate residues that form a putative nuclear localization signal. Figure panels created with BioRender.com.

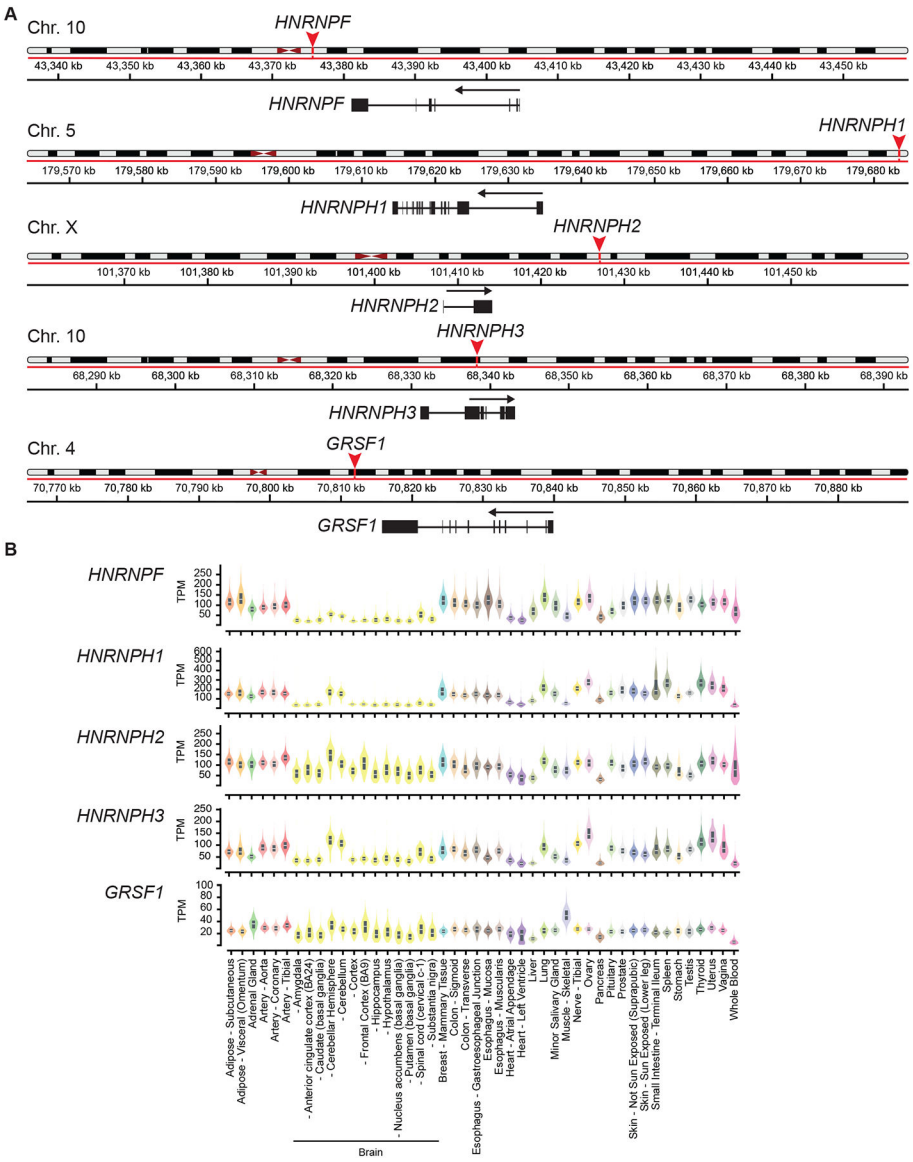

Figure 2. An overview of the HNRNPF/H genes.

(**A**) Schematics of the location and organization of genes encoding members of the HNRNPF/H proteins (GTEx Integrated Genome Viewer). (**B**) An overview of gene level expression of each HNRNPF/H family member assessed using bulk RNA sequencing of selected human tissues. Data extracted from GTEx Analysis Release V8 (dbGaP Accession phs000424.v8.p2; please note the sampling biases in these datasets discussed in (Garsetti et al., 2022).

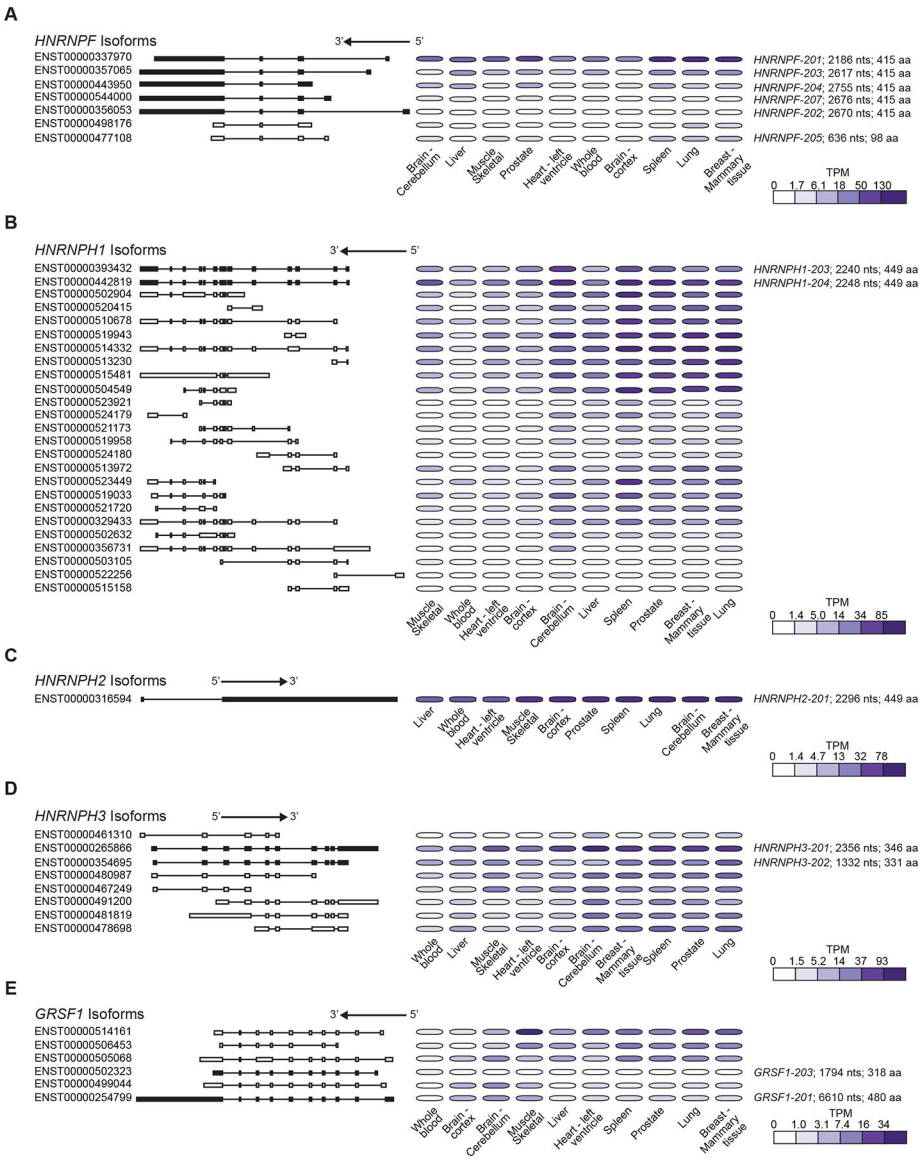

Figure 3. *HNRNPF/H* transcript variant expression.

The organization and expression of selected (**A**) *HNRNPF*, (**B**) *HNRNPH1*, (**C**) *HNRNPH2*, (**D**) *HNRNPH3*, and (**E**) *GRSF1* transcript variants in selected tissues. Data extracted from GTEx Analysis Release V8 (dbGaP Accession phs000424.v8.p2; please note the sampling biases in these datasets discussed in (Garsetti et al., 2022). The arrow indicates the direction of expression.

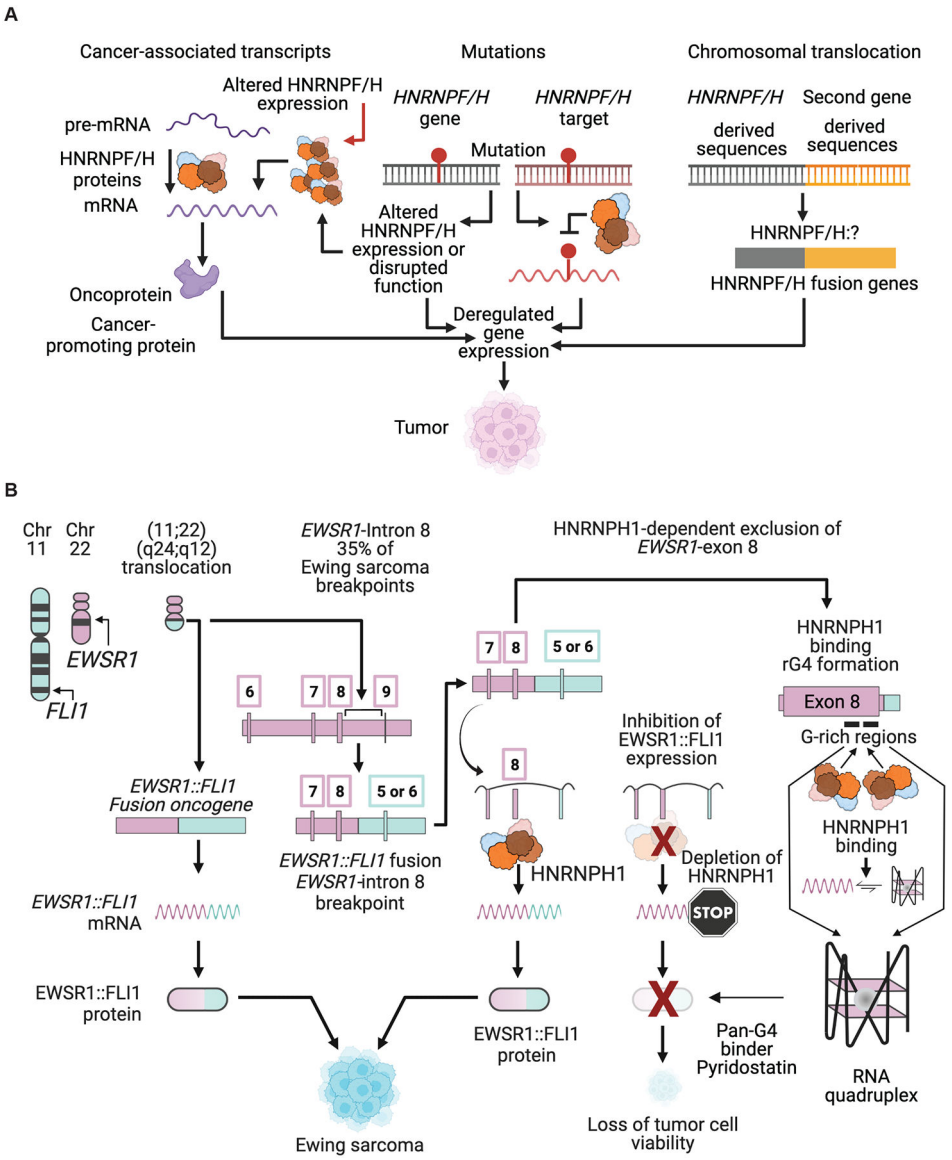

Figure 4. HNRNPF/H proteins and the expression of cancer-specific transcripts.

(**A**) Schematics of the mechanisms by which HNRNPF/H genes or proteins can contribute to tumorigenesis. Mechanisms shown are the HNRNPF/H-dependent processing of cancer-specific transcripts, the effect of mutations that disrupt HNRNPF/H protein expression or function or the binding sites of these proteins, and the involvement of HNRNPF/H genes in chromosomal translocations. (**B**) Schematic of the HNRNPH1-dependent exclusion of *EWSR1*-exon 8 present in the *EWSR1::FLI1* transcripts expressed in a subset of the pediatric tumor Ewing sarcoma (see Grohar et al., 2016; Neckles et al., 2019; Vo et al., 2022) for further details). *In vitro* analysis has demonstrated that HNRNPH1 binds *EWSR1*-exon 8 G-rich sequences in non-G4 and G4 states, and that its binding favors accumulation of the bound RNA in an unfolded state (Vo et al., 2022). Figure panels created with BioRender.com.

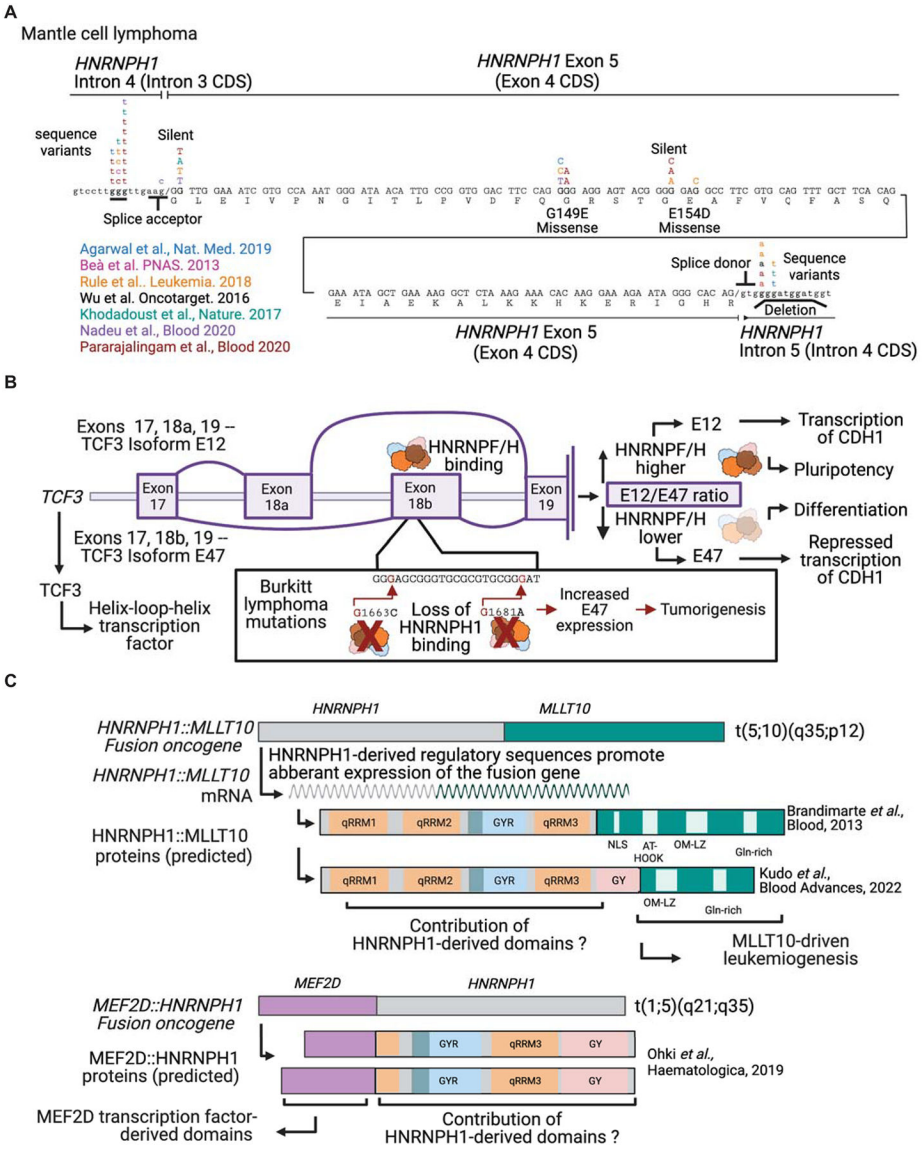

Figure 5. Cancer-associated mutations in HNRNPF/H genes.

(**A**) A schematic of the position of mutations in the *HNRNPH1* gene identified in mantle cell lymphomas reported by (Nadeu et al., 2020; Pararajalingam et al., 2020), with the latter study including reanalysis of the following studies (Agarwal et al., 2019; Beà et al., 2013; Khodadoust et al., 2017; Rule et al., 2018; Wu et al., 2016). (**B**) A schematic of the HNRNPF/H-dependent processing of TCF3 (transcription factor 3) transcript variants that express isoforms with different phenotypic outcomes and the effect of mutations that alter HNRNPF/H binding sites based on findings reported in Burkitt's lymphoma(Yamazaki et al., 2020; Yamazaki et al., 2018; Yamazaki et al., 2019). (**C**) Schematics of *MLLT10-HNRNPH1* fusion genes identified in pediatric T-acute lymphoblastic leukemia (Brandimarte et al., 2013) and pediatric acute myeloid leukemia (Kudo et al., 2022) and *HNRNPH2-MEF2D* identified in a case of pediatric B-cell precursor acute lymphoblastic leukemia (Ohki et al., 2019). MMT10 = MLLT10 histone lysine

methyltransferase DOT1L cofactor; MEF2D = myocyte enhancer factor 2D. Figure panels created with BioRender.com.

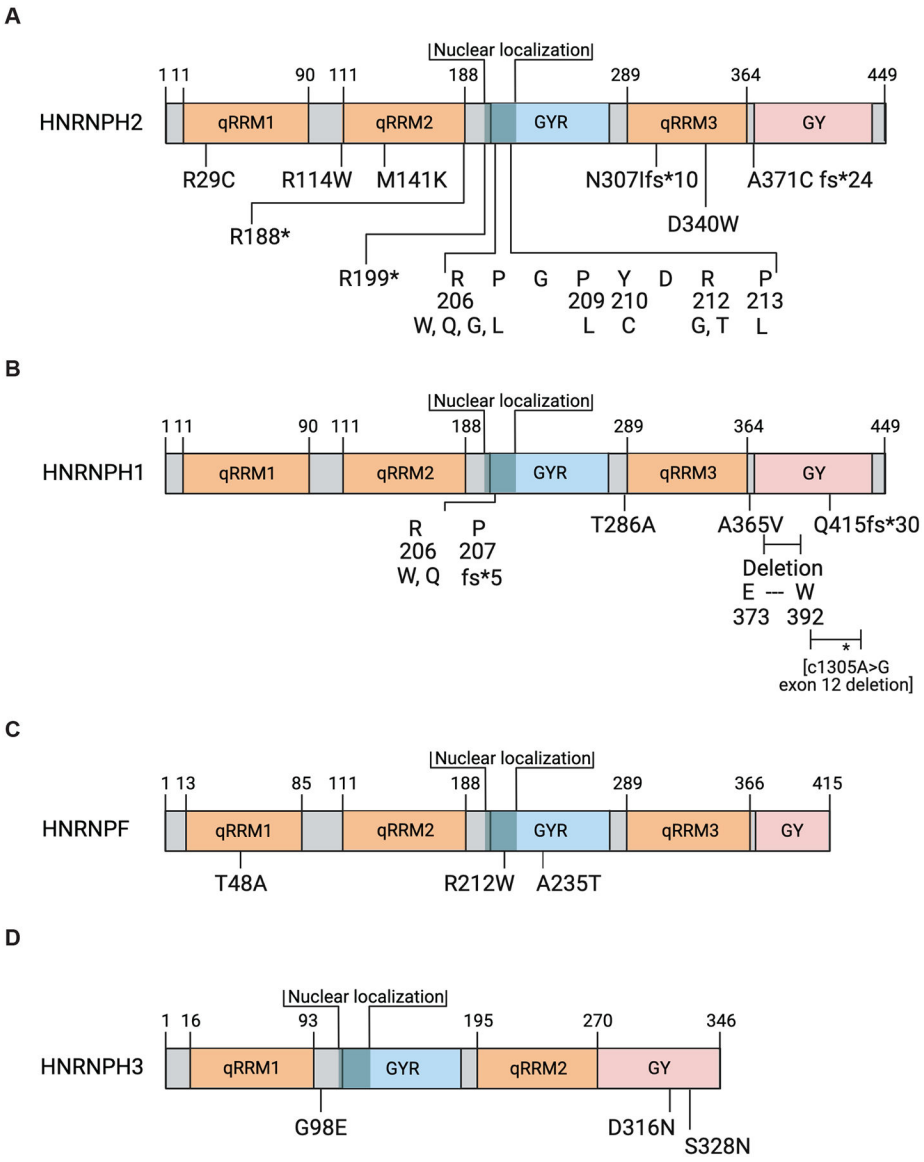

Figure 6. Mutations in *HNRNPF/H* genes and neurodevelopment disorders.

Schematics of **(A)** HNRNPH2, **(B)** HNRNPH1, **(C)** HNRNPF, and **(D)** HNRNPH3 proteins indicating the locations of amino acid changes predicted based on mutations identified in individuals diagnosed with a neurodevelopmental disorder and reported in the following studies: Bain et al., 2016; Bain et al., 2021; Epi et al., 2013; Gillentine et al., 2021; Harmsen et al., 2019; Jepsen et al., 2019; Kreienkamp et al., 2022; Peron et al., 2020; Pilch et al., 2018; Reichert et al., 2020; Somashekar et al., 2020; White-Brown et al., 2021. Figure panels created with BioRender.com.