Molecular basis for GIGYF–Me31B complex assembly in 4EHP-mediated translational repression

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GIGYF [Grb10-interacting GYF [glycine–tyrosine–phenylalanine domain]] proteins coordinate with 4EHP (eIF4E [eukaryotic initiation factor 4E] homologous protein), the DEAD (Asp–Glu–Ala–Asp)-box helicase Me31B/DDX6, and mRNA-binding proteins to elicit transcript-specific repression. However, the underlying molecular mechanism remains unclear. Here, we report that GIGYF contains a motif necessary and sufficient for direct interaction with Me31B/DDX6. A 2.4 Å crystal structure of the GIGYF–Me31B complex reveals that this motif arranges into a coil connected to a β hairpin on binding to conserved hydrophobic patches on the Me31B RecA2 domain. Structure-guided mutants indicate that 4EHP–GIGYF–DDX6 complex assembly is required for tristetraprolin-mediated down-regulation of an AU-rich mRNA, thus revealing the molecular principles of translational repression.

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Initiation of translation by the eukaryotic initiation factor 4E (eIF4E) is regulated by competitor cap-binding proteins of the eIF4E family, such as the elf4E homologous protein (4EHP; also known as elf4E2) [Kong and Lasko 2012]. 4EHP is responsible for the assembly of translational repressor complexes that inhibit mRNA expression in different biological contexts [Cho et al. 2005, 2006; Villaescusa et al. 2009; Chapat et al. 2017]. 4EHP specifically associates with Grb10-interacting GYF [glycine–tyrosine–phenylalanine domain] protein 1 (GIGYF1) and GIGYF2. These proteins possess an N-terminal 4EHP-binding region (4EHP-BR) and a central compacted GYF domain [Supplemental Fig. S1A; Ash et al. 2010; Peter et al. 2017] that mediates the interaction with ZN5F98, tristetraprolin [TTP], or the microRNA (miRNA)-induced silencing complex-associated TNRC6 proteins [Morita et al. 2012; Fu et al. 2016; Schopp et al. 2017]. These RNA-associated proteins recruit the 4EHP–GIGYF2 complex to specific mRNAs important for mouse embryonic development, cytokine mRNA expression, or repression of miRNA targets, respectively [Morita et al. 2012; Fu et al. 2016; Tollenare et al. 2019].

GIGYF proteins do not simply bridge 4EHP to the RNA-associated proteins but rather participate directly in the repression mechanism [Peter et al. 2017]. Human GIGYF2 regulates the expression of a subset of mRNAs via the recruitment of the CCR4–NOT complex [Amaya Ramirez et al. 2018]. GIGYF proteins also associate with DDX6 (Me31B in Drosophila melanogaster [Dm] and Dhh1p in yeast) [Amaya Ramirez et al. 2018; Ruscica et al. 2019], an important regulator of gene expression [Ostareck et al. 2014; Wang et al. 2015; Lumb et al. 2017] that acts as translational repressor and enhancer of mRNA decapping [Coller et al. 2001; Radhakrishnan et al. 2016].

DDX6 orthologs are RNA-dependent ATPases of the DEAD (Asp–Glu–Ala–Asp)-box family that feature two globular RecA-like domains [Reca1 and Reca2] connected by a flexible linker. DEAD-box proteins use ATP binding and hydrolysis coupled to RNA binding to promote conformational transitions and remodeling of RNA and/or ribonucleoprotein particles (mRNPs) [Ozgur et al. 2015b]. DDX6 has restricted conformational flexibility and limited ATPase activity and requires stimulation by interacting factors [Mathys et al. 2014].

DDX6 assembles in mutually exclusive complexes with P-body components such as EDC3, LSM14A, PatL1, and the elf4E transporter protein (4E-T) [Jonas and Izaurralde 2013]. These proteins use different short linear motifs to associate with two binding pockets in the RecA2 domain of DDX6, referred to here as Phe–Asp–Phe (FDF) and Trp [W] pockets [Tritschler et al. 2008; Sharif et al. 2013; Ozgur et al. 2015a; Brandmann et al. 2018].

To elucidate how GIGYF proteins function together with DDX6 in the regulation of mRNA expression, we determined the crystal structure of an N-terminal conserved motif from Dm GIGYF that mediates direct binding to Me31B [Fig. 1A; Supplemental Fig. S1A]. This binding motif, characterized by a Pro–Glu–Trp (PEW) sequence and a “split” FDF sequence, binds to Me31B in a unique manner. We further show that recruitment of DDX6 via GIGYF2 is required in human cells for efficient translational repression of an AU-rich reporter mRNA by TTP. Collectively, these data have advanced our understanding of the molecular principles governing the assembly of mRNPs that rely on the 4EHP–GIGYF complex and DDX6 proteins to posttranscriptionally regulate gene expression.
Figure 1. GIGYF proteins contain a conserved Me31B/DDX6-binding motif (MBM). [A] Sequence alignment of the MBM of Dm and Homo sapiens (Hs) GIGYF with the CUP homology domain (CHD) of Dm and Hs 4E-T and Dm CUP. Residues with >70% similarity are shown with a light-colored background. Conserved residues are highlighted with a darker background and are printed in white. Secondary structure elements based on the structures presented in this study are indicated above the Dm GIGYF sequence. Boxed residues highlight the PEW [green] and FDF/IEL [black] motifs. The asterisk indicates the polar residue preceding the FDF motif. [B] The binding of HA-Dm GIGYF (FL or the indicated proteins) to Me31B was analyzed in coimmunoprecipitation (co-IP) assays using anti-HA antibodies upon S2 cell transfection. HA-MBP served as a negative control. The input [1.5% for the HA proteins and 0.2% for Me31B] and immunoprecipitated [30% for the HA proteins and 45% for Me31B] fractions were analyzed by Western blotting using anti-HA and anti-Me31B antibodies. (C) The interaction between GFP-Dm Me31B (FL or the indicated RecA domains) and HA-Dm GIGYF N-terminal expressed in S2 cells was analyzed in co-IP assays using anti-GFP antibodies. GFP-MBP served as a negative control. Input [3% for the GFP proteins and 1% for the HA proteins] and immunoprecipitated [15% for the GFP proteins and 30% for the HA proteins] fractions were analyzed by Western blotting using anti-GFP and anti-HA antibodies. [D] GST pull-down assay showing the interaction between the GST-Me31B RecA2 domain and the MBP-tagged Dm GIGYF and human GIGYF1/2 MBM by pull-down [Fig. 1D; Supplemental Fig. S1F,G]. The MBM thus has a crucial role in mediating a direct and stable interaction between GIGYF and DDX6.

Results and Discussion

The GIGYF linear motif is necessary and sufficient to directly bind Me31B/DDX6

The GIGYF orthologs contain a short conserved sequence motif with partial similarity to the CUP homology domain (CHD) present in 4E-T proteins [Fig. 1A; Kamencka et al. 2014; Ruscica et al. 2019]. Deletion of this Me31B/DDX6-binding motif (MBM) abrogated the interaction of Me31B/DDX6 with transiently expressed and tagged GIGYF [Dm GIGYF and Homo sapiens (Hs) GIGYF1/2] in Drosophila and human cells [Fig. 1B; Supplemental Fig. S1B,C; Ruscica et al. 2019]. The MBM alone interacted with Me31B/DDX6 as efficiently as full-length (FL) GIGYF or the N-terminal fragment of GIGYF [Fig. 1B; Supplemental Fig. S1B,C], indicating that the MBM is necessary and sufficient for a stable interaction between the proteins.

In coimmunoprecipitation (co-IP) assays, GIGYF proteins associated with the RecA2, but not the RecA1, domain of Dm Me31B and human DDX6 [Fig. 1C; Supplemental Fig. S1D,E], as observed previously for other DDX6-interacting factors [Tritschler et al. 2009; Sharif et al. 2013; Ozgur et al. 2015a; Brandmann et al. 2018]. The purified recombinant GST-tagged RecA2 domain of Me31B/DDX6 associated with MBP-tagged Dm GIGYF and human GIGYF1/2 MBM by pull-down [Fig. 1D; Supplemental Fig. S1F,G]. The MBM thus has a crucial role in mediating a direct and stable interaction between GIGYF and DDX6.

The Dm GIGYF MBM interacts with Me31B using a bipartite mode

We hypothesized that the GIGYF MBM binds to the W pocket of DDX6 via the conserved PEW motif because of the apparent sequence similarity to the CHD region of 4E-T [Fig. 1A; Ozgur et al. 2015a]. However, the presence of alanine or serine in place of the second phenylalanine in the FDF-like motif [FDA/S] and the absence of an Ile–Glu–Leu [IEL] motif as observed in 4E-T suggest that the binding mode to the conserved hydrophobic D pocket of DDX6 may have diverged. To investigate this further, we determined the crystal structure of the Dm GIGYF MBM [residues D343–G369] in complex with the RecA2 domain of Me31B [residues E264–V431] to 2.4 Å resolution [Supplemental Table S1].

The RecA2 domain of Me31B adopts a typical α/β-fold characterized by a central six-stranded parallel β sheet covered by α-helical layers on either side [Fig. 2A; Cheng et al. 2005]. In the structure of the complex, the GIGYF MBM curves around helices a10 and a11 of Me31B to engage the conserved DFX and W pockets—known binding sites for Hs 4E-T and Saccharomyces cerevisiae [Sc] Edc3, Sc Pat1, and Hs LSM14A [Fig. 2A; Supplemental Fig. S2A–F; Tritschler et al. 2008; Sharif et al. 2013; Ozgur et al. 2015a; Brandmann et al. 2018]. Two distinct structured elements can be identified in the MBM: a short coiled running along helix a11 of Me31B and a β hairpin containing a “split” β motif [DFx,β] [Fig. 2B–D].

The N-terminal PEW [P347, E348, and W349] peptide trio of the GIGYF MBM initiates a short coil that inserts the aromatic side chain of W349GIGYF into the hydrophobic pocket formed by residues V283, L310, L311, and F370 between helices a10 and a11 of Me31B [Fig. 2B; Supplemental Fig. S3A]. Other DDX6 interactors also feature a large aromatic residue [W91 in Sc Edc3, F192 in Hs EDC3, F42 in Sc Pat1, or F396 in Hs LSM14A] inserted at the equivalent pocket of Dhh1/DDX6 [Supplemental Figs. S3, S4A–C]. Hydrogen bonding between the side chains of Q306Me31B and K314Me31B and the backbone oxygens of N345GIGYF and A350GIGYF lends additional stability to the interface [Fig. 2B].

The PEW sequence of the GIGYF MBM is then connected via a flexible linker to a β-hairpin structure formed at the DFX pocket of Me31B [Fig. 2A,C,D]. The β hairpin serves to orient the FDF motif [F361, D362, and F367GIGYF] to optimally engage Me31B. The F361 and F367GIGYF are in positions structurally equivalent to those observed previously in other DDX or IEL sequences [Supplemental Fig. S5; Tritschler et al. 2008; Sharif et al. 2013; Ozgur et al. 2015a; Brandmann et al. 2018]. The
The GIGYF FD$_{x4}$F motif does not block NOT1 binding to DDX6

NOT1, the scaffold protein of the CCR4–NOT deadenylase complex, interacts with a surface of RecA2 domain adjacent to but not overlapping with the surface engaged by the other DDX6 interactors (Supplemental Fig. S2A). Consistently, these binding studies are consistent with a differential contribution of the two binding pockets in DDX6 toward promoting stable interactions with various factors. Reported differences in the binding affinities further support this model: Both Sc Pat1 and Sc EDC3 are high-affinity binders of Sc Dhh1 ($K_{D}$ of 50 nM and 200 nM, respectively) [Sharif et al. 2013]; human DDX6 interactors are rather more diverse in their affinities, with reported $K_{D}$s of 230 nM for PatL1, 390 nM for 4E-T, 410 nM for EDC3, and 1.62 µM for LSM14A [Brandmann et al. 2018].

The bipartite binding mode is essential for GIGYF–DDX6 complex assembly

Guided by the structural analysis of the binding interfaces, we next substituted key residues on the W (LK-AA mutant) or FDF (CL-AA mutant) pockets in Me31B/DDX6 [Supplemental Table S2; Supplemental Fig. S4D] and tested binding by co-IP following transient expression in either Dm S2 or human cells. The interaction of GIGYF with Me31B/DDX6 was strongly impaired by individual or combined pocket mutations [Fig. 3A,B], pointing to a crucial functional role for both binding pockets in stabilizing the association between the proteins.

Conversely, we also analyzed the effect of amino acid substitutions in GIGYF on the interaction with DDX6. Tryptophan substitution by alanine in the PEW motif (W* mutant), of both phenylalanines in the FD$_{x4}$F motif (FF* mutant), or in combination (WFF* mutant) [Supplemental Table S2] abolished the interaction of Dm and human GIGYF with Me31B/DDX6 in cells (Fig. 3C,D, Supplemental Fig. S6A).

DDm HPat and human PatL1 do not contain an FDF motif but rather contain a DW sequence motif that interacts with Me31B/DDX6 [Supplemental Fig. S4A]. Interestingly, the mutations in the W and FDF pockets of Me31B/DDX6 also strongly reduced binding to HPat/PatL1, which is consistent with previous observations (Fig. 3A, B; Sharif et al. 2013). However, the disruption of the FDF pocket (CL-AA mutant) did not affect the association of Me31B/DDX6 with 4E-T or LSM14A and only mildly impaired binding to EDC3 [Dm and Hs] [Fig. 3A, Supplemental Fig. S6B–D]. In contrast, the substitutions in the W pocket strongly reduced binding to Dm and human 4E-T, EDC3, and LSM14A [Fig. 3A; Supplemental Fig. S6B–D].

Collectively, these binding studies are consistent with a differential contribution of the two binding pockets in DDX6 toward promoting stable interactions with various factors. Reported differences in the binding affinities further support this model: Both Sc Pat1 and Sc EDC3 are high-affinity binders of Sc Dhh1 ($K_{D}$ of 50 nM and 200 nM, respectively) [Sharif et al. 2013]; human DDX6 interactors are rather more diverse in their affinities, with reported $K_{D}$s of 230 nM for PatL1, 390 nM for 4E-T, 410 nM for EDC3, and 1.62 µM for LSM14A [Brandmann et al. 2018].
To explore the functional relevance of the GIGYF–DDX6 interaction, we investigated the regulation of mRNA expression by TTP in human cells. TTP represses the expression of AU-rich transcripts via the recruitment of the 4EHP–GIGYF2 complex ([Po et al. 2016; Po et al. 2017]). To test the TTP-mediated repression in a reporter assay, we chose a Renilla luciferase (R-Luc) mRNA with two copies of the TNF-α mRNA AU-rich element (ARE) in the 3′ untranslated region (UTR) [Supplemental Fig. S7A]. To distinguish the consequences of translational repression from degradation, an internal polyadenosine sequence of 90 nucleotides was “tailed” by a noncoding RNA MALAT1 sequence at the 3′ end, which is generated by RNase P endonuclease cleavage, rendering this reporter mRNA resistant to 5′–3′ decay [R-Luc-ARE-A90-MALAT1] [Peter et al. 2017]. A plasmid encoding firefly luciferase (F-Luc-GFP) was included as a transfection and normalization control.

To bypass the recruitment of DDX6 via NOT1, we transiently expressed a TTP construct lacking the NOT1-binding motif (ΔCIM) [Fabian et al. 2013]. We observed that TTP ΔCIM efficiently repressed the expression of the R-Luc reporter without altering its mRNA abundance in control cells [Fig. 4A; Supplemental Fig. S7B,C]. By comparison, TTP-induced translational repression was alleviated in GIGYF1/2-null cells (GIGYF1/2 knockdown) even though the observed level of TTP expression was comparable with that in the control cells [Fig. 4, A, B, lane 4 vs. lane 2]. In GIGYF1/2-null cells, TTP-mediated translational repression was restored by coexpression of GIGYF2 and its stabilizing partner, 4EHP [Fig. 4A,B]. However, the repressive function of TTP could be selectively impaired when 4EHP was coexpressed with the GIGYF2 mutants that do not bind to DDX6 [WF∗] or TTP [WF∗] [Fig. 4A,B, Supplemental Fig. S7D]. The repressive function of TTP was critically dependent on the ARE, as none of the factors influenced the expression of a reporter lacking this sequence [R-Luc-A95-MALAT1] [Supplemental Fig. S7E,F]. Collectively, these data support a model in which the assembly of the 4EHP–GIGYF2–DDX6 complex is a prerequisite for TTP-mediated translational control of AU-rich transcripts.

**Concluding remarks**

In this study, we showed that GIGYF proteins interact directly with the RNA-dependent ATPase DDX6 via a short motif. This interaction is mutually exclusive with other DDX6-binding partners such as 4E-T, EDC3, LSM14A, and PatL1 and has an important functional role in posttranscriptional regulation [Fig. 4C]. We showed that GIGYF2 is a direct link between DDX6 and TTP, which explains at the molecular level why DDX6 is required for ARE mRNA translational repression [Qi et al. 2012]. The GIGYF–4EHP complex can also be part of TTP-independent mRNPs via direct mRNA binding [Amaya Ramirez et al. 2018] or the interaction with ZNF598 and TNRC6 proteins [Morita et al. 2012; Schopp et al. 2017]. As the latter are important in ribosome quality control [Garzia et al. 2017; Sundaramoorthy et al. 2017; Juszkiwicz et al. 2018] and miRNA-mediated gene silencing [Chapat et al. 2017], respectively, the control of mRNA expression by the 4EHP–GIGYF–DDX6 complex is relevant for a wide range of cellular transcripts. Furthermore, as all of the components of the complex have been implicated to function in miRNA-mediated translational repression [Chen et al. 2014; Mathys et al. 2014; Chapat et al. 2017; Schopp et al. 2017], the 4EHP–GIGYF–DDX6 complex is likely to have an important role in miRNA-mediated mechanisms.

**Materials and methods**

**DNA constructs**

The DNA constructs used in this study are described in the Supplemental Material and listed in Supplemental Table S2. All of the constructs and mutations were confirmed by sequencing.

**Protein production and purification**

The experimental procedures for the production and purification of recombinant proteins are described in the Supplemental Material.
HEK293T cells [wild-type or GIGYF1/2-null cells] were seeded in six-well plates (0.6 x 10^6 cells per well) and transfected using Lipofectamine 2000 [Invitrogen]. The transfection mixtures contained 1 µg of the R-Luc reporter and 0.25 µg of the F-Luc control in the presence of 50 ng of XN-HA-TPPΔCIM, 0.2 µg of GFP-MBP, 1 µg of GFP-GIGYF2;[wild type or mutants], or 0.5 µg of XN-HA-4EHP. F-Luc and R-Luc activities were measured 2 d after transfection using the dual-luciferase reporter assay system [Promega]. R-Luc activity was normalized to that of the F-Luc control and set to 100% in the absence of TTP in wild-type and GIGYF1/2-null cells. Total RNA was isolated using TriFast [Peqlab Biotechnologies], and the RNA samples were analyzed by Northern blot as described previously [Behm-Ansmant et al. 2006].

Data availability

Atomic coordinates and structure factors have been deposited in the Protein Data Bank under the accession codes 6S8R [Dm Me31B-GIGYF] and 6SSS [Hs DDX6-EDC3].

Acknowledgments

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Author contributions: D.P. purified, crystallized, collected the data, and determined the structure of the complex. E.V. contributed to structural data analysis. R.W. performed the complementation assay. P.B., S.H., and V.R. performed co-IP or pull-down assays. P.B. generated several of the constructs used in this study, and V.R. contributed to complex purification. C.I. coordinated the project. E.I. was the principal investigator. D.P., E.V., and C.I. wrote the manuscript. All authors corrected the manuscript.

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Crystalization, data collection, and structure determination

Detailed descriptions of the crystallization conditions and of the structure determination are in the Supplemental Material.

Co-IP assays and Western blotting

Co-IP assays in HEK293T and Schneider S2 cells were performed in the presence of RNase A as described previously [Peter et al. 2015a]. All Western blots were developed using the ECL Western blotting detection system [GE Healthcare]. The antibodies used in this study are listed in Supplemental Table S3.

Pull-down assays

The in vitro pull-down assays were performed as described previously [Igreja et al. 2014; Peter et al. 2015a,b]. The details are in the Supplemental Material.
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