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INTRINSICALLY DISORDERED REGION 2 OF G3BP1 IS REQUIRED FOR RNA RECRUITMENT BY G3BP1-CAPRIN1 COMPLEX

Aim. *The stress granule assembly requires coordinated interactions between RNA-binding proteins and mRNA, yet how the intrinsically disordered regions (IDRs) of stress granule proteins regulate RNA recruitment remains incompletely understood. The aim of this study was to characterize a role of the Intrinsically Disordered Region 2 (IDR2) of Ras GTPase-activating protein-binding protein 1(G3BP1) in mRNA recruitment within the G3BP1-Caprin1 complex.* ***Methods.*** *Protein-protein and protein-RNA interactions in cells were analyzed as colocalization of their fluorescent signals on microtubules (microtubule bench recruitment and mixing assay). Nucleic acid-binding properties of G3BP1, G3BP1-ΔIDR2, a G3BP1 variant with deletion of IDR2, and G3BP1-Caprin1 complex were assessed in vitro by electrophoretic mobility shift assays.* ***Results.*** *Deletion of IDR2 strongly impaired mRNA recruitment by G3BP1 despite the presence of intact RNA-binding domains. G3BP1-ΔIDR2 retained the ability to interact with Caprin1 and exhibited robust heterotypic mixing in the cellular context. However, neither Caprin1 nor other G3BP1 partners were able to compensate for the loss of mRNA recruitment. In vitro assays confirmed reduced nucleic acid binding by G3BP1-ΔIDR2 and showed that Caprin1 did not compensate for the lossof G3BP1-ΔIDR2 nucleic acid binding. These findings indicate that impaired mRNA recruitment is not caused by the altered compartmentalization but reflects an intrinsic defect associated with IDR2 loss.* ***Conclusions.*** *Our results demonstrate that IDR2 plays a critical, indirect role in G3BP1-mediated mRNA recruitment by maintaining an RNA-binding-competent conformation of this protein. This study highlights the importance of intrinsically disordered regions in regulating mRNA engagement through conformational and structural effects rather than direct RNA binding and provides new insight into the molecular principles underlying stress granule assembly.*

Keywords: *messenger RNA-binding proteins, G3BP1, Caprin1, protein-protein interactions, protein-mRNA interactions.*

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Introduction

Stress granules (SG) are ribonucleoprotein condensates that form in cells in response to various stress conditions. SGs form due to weak multivalent interactions between SG RNA-binding proteins and mRNA, and function as hubs for mRNA protection and rerouting translation from housekeeping proteins to cellular defense proteins [1, 2]. Most RNA-binding proteins implicated in stress granule formation and function contain both structured domains and intrinsically disordered regions (IDRs) [1].

G3BP1 is one of the central SG proteins and is recruited early in pre-SGs [3]. Its key role in SG assembly is well-evidenced, as knockdown [4] or knockout [5] of G3BP1 leads to inhibition or abolishment of stress granule assembly, respectively, and overexpression, in turn, leads to SG nucleation[3]. It consists of five distinct domains: the NTF2-L domain at the N-terminus, the negatively charged acidic-rich region (IDR1), the positively charged proline-rich region (IDR2), the RNA Recognition Motif (RRM), and the RGG domain (IDR3) at the C-terminus (Fig. 1). NTF2-L is involved in protein-protein interactions, RRM binds RNA, and the interplay of three IDRs facilitates G3BP1`s conformational changes (reviewed in [6]). Caprin1 is a prominent partner protein of G3BP1 [7]. The study by Kedersha *et al.* [8] describes Caprin1 as a non-essential component of SG function. Within SGs, Caprin1 binds G3BP1 in competition with USP10. Caprin1-G3BP1 interaction promotes SG assembly by lowering the threshold of liquid-liquid phase separation (LLPS), while USP10 maintains G3BP1 in the soluble state and facilitates SG disassembly.

In our previous study [9], we explored the interplay between G3BP1 and its prominent partner Caprin1 in mRNA recruitment, both *in vitro* and in a cellular context. Notably, we found that Caprin1 improved the mRNA recruitment in SGs orchestrated by G3BP1. In addition, the removal of the RRM or NTF2-L domain of G3BP1 results in a strong decrease in G3BP1 and mRNA recruitment in SGs. Interestingly, we observed that the absence of the IDR2 in G3BP1 did not impede its recruitment to SGs, while the recruitment of mRNA was disrupted. This phenotype presents an intriguing model of the indirect effect of domain loss on protein function. Here, we aim to expand on the characterization of G3BP1-ΔIDR2 and further clarify the involvement of G3BP1`s IDR2 in RNA recruitment of the G3BP1-Caprin1 complex.

Materials and methods

Plasmid preparation

Plasmid construction, cloning approaches, generation of fluorescently tagged proteins, and microtubule-binding domain (MBD) fusion constructs were performed as previously described [9]. Constructs encoding G3BP1, G3BP1-ΔIDR2, Caprin1, G3BP2, and YBX1 were generated using standard PCR-based cloning for fluorescently tagged proteins and the Gateway recombination strategy for GFP/RFP-MBD fusion constructs.

Site-directed mutagenesis of the residue L378P of human Caprin1was carried out directly on the Caprin1-GFP-MBD-pEF-DEST51 expression plasmid by using the 'Quikchange II XL site-directed mutagenesis kit' (Stratagene) and appropriate oligonucleotides (Eurofins Genomics).

Sequences of all obtained plasmids and introduced point mutations were verified by DNA sequencing (Eurofins Genomics)

Microtubule bench assay: Recruitment and mixing experiments

Microtubule bench (MT-bench) recruitment and mixing experiments were performed as described previously [9]. Briefly, for recruitment experiments, fluorescently tagged prey proteins and RFP/GFP-MBD-fused bait proteins, and for mixing experiments, pairs of RFP/GFP-MBD-fused partner proteins were transiently expressed in HeLa cells using Lipofectamine 2000 according to the manufacturer's instructions. Afterwards, cells were fixed with ice-cold methanol, followed

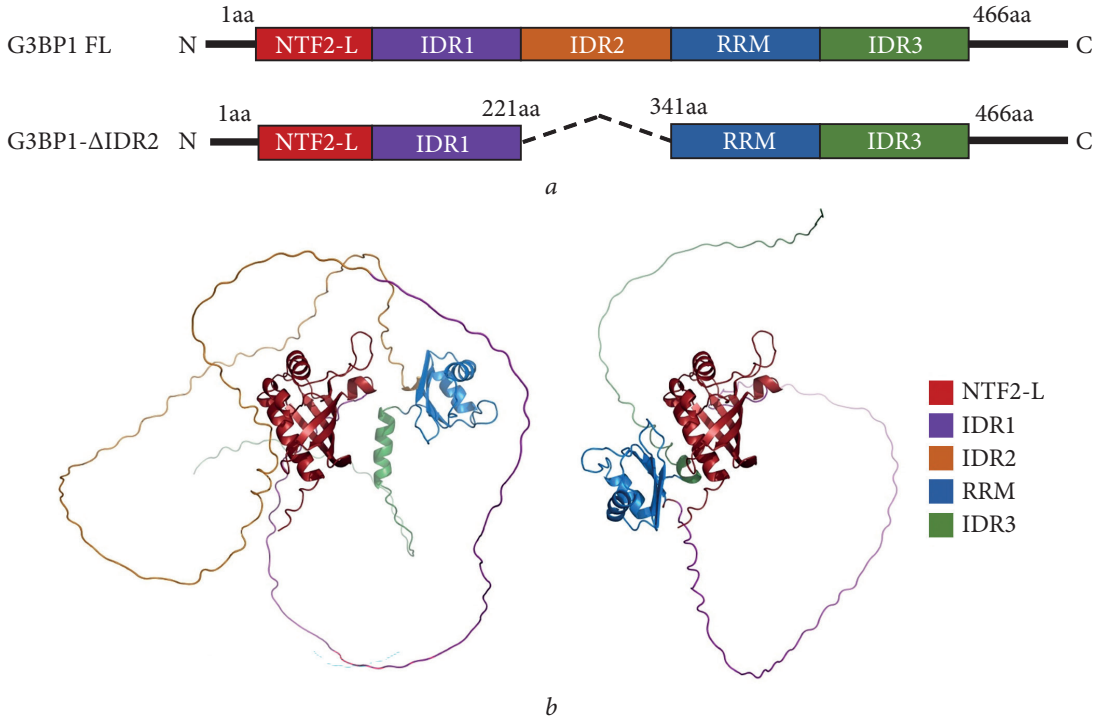

Fig. 1. *a* — Schematic representation of the G3BP1 constructs, FL — full-length, IDR — intrinsically disordered region (adapted from [9]). *b* — Predicted structural models of G3BP1 and G3BP1-ΔIDR2. AlphaFold-predicted models of full-length G3BP1 (left) and the G3BP1-ΔIDR2 variant (right). Domains are colored as indicated. The models illustrate the position of IDR2 within the protein and its removal in the truncated construct. Given the intrinsic disorder of these regions, the models should be interpreted as schematic representations rather than precise structural conformations

by 4% paraformaldehyde (PFA). Endogenous proteins were stained with appropriate antibodies, and RNA *in situ* hybridization was performed using oligo(dT)-Cy5 fluorescent probes (Molecular Probes, Life Technologies). For both MT bench recruitment and mixing experiments, cell images were acquired using the Opera Phoenix Plus High-Content Screening System (PerkinElmer), and fluorescence intensities were quantified using Harmony 5.2 software. Recruitment scores were calculated as correlation coefficients between bait and prey fluorescence intensities along microtubules, and mixing scores were calculated as squared correlation coefficients between co-expressed MBD-fused proteins. The datasets obtained from the Harmony 5.2 software analysis were presented as scatter plots in MATLAB R2021b. All statistical tests were performed using MATLAB R2021b software. Images from the Servier Medical Art image database were used to create experiment schematics.

Protein production and purification

Recombinant human proteins Caprin-1 (1—709 aa), G3BP1 (1—466 aa), and G3BP1-ΔIDR2 were expressed in *E. coli* BL21(DE3) and purified by Ni^{2+}-NTA affinity chromatography using the procedure described in [10].

Electrophoretic mobility shift assay (EMSA)

Purified G3BP1, G3BP1-ΔIDR2, and Caprin1 proteins were serially diluted in HEPES buffer (HEPES 50 mM, KCl 25 mM, TCEP 1 mM, 300 mM Urea pH 7.6). Urea was included to maintain protein

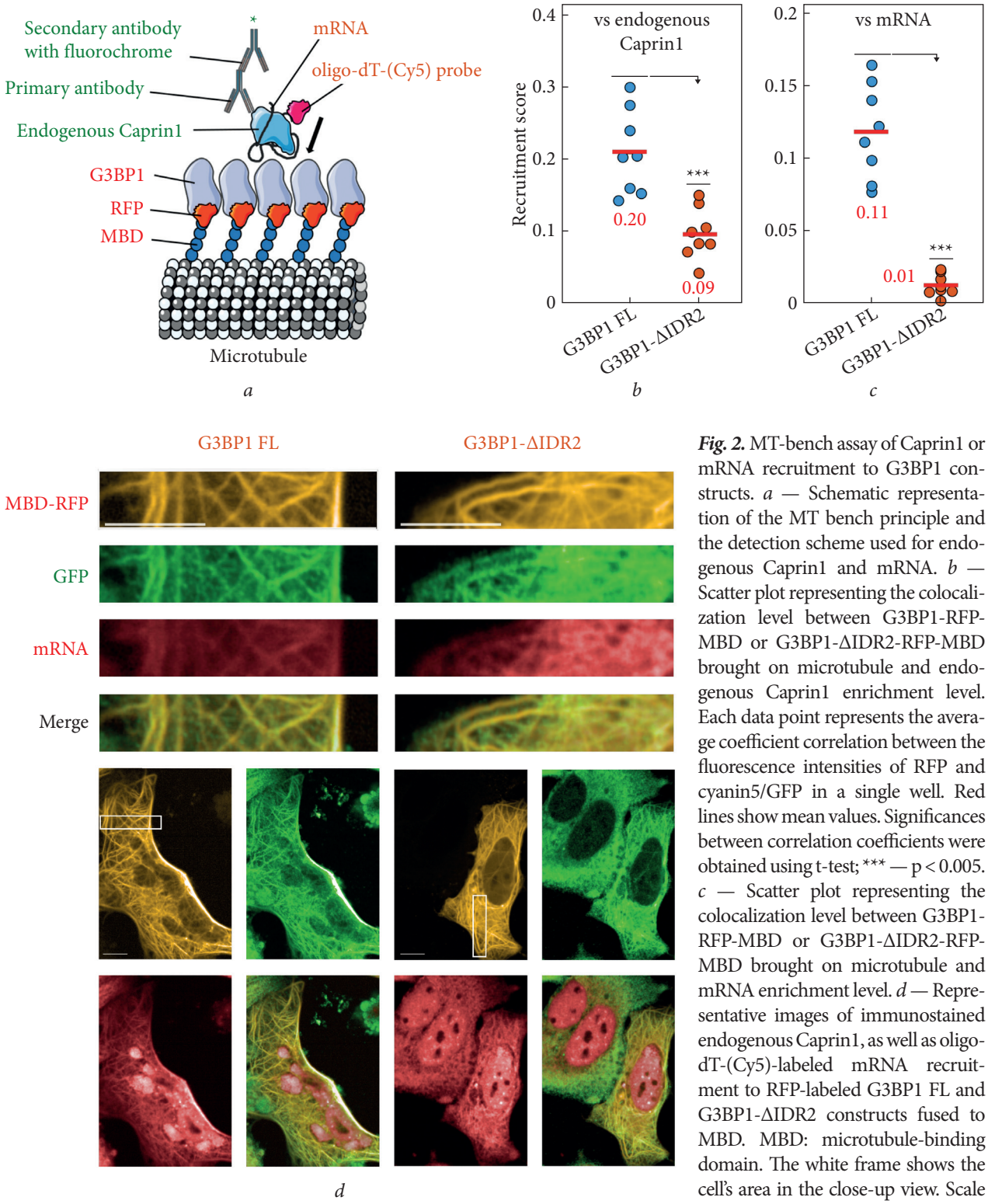

Fig. 2. MT-bench assay of Caprin1 or mRNA recruitment to G3BP1 constructs. *a* — Schematic representation of the MT bench principle and the detection scheme used for endogenous Caprin1 and mRNA. *b* — Scatter plot representing the colocalization level between G3BP1-RFP-MBD or G3BP1-ΔIDR2-RFP-MBD brought on microtubule and endogenous Caprin1 enrichment level. Each data point represents the average coefficient correlation between the fluorescence intensities of RFP and cyanin5/GFP in a single well. Red lines show mean values. Significances between correlation coefficients were obtained using t-test; *** — $p < 0.005$. *c* — Scatter plot representing the colocalization level between G3BP1-RFP-MBD or G3BP1-ΔIDR2-RFP-MBD brought on microtubule and mRNA enrichment level. *d* — Representative images of immunostained endogenous Caprin1, as well as oligo-dT-(Cy5)-labeled mRNA recruitment to RFP-labeled G3BP1 FL and G3BP1-ΔIDR2 constructs fused to MBD. MBD: microtubule-binding domain. The white frame shows the cell's area in the close-up view. Scale bar: 10 μm

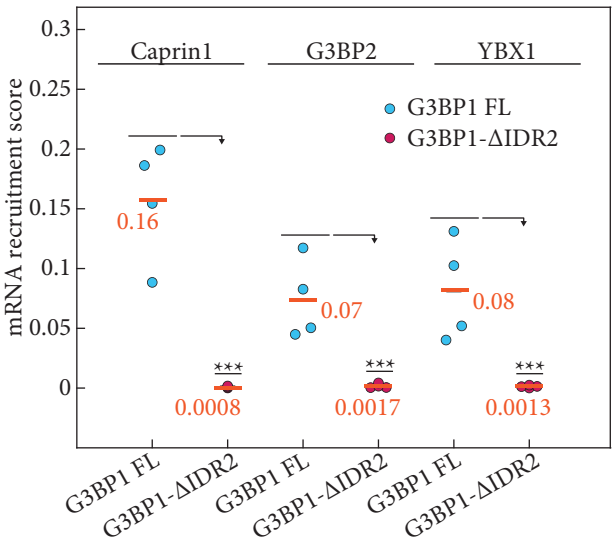

Fig. 3. MT-bench assay mRNA recruitment to G3BP1 or G3BP1-ΔIDR2 in the presence of G3BP1 partner proteins. HeLa cells were co-transfected with the plasmids encoding G3BP1-RFP-MBD or G3BP1-ΔIDR2-RFP-MBD and the plasmid expressing the full-length Caprin1, G3BP2 or YBX1 all fused to GFP. Scatter plot representing the colocalization level of MBD-fused G3BP1-RFP or G3BP1-ΔIDR2 with mRNA in presence of overexpressed partner proteins. Each data point represents the average correlation coefficient between fluorescence intensities from RFP and cyanin5 channels in one well. The plot shows the data from four independent experiments. Red lines show mean values. Significances between correlation coefficients were obtained using t test; *** — $p < 0.001$

solubility and minimize nonspecific aggregation during the binding assay. 100 ng of M13mp18 single-stranded DNA (New England Biolabs) per reaction, heat-denatured, cooled on ice, and incubated with proteins at increasing molar ratios. For mixtures of G3BP1 and Caprin1, the proteins were pre-incubated before the addition of nucleic acid. Samples were resolved on a 0.5% agarose gel and visualized by ethidium bromide staining

Results and Discussion

To reinforce our previous data on G3BP1-ΔIDR2 colocalization with mRNA, we performed an MT-bench recruitment experiment [11] (Fig. 2). In short, to observe and quantify the interaction between two proteins *in vivo*, the bait protein, labeled with an RFP tag, is brought to microtubules via fusion to a microtubule-binding domain (MBD), and the prey protein is labeled or antibody-stained with GFP fluorophore (see Fig. 2*a* for the experimental principle). This way, we can track the protein-protein or protein-RNA interactions along the microtubules by observing the appearance of microtubules in the GFP and Cy5 channels, which indicates the presence of the interaction. In this experiment, we used a transiently expressed full-length G3BP1 (control condition) and a G3BP1-ΔIDR2 construct fused with RFP and a microtubule-binding domain (MBD) as bait, with a cyanine 5-oligo dT probe-labeled cellular mRNA as prey. In parallel, we measured the presence of endogenous Caprin1 on the microtubules using anti-Caprin1 antibodies and selected secondary antibodies with a fluorochrome that emits in the (500—550 nm) spectrum. The recruitment score is measured and quantified as the correlation between the increase in fluorescence of the bait and the prey tracked in the microtubule compartment. Here, we confirm previously obtained data, as in the case of G3BP1-ΔIDR2 being used as bait on microtubules, we observed a drastic decrease in mRNA binding of this protein.

Looking at the domain composition of G3BP1, the removal of central IDR2 should not interfere with NTF2-L-related protein interactions, such as Caprin1 or G3BP2, nor with RNA binding through domains located at the C-terminus. In the cellular context, however, we observe that G3BP1-ΔIDR2 exhibits a near-complete absence of mRNA recruitment (Fig. 2*b*). In parallel with tracking mRNA, we detected the recruitment of the G3BP1 partner protein, Caprin1, as there is an undeniable link between RNA recruitment and Caprin1 recruitment by G3BP1.The interaction between G3BP1 and Caprin1 is independent of RNA, but Caprin1 enhances the recruitment of RNA by the G3BP1-Caprin1 complex [9]. While the removal of IDR2 caused a significant reduction in RNA recruitment, the presence of endogenous Caprin1 on microtubules decreased by only around 50%

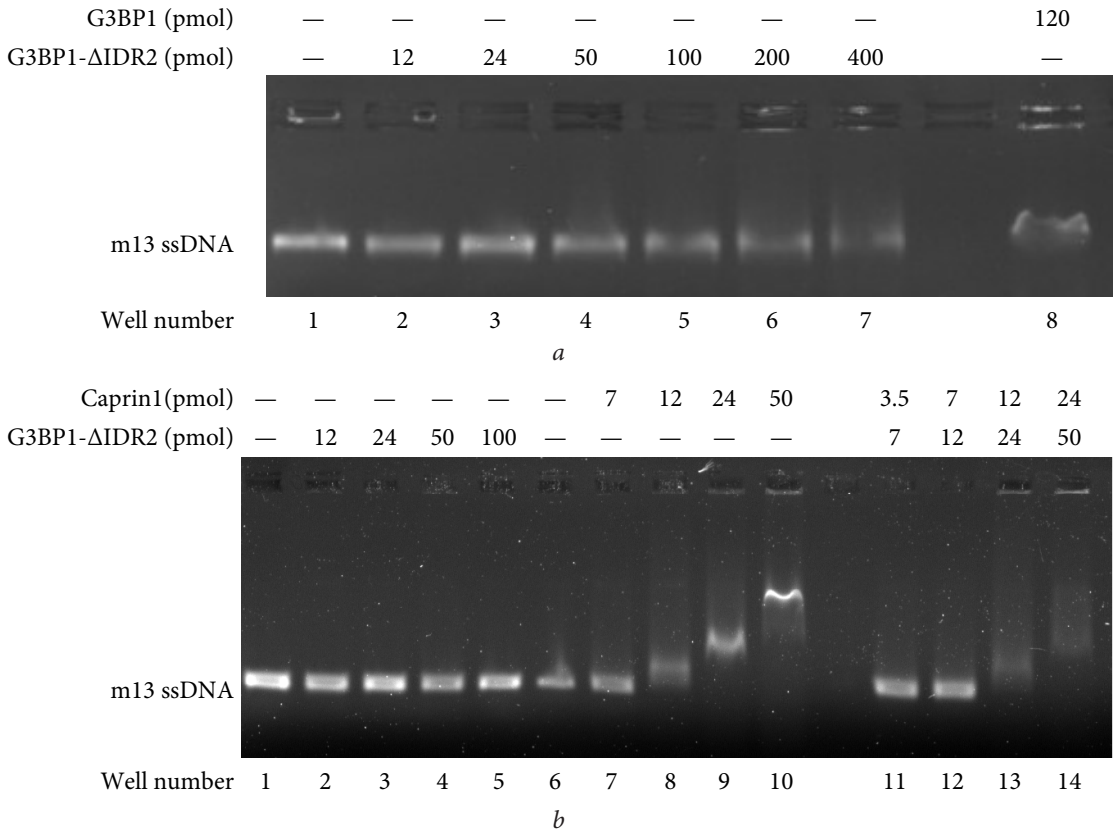

Fig. 4. Electrophoretic mobility shift assay demonstrating the difference in ssDNA binding between G3BP1 and G3BP1-ΔIDR2. *a* — G3BP1-ΔIDR2 (wells 2—7) has decreased nucleic acid binding ability compared to full-length G3BP1 (well 8). One hundred nanograms of m13mp18 ssDNA were incubated with gradually increased concentrations of G3BP1-ΔIDR2and compared to the ssDNA gel-mobility reduction ability of 120 pmol of G3BP1. *b* — Caprin1 does not enhance G3BP1-ΔIDR2 binding to nucleic acids. Proteins were mixed with a constant amount of m13mp18 ssDNA and an increasing amount of Caprin1 and G3BP1-ΔIDR2 (wells 2—6) and compared to equimolar amounts of Caprin1 (wells 7—10) and G3BP1-ΔIDR2 (wells 11—14)

(Fig. 2*c*), which coincides with the previously observed behavior [9] of the G3BP1-Caprin1-RNA complex in the cellular environment and suggests the disruption of the ternary complex.

While Caprin1 is a major partner of G3BP1, it is by no means the only one; hence, we examined the mRNA recruitment behavior of G3BP1-ΔIDR2 in the presence of several other protein partners of G3BP1 that are overexpressed in the cell. To that end, we performed an MT bench recruitment experiment where we overexpressed several GFP-labeled proteins, such as G3BP2, and YBX1 — both known partners of G3BP1 [12, 13] and observed the mRNA recruitment to G3BP1-ΔIDR2 on microtubules compared to the same interaction of full-length G3BP1, while using Caprin1 as a positive control (Fig. 3). Consistent with the behavior of the G3BP1-ΔIDR2-Caprin1 complex, over expression of G3BP1 partner proteins did not restore mRNA recruitment by G3BP1-ΔIDR2. This indicates that the RNA-binding deficiency of this construct is intrinsic and cannot be compensated for by partner proteins.

To further explore the RNA binding of G3BP1-ΔIDR2 in conditions where no mRNA or G3BP1 partners will influence the result, we conducted

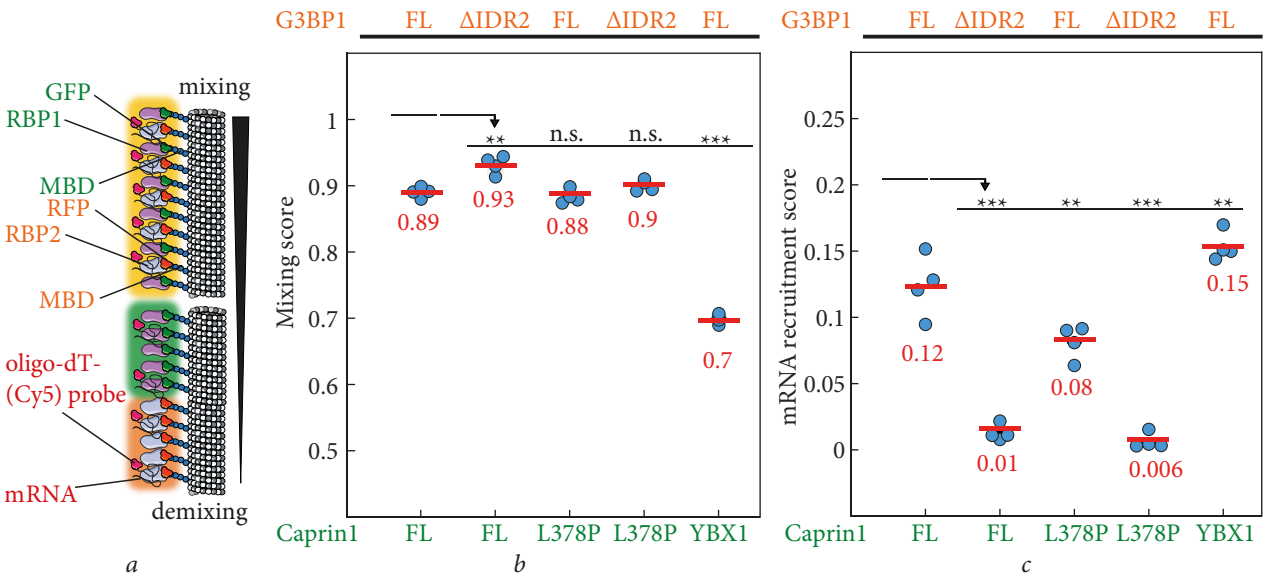

Fig. 5. Mixing and mRNA recruitment of G3BP1 or G3BP1-ΔIDR2 with Caprin1 in the microtubule compartment. *a* — Principle of the microtubule bench mixing assay used to measure the mixing score of two GFP- or RFP-MBD labeled proteins in HeLa cells. Pairs of tested RBPs fused to MBD were co-expressed to automatically measure their mixing along microtubules. The mixing score was measured for different protein pairs using an HCS imager and an automated pipeline. MBD: microtubule-binding domain. *b* — The mixing of G3BP1 with Caprin1 is not disrupted whenever the point mutation L378P in Caprin1 GIM is introduced. *c* — The mRNA recruitment to G3BP1 is partially disrupted by the introduction of the L378P point mutation in Caprin1. * — $p < 0.05$, paired t-test. n.s., non-significant. Each dot represents the mean value of single-cell analysis in a single well

a series of Electrophoretic Mobility Shift Assays (EMSA) experiments. Firstly, we progressively increased the amount of purified G3BP1-ΔIDR2 while maintaining the same nucleic acid quantity, thereby increasing the protein-to-nucleic acid ratio, and compared this to the protein-to-nucleic acid ratio at which the electrophoretic mobility shift occurs for full-length G3BP1 (Fig. 4*a*). Consequently, we observed the differences in RNA binding between G3BP1 FL and G3BP1-ΔIDR2 *in vitro*. G3BP1-ΔIDR2 construct was unable to decrease the electrophoretic mobility of nucleic acid at the same protein-to-nucleotide ratio as full-length G3BP1, and the earliest point of the mobility decrease happened at 400 pmol of G3BP1-ΔIDR2 (Fig. 4*a*, well 7). EMSA analysis showed that G3BP1-ΔIDR2 produced substantially weaker DNA binding compared to full-length G3BP1. For example, 100 pmol of G3BP1-ΔIDR2 (well 5) produced little to no detectable shift, while 120 pmol of full-length G3BP1 (well 8) caused a pronounced shift.

We then analyzed the interplay between G3BP1-ΔIDR2 and Caprin1 for nucleic acid binding. Whenever the interaction between nucleic acid, G3BP1-ΔIDR2, and Caprin1 is forced in an EMSA experiment, we observe that the EM shift profile of the resulting complex is similar to that of Caprin1 alone, suggesting that G3BP1-ΔIDR2 does not form well-defined nucleoprotein complexes in EMSA. At higher concentrations of G3BP1-ΔIDR2 (Fig. 4*b*, wells 13 and 14), the DNA signal becomes diffuse, consistent with the formation of heterogeneous protein-DNA complexes or aggregates with variable electrophoretic mobility.This diffuse character of the bands suggests the formation of heterogeneous nucleoprotein assemblies rather than discrete, stable complexes.

The property of G3BP1, as well as other SG proteins, to undergo Liquid-Liquid Phase Separation (LLPS) and, consequently, form dynamic, reversible liquid-like condensates through self-association or interactions with partner proteins is largely driven by their IDRs [1]. The microtubule bench assay can be used to assess whether a pair of proteins partitions into the same condensed phase or segregates into distinct phases. In this assay, both proteins fused to MBD are co-expressed in cells, resulting in increased local concentration along microtubules and promoting condensate formation along the filament. This enables visualization of their spatial distribution: overlapping compartments indicate mixing within a common condensate, whereas distinct, non-overlapping compartments indicate demixing and the coexistence of separate condensed phases [14] (Fig. 5*a*). We performed such a mixing assay to determine the impact of IDR2 on the G3BP1-Caprin1 mixing in a cellular context and compare it to changes in mixing if Caprin1 has disrupted interaction with G3BP1 via a point mutation in the L378 residue. This residue is located in the G3BP1 interacting motif (GIM) of Caprin1 and was shown to weaken the interaction between G3BP1 and Caprin1 [15]. We observe that G3BP1 mixes well with Caprin1, and the mixing score remains largely unchanged when Caprin1 L378P mutant is expressed. If G3BP1-ΔIDR2 is overexpressed instead of full-length G3BP1, we observe a meaningful increase in mixing of these proteins (Fig. 5*b*). Next, we assessed mRNA recruitment within these mixed compartments. Here, while the introduction of the L378P point mutation in Caprin1 had a minor negative effect on RNA recruitment to full-length G3BP1-Caprin1 phase, G3BP1-ΔIDR2-Caprin1 phase once again had almost absent RNA recruitment (Fig. 5*c*). Regardless of modifications made to G3BP1 or Caprin1, the pair maintains high levels of mixing, while the level of RNA recruitment to such phase remains drastically decreased in cases with G3BP1-ΔIDR2 present. These results demonstrate that the impairment of mRNA recruitment is not a consequence of altered compartmentalization between G3BP1 and Caprin1, but a consequence of IDR2 loss in G3BP1.

Conclusions

The G3BP1-ΔIDR2 construct provides a clear example of structural integrity being a key factor in G3BP1 functionality.G3BP1-ΔIDR2 fails to recruit mRNA despite both the RRM and RGG-box RNA-binding domains being present (Fig. 2, 4*a*). G3BP1-ΔIDR2 retains the ability to recruit Caprin1, albeit to a lesser extent than full-length G3BP1; however, neither Caprin1 nor other G3BP1 partner proteins can even partially compensate for the decreased mRNA binding of IDR2-deficient G3BP1 (Fig. 3, 4*b*). This observation is notable, as G3BP1 and Caprin1 mix efficiently in the common compartment regardless of IDR2 loss or introduction of a point mutation in the GIM of Caprin1 (Fig. 5).

While IDR2 contains positively charged residues and may contribute weak or transient electrostatic interactions with RNA, our findings indicate that its primary role is to maintain a confirmation of G3BP1 that enables effective engagement of its canonical RNA-binding domains.

Consequently, we propose that mRNA recruitment to G3BP1-ΔIDR2 is impaired due to several non-exclusive mechanisms. First, G3BP1 undergoes conformational changes, thereby preventing mRNA from having access to the RRM when IDR2 is removed. Second, the regulation of G3BP1 conformation relies on the interplay between its three intrinsically disordered regions; therefore, the removal of flexible IDR2 forces negatively charged IDR1 and RRM into closer proximity, preventing RRM from binding mRNA targets. Third, since both Caprin1 and G3BP1 have moderate affinity (in the micromolar range) for mRNA, slight modifications in their interactions within the complex may have detrimental consequences for mRNA recruitment to this complex.

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ВНУТРІШНЬО НЕВПОРЯДКОВАНА ДІЛЯНКА 2 БІЛКА G3BP1
НЕОБХІДНА ДЛЯ ЗАЛУЧЕННЯ РНК КОМПЛЕКСОМ G3BP1–CAPRIN1

Мета. Формування стресових гранул потребує скоординованих взаємодій між РНК-зв'язувальними білками та мРНК, однак механізми, за допомогою яких внутрішньо невпорядковані ділянки (IDRs) білків стресових гранул регулюють залученням РНК, залишаються недостатньо з'ясованими. Метою цього дослідження було охарактеризувати роль Внутрішньо Невпорядкованої Ділянки 2 (IDR2) білка G3BP1 у залученні мРНК до комплексу G3BP1-Caprin1. ***Методи.*** Взаємодії білок-білок і білок-РНК у клітинах аналізували шляхом оцінки колокалізації їхніх флуоресцентних сигналів на мікротрубочках із використанням аналізу залучення та змішування на мікротрубочках (microtubule bench recruitment and mixing assay). Здатність G3BP1, G3BP1-ΔIDR2 та комплексу G3BP1-Caprin1 до зв'язування з нуклеїновими кислотами оцінювали *in vitro* методом аналізу зсуву електрофоретичної рухливості (EMSA). ***Результати.*** Видалення IDR2 суттєво порушувало залучення мРНК білком G3BP1, незважаючи на наявність інтактних РНК-зв'язувальних доменів. G3BP1-ΔIDR2 зберігав здатність взаємодіяти з Caprin1 і демонстрував виражене гетеротипне змішування в клітинному контексті. Однак ані Caprin1, ані інші партнери G3BP1 не могли компенсувати втрату цим делеційним варіантом здатності до залучення мРНК. Дослідження *in vitro* підтвердили зниження зв'язування нуклеїнових кислот варіантом G3BP1-ΔIDR2 та показали, що Caprin1 не компенсує втрату цієї здатності. Отримані дані свідчать, що порушення залучення мРНК не зумовлене зміною компартменталізації, а відображає внутрішній дефект, пов'язаний із втратою IDR2. ***Висновки.*** Наші результати демонструють, що IDR2 відіграє критичну, непряму роль у G3BP1-опосередкованому залученні мРНК, підтримуючи конформацію білка, здатну до ефективного зв'язування з мРНК. Дослідження підкреслює важливість внутрішньо невпорядкованих ділянок у регуляції взаємодії з мРНК через конформаційні та структурні ефекти, а не через безпосереднє зв'язування з РНК, і надає нові уявлення про молекулярні принципи, що лежать в основі формування стресових гранул.

***Ключові слова*:** мРНК-зв'язувальні білки, G3BP1, Caprin1, білок-білкові взаємодії, білок-мРНК взаємодії.