Has AlphaFold 3 reached its success for RNAs?

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Predicting the 3D structure of RNA is a significant challenge despite ongoing advancements in the field. Although AlphaFold has successfully addressed this problem for proteins, RNA structure prediction raises difficulties due to fundamental differences between proteins and RNAs, which hinder direct adaptation. The latest release of AlphaFold, AlphaFold 3, has broadened its scope to include multiple different molecules like DNA, ligands and RNA. While the article discusses the results of the last CASP-RNA dataset, the scope of performances and the limitations for RNAs are unclear. In this article, we provide a review of the performance of AlphaFold 3 in the prediction of RNA 3D structures. Through an extensive benchmark over four different test sets, we discuss the performances and limitations of AlphaFold 3. We also compare its performances with ten existing state-of-the-art ab initio, template-based and deep-learning approaches.
Our results are freely available on the EvryRNA platform: `https://evryrna.ibisc.univ-evry.fr/evryrna/alphafold3/`.

RNA 3D structure | AlphaFold 3 | Deep Learning | Structure evaluation
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1. Introduction

RNA molecules play a crucial role in the living. While its function is directly linked to its structure, the prediction of the latter remains an open challenge to be addressed. Knowing the structure of RNA could be of great interest for drug design or the comprehension of biological processes like cancer (1). While experimental methods like X-ray crystallography, NMR or cryo-EM (2) can determine the RNA 3D structures, their use is costly (in time and resources) and hardly scalable for the number of RNAs in the living. Computational approaches have emerged with *ab initio*, template-based and deep learning procedures. *Ab initio* methods (3–12) tend to reproduce the physics of the system, with force field applied to a coarse-grained representation (low-resolution where a nucleotide is replaced by some of its atoms). The template-based approaches (13–22) create a mapping between sequences and fragments of structure before refining the assembled structures.

Recently, with the recent success of AlphaFold for proteins (23, 24), approaches have been made to replicate its success in RNAs. Deep learning approaches like DeepFoldRNA (25), RhoFold (26), DrFold (27), NuFold (28), trRosettaRNA (29) employ strategies related to AlphaFold 2. They consider coarse-grained representation and predict Euclidean transformation before reconstructing the full-atom structure. The use of torsional angles is also adapted to RNAs, with either the standard torsional angles or angles from their coarse-grained representations.

While being better than existing template-based or *ab initio* methods, deep learning approaches do not solve the prediction of RNA structures yet (30), as shown in our recent benchmark State-of-the-RNArt (31). It has also been demonstrated in the CASP-RNA challenge (32), where the top four best predictions are not deep learning-based.

The recent release of AlphaFold, AlphaFold 3, extends the prediction to ligands, DNA and RNA. It uses as inputs the sequence and multiple sequence alignment (MSA) and it also changes a part of the architecture to better adapt to the variety of its inputs. It no longer relies on torsional angles to prevent the restriction to specific molecules. It directly predicts atom coordinates with the use of a multi-cross diffusion model. Through a benchmark on CASP-RNA (32), the article mentioned good results but did not outperform human-helped methods. Nonetheless, it is not clear what the current limitations are and how well it performs compared to state-of-the-art solutions.

The aim of this article is to provide a comprehensive extension on the benchmark of AlphaFold 3 for RNAs. We first describe the main differences between RNA and proteins, before discussing the benchmark we did to evaluate AlphaFold 3. Then, we describe the results of AlphaFold 3 and the current limitations of the model. The results and the data are freely available and usable in the EvryRNA platform: `https://evryrna.ibisc.univ-evry.fr/evryrna/alphafold3/`.

2. RNA vs proteins

RNA and proteins are both molecules that play crucial roles in the living (33). They share the characteristic of having a 3D structure that directly defines their function. In this part, we discuss the differences that have prevented protein algorithms from being adaptable to RNAs.

RNAs comprise four nucleotides (A, C, G and U), whereas proteins comprise 20 amino acids. This difference has a high consequence on the adaptation of protein algorithms to RNA. The vocabulary available for RNA is limited to four unique elements, making the use of protein vocabulary not directly adaptable. The sequence length of RNA molecules also has a high variability (from a dozen to thousands of nucleotides) compared to proteins (around a hundred

amino acids).

A major difference between RNAs and proteins lies in the folding stabilisation. RNA structure is maintained by base pairing and base stacking, while protein structure is supported by hydrogen interactions in the skeleton. The protein backbone is also modelled by torsion angles (Φ and Ψ) for each amino acid because the peptide bond is planar. This is not the case for RNA, where each nucleotide can be described by six torsion angles (α, β, γ, δ, ϵ, ξ) and the sugar-pucker conformation (χ). An approximation usually involves pseudo-torsion η and θ (**?**). Protein models learn a conformational mechanism fundamentally different from the RNA folding process.

The nature of pairwise interactions of RNA 3D molecules differ from those of proteins. The Watson-Crick (WC) and non-WC interactions can be made through three different edges of the RNA base: WC edge, Hoogsten edge and sugar edge, shown in Figure 1. In addition, the orientation of the glycosidic bonds gives another property to an interaction: *cis* or *trans*. The combination of edge and orientation gives 12 possibilities of interaction between bases. The standard WC base pair corresponds to the cis WC/WC pairing. Given the orientations (*cis* or *trans*), the edges (WC-WC, WC-S, WC-H, S-WC, S-S, S-H, H-WC, H-S, H-H) and the base pairing (AA, GG, CC, UU, AG, AC, AU, GA, GC, GU, CA, CG, CU, UA, UG, UC), there are more than 200 possible base pairs. Only the standard WC pairs (cis WC/WC) of AU and CG (with GU wobble pair) are used for the 2D structure representation. Non-standard interactions play a crucial role in the overall topology of the RNA folding process. They help stabilise the structure and can not be ignored when working on RNA 3D structures.

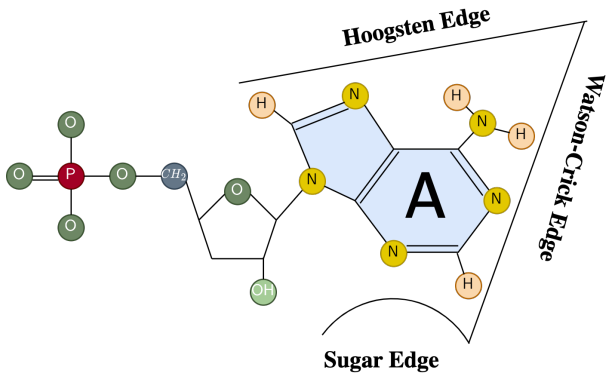

Figure 1. Description of the three different edges of the adenine RNA base: Watson-Crick edge, Hoogsten edge and Sugar edge. The edges are similar to the other nucleotides.

The stability of the RNA and protein structures is different. Proteins tend to have a stable structure corresponding to a minimum in the energy landscape. On the other hand, RNAs can have multiple conformations depending on the environmental condition. Those foldings are of equivalent validity as they are all local minimums in the energy landscape. Ideally, finding an RNA 3D structure means finding the native foldings of RNA, and should output multiple structures with confidence scores. However, the available structures found in the PDB do not provide access to this diversity of structures.

There is a huge disparity in the protein and RNA data. Even if there is a higher proportion of RNAs than proteins in the living, this is not reflected in the available data: only a small amount of 3D RNA structures are known. Up to June 2024, 7,759 RNA structures were deposited in the Protein Data Bank (34), compared to 216,212 protein structures. The quality and diversity of data are also different: a huge proportion of RNAs come from the same families. It implies several redundant structures that could prevent a model from being generalized to other families. In addition, a huge amount of RNA families have not yet solved structures in the PDB. This means there is no balanced and representative proportion of RNA families through the known structures.

Finally, no standard dataset has been used through the community for RNAs. Each research group uses its dataset with different preprocessing associated. It prevents using deep learning methods, as a lot of work is needed for a clean dataset. While the community agrees to use RNA-Puzzles (35–39) or the newly-RNA (32) to test the generalization of proposed models, no clear training set is available. RNANet (40) has recently been developed to solve this issue. A new approach, RNA3DB (41), tries to create independent datasets to use for deep learning approaches where clustering is done based on sequence and structure disparity.

3. Materials & methods

In this section, we describe the benchmarked models, metrics, and datasets used to evaluate AlphaFold 3 performances.

A. Datasets

To evaluate the prediction of RNA structures, we considered four test sets, with three of them from our previous work (31).

- **RNA-Puzzles**: the first dataset is composed of the single-stranded structures from RNA-Puzzles (35–39), a community initiative to benchmark RNA structures. We considered only single-stranded solutions to have a fair comparison between the benchmarked models. It is composed of 22 RNAs of length between 27 and 188 nucleotides with a mean of 85 nucleotides.

- **CASP-RNA**: the second test set is CASP-RNA (32) structures, which is a collaboration between the CASP team and RNA-Puzzles. It is composed of 12 RNAs with wide-range sequences, from 30 to 720 nucleotides (with a mean of 210 nucleotides).

- **RNASolo**: the third test set is a custom test set composed of independent structures from RNAsolo (42). We downloaded the representative RNA molecules from RNAsolo (42) with a resolution below 4 Å and removed the structures with sequence identity higher than 80%. Then, we considered only the structures with a unique Rfam family ID (43), leading to 29 non-redundant RNA molecules, with a sequence between 40 and 298 nucleotides (and a mean of 96 nucleotides). It can not be ensured that the structures from this dataset were not used in the training set of

AlphaFold 3. We keep this dataset for comparison, as we already have the results for the benchmarked methods.

- **RNA3DB**: the last dataset is composed of a non-redundant set of structurally and sequentially independent structures from RNA3DB (41). It comprises the component #0, which is composed of orphan structures that are advised to be used as a test set. These structures do not have Rfam family (43) and include synthetic RNAs, small messenger RNA crystallized as part of larger complexes. After removing structures with sequences below ten nucleotides, we ended up with a dataset of 359 structures from 10 nucleotides to 339 nucleotides (with a mean of 51 nucleotides).

For the rest of the article, we mention each dataset by RNA-Puzzles, CASP-RNA, RNASolo and RNA3DB to refer them.

B. State-of-the-art methods

Existing solutions for the predictions of RNA 3D structures are based on three main types of methods: *ab initio*, template-based and deep-learning ones. As discussed previously in our work(31), *ab initio* methods (3, 5, 7) integrate the physics of the system by usually simplifying the representation of nucleotide (coarse-grained). Instead of using all the atoms for one nucleotide, they create a low-resolution representation that simplifies the computation time while losing information. They use approaches like molecular dynamics (44) or Monte Carlo (45) to perform sampling in the conformational space and use a force field to simulate real environment conditions. On the other hand, template-based methods (13, 17–19, 46) create a mapping between sequences and known motifs with, for instance, secondary structure trees (SSEs) before reconstructing the full structure from its subfragments. Finally, the recent methods tend to incorporate deep learning methods (25–29) by using attention-based architecture with self-distillation, recycling as done in AlphaFold 2 (24).

To compare the performances of AlphaFold 3 (47), we benchmarked ten different previously mentioned approaches on three of the four test sets: the ones used in our previous work (31). We used the web servers with default parameters to compare available models fairly, where each user could reproduce our experiments. For the *ab initio* methods, we benchmarked SimRNA (7), IsRNA1 (5) and RNAJP (3). Only RNAJP was used locally. For the template-based approaches, we used MC-Sym (46), Vfold3D (19), RNAComposer (18), 3dRNA (17) and Vfold-Pipeline (13). For the deep learning methods, we benchmark trRosettaRNA (29) and RhoFold (26). More details on each method are provided in our previous article (31). As me made most of the predictions using web servers, the predictions on the last test set RNA3DB were hardly applicable for all the methods. Therefore, for the RNA3DB dataset, we only benchmarked AlphaFold 3, which returns predictions very fast.

C. Evaluation metrics

To compare the predictions, we used the RNAdvisor tool (48) developed by our team, which enables the computation of a wide range of existing metrics in one command line. For the evaluation of RNA 3D structures, a general assessment of the folding of the structure can be done with either the root-mean-square deviation (RMSD) or its extension adding RNA features ϵRMSD (49). Protein-inspired metrics can also be adapted to assess structure quality like the TM-score (50, 51) of the GDT-TS (52) (counts the number of superimposed atoms). There are also the CAD-score (53) (which measures the structural similarity in a contact-area difference-based function) and the lDDT (54) (which assesses the interatomic distance differences between a reference structure and a predicted one). Finally, there are RNA-specific metrics that have been developed, like the P-VALUE (55) (which assesses the non-randomness of a given prediction). The INF and DI (56) have been developed to consider the RNA-specific interactions. The INF score incorporates canonical and non-canonical pairing with Watson-Crick (INF-WC), non-Watson-Crick (INF-NWC), and stacking (INF-STACK) interactions. The consideration of torsional angles has been developed with the mean of circular quantities (MCQ) (57). As discussed in (48), these metrics are complementary and can infer different aspects of RNA 3D structure behaviour.

4. Results

A. AlphaFold 3 compared to the state-of-the-art

We compare the predictions of ten existing methods and AlphaFold 3 on three of the four test sets. Figure 2 shows the different normalised metrics for the different models averaged over three test sets. All the metrics are normalised by the maximum values and converted to be better where near to 1 and worst when near to 0. Details of the results for each dataset are shown in Figure S1 of the Supplementary file. The results show that AlphaFold 3 has the best cumulative scores, which means it outperforms the other models when considering all the metrics. It has the highest MCQ value, indicating it returns structures which are even more physically plausible than *ab initio* methods (that use physics properties in their predictions). AlphaFold 3 does not have the best RMSD (beaten by Rhofold) but has the best lDDt and TM-score. This suggests AlphaFold does not always have the best alignment (in terms of all atoms). AlphaFold 3 has, for this benchmark, better results than the other existing approaches but does not outperform them in all existing metrics.

B. AlphaFold 3 on the different test sets

We also predicted structures from AlphaFold 3 on the last test set from RNA3DB (41). We report the TM-score, lDDT, DI and MCQ distributions for each of the four test sets in Figure 3. Distributions for other metrics are shown in Figure S2 of the Supplementary file. We observe the same behaviour for the four metrics: AlphaFold 3 has more difficulty in predicting good predictions for the dataset from RNA3DB and CASP-RNA. It has slightly better results for

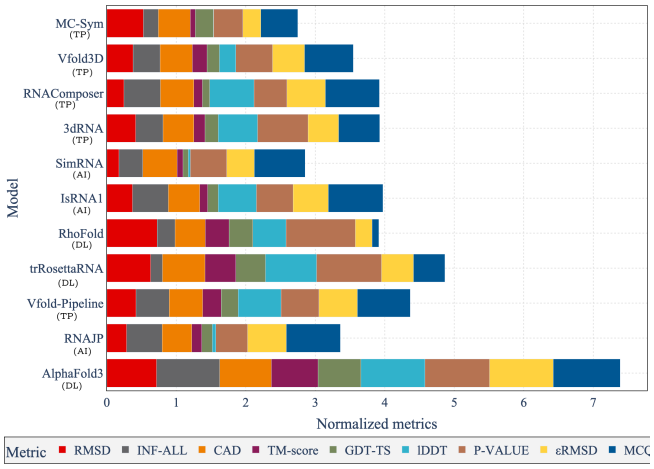

Figure 2. Cumulative normalised metrics for each of the benchmark models (3, 5, 7, 13, 17–19, 26, 29, 46, 47) averaged over the three test sets (RNA-Puzzles (35–39), CASP-RNA (32) and RNAsolo (42)). Each metric is normalised by the maximum value over the four test sets, and the decreased metrics are inversed to have better values close to 1.

RNA-Puzzles, and good results for RNASolo (which makes sense as we could not ensure the structures were not leaked in the training set). These results show the difficulty of AlphaFold 3 in predicting complex structures (with either long sequences of CASP-RNA or orphan structures of RNA3DB).

We detail the two best and worst predictions for each dataset from AlphaFold 3 in Figure 4. The worst performances are for structures of long sequences for CASP-RNA and RNA-Puzzles (sequences of 71, 185, 363 and 720 nucleotides). For the RNA3DB dataset, the worst performances are for RNA with non-common structures, which might not be found in the training set of AlphaFold 3.

C. Dependance on sequence length

The prediction of long RNAs (RNAs with sequence lengths higher than 200 nucleotides) is a major issue for current approaches. Indeed, the *ab initio* methods fail to predict long interactions as the computation time highly increases with the sequence length. The template-based approaches are limited by the small amount of long RNAs, as well as the deep learning methods, as shown in (31).
To observe the relation between sequence length and AlphaFold 3 performances, we report its link with the DI and lDDT metrics in Figure 5. We considered the DI as it considers both global conformation and key RNA interactions and the lDDT as it gives a general idea of atomic distances. Links between the other metrics and the sequence length are available in Figures S3, S4 and S5 of the Supplementary file. Figure 5 indicates that the DI highly increases for the RNA3DB dataset for sequences between 0 and 100. There is also a lower slope of increase for the other test sets (except for RNAsolo), meaning that the DI slightly increases with the sequence length. Indeed, it becomes harder to predict both the folding and the canonical and non-canonical interactions when the sequence is increasing. We also observe a tendency of decrease for the lDDT when the structures have sequences higher than 100 nucleotides.

D. Non-canonical interactions

To understand the comportment of AlphaFold 3 on RNA-specific interactions, we study the Watson-Crick (WC), non-Watson-Crick (nWC) and stacking (STACK) interactions are shown for each dataset in Table 1. All test sets have similar mean nWC interactions (except for the RNA3DB dataset). As the CASP-RNA has structures with longer sequences, it also has more stacking and WC interactions.

Table 1. Information about Watson-Crick (WC), non-Watson-Crick (nWC) and Stacking (STACK) interactions for the four test sets. The mean nWC is counted for structures with more than one nWC interaction. All test sets have more than one nWC interaction (except from RNA3DB, which has 56% of structures with at least one nWC interaction). All the datasets have at least one stacking and one WC interaction.

	RNA3DB	CASP	RNA-Puzzles	RNASolo
Mean nWC	4.7	7.6	7.5	7.8
Max. nWC	18.0	17.0	16.0	22.0
Mean STACK	31.3	160.4	65.8	73.4
Max. STACK	187.0	553.0	155.0	207.0
Mean WC	16.0	83.1	27.7	30.2
Max. WC	77.0	320.0	66.0	95.0

To evaluate the ability of AlphaFold 3 to predict non-canonical structures, we depict the scatter plots between non-Watson-Crick INF (INF-NWC) and Watson-Crick INF (INF-WC) in Figure 6. The size of the points is proportional to the RMSD of structures and, thus, to their alignment. We include the RMSD to highlight the global alignment. We observe a tendency to have a high RMSD (small points) whenever the INF-WC and INF-nWC are high. There is also a large number of structures with an INF-NWC of 0, implying that AlphaFold 3 does not predict any of the non-Watson-Crick interactions. Examples of successful and missing non-Watson-Crick interactions are shown in the Figure. For the results on stacking interactions, there are predictions where AlphaFold 3 does not predict the Watson-Crick interactions well but still predicts the stacking ones. It can be explained by good skeleton predictions while lacking the base conformations that produce the WC interactions. Secondly, there is an increased tendency between the INF-STACK and INF-WC: when AlphaFold 3 predicts the WC interactions well, it also tends to estimate the stacking well. Indeed, whenever the WC are respected, the stacking can be more easily inferred. These results show the limitations of AlphaFold 3 for predicting complex RNA interactions.

5. Conclusion

AlphaFold 2 had a huge success in the prediction of protein folding and has changed the field by the quality of its predictions. The new release of AlphaFold, named AlphaFold 3, has extended the model to predict all molecules from the PDB, like ions, ligands, DNA or RNA.

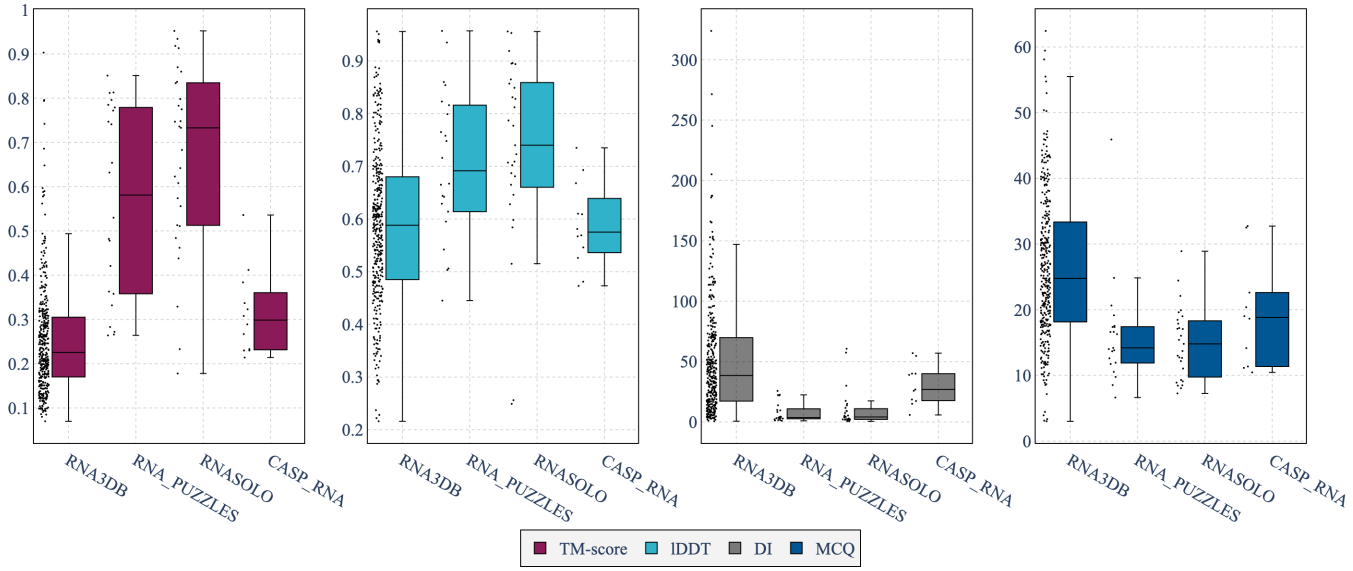

Figure 3. TM-score (58), lDDT (54), DI (56) and MCQ (57) distributions for each of the four test sets of the AlphaFold 3 (47) predictions. TM-score and lDDT are ascending: the higher, the better. DI and MCQ are descending: the lower, the better.

Through an extensive benchmark on four different robust test sets, we have evaluated the quality of prediction of AlphaFold 3 for RNA molecules. We have also compared its predictions to ten existing methods, which are easily reproducible as we considered only models with web servers.

Our results show that AlphaFold 3 has not yet reached the same success as proteins for RNAs. Its new architecture allows the prediction of wide molecules but remains limited and hardly predicts non-Watson-Crick interactions.

The predictions of AlphaFold 3 remain of competitive quality, as it outperforms most of the existing solutions and returns more physically plausible structures than *ab initio* methods. It outclasses existing deep-learning approaches but does not generalize well on orphan structures or long RNAs. It also returns predictions very quickly compared to *ab initio* or current template-based approaches.

The prediction of atom coordinates instead of base frames in AlphaFold 2 allows the extension of predictions for a wide range of molecules but prevents the generalisation of RNA-specific interactions. The lack of data is also a limitation that prevents the robustness of deep learning methods in general, and so is AlphaFold 3.

ACKNOWLEDGEMENTS

This work is supported in part by UDOPIA-ANR-20-THIA-0013, Labex DigiCosme (project ANR11LABEX0045DIGICOSME), performed using HPC resources from GENCI/IDRIS (grant AD011014250), and operated by ANR as part of the program "Investissement d'Avenir" Idex ParisSaclay (ANR11IDEX000302).

Conflict of Interest: None is declared.

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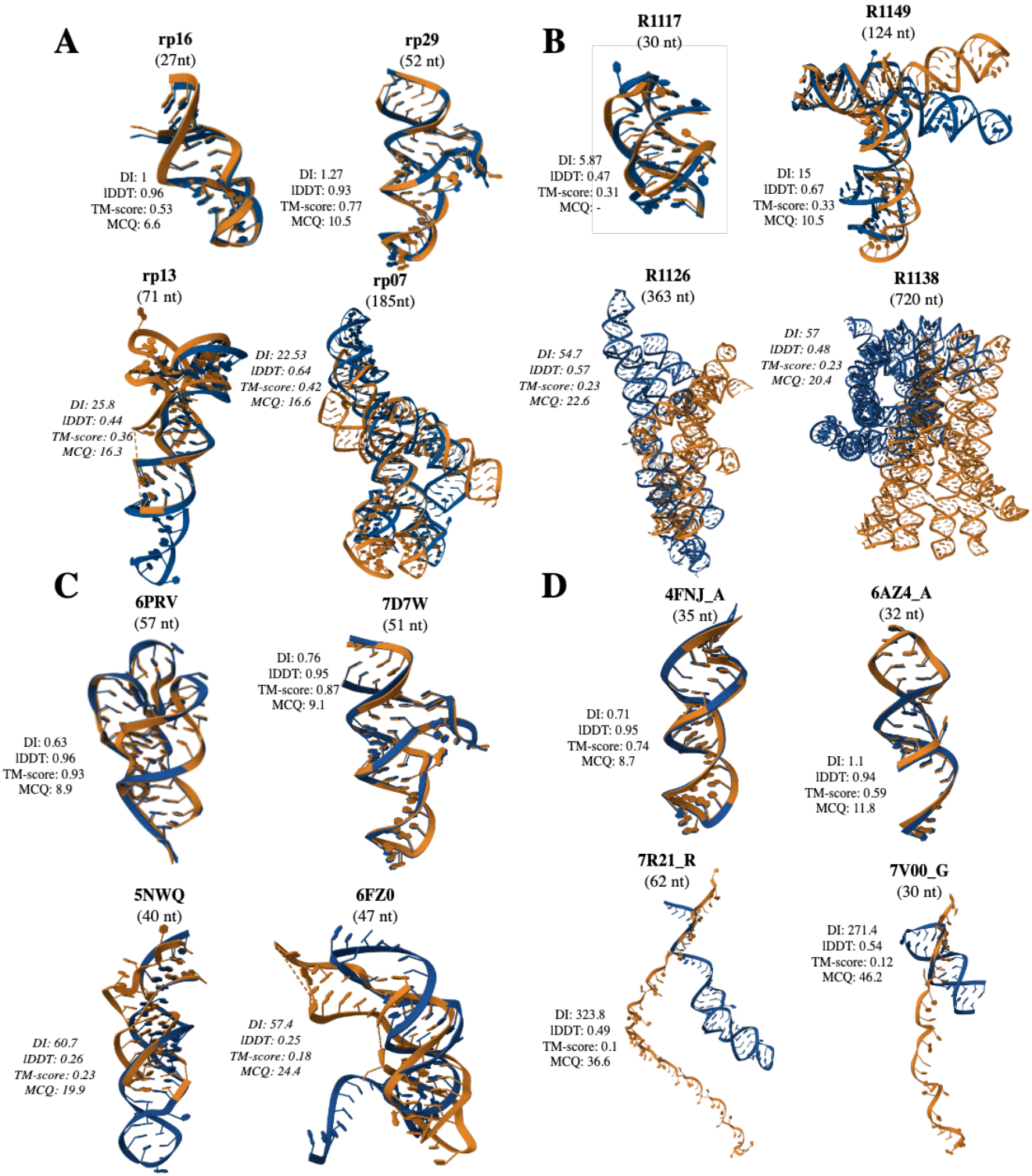

Figure 4. The two best and two worst predictions from AlphaFold 3 (47) for A) RNA-Puzzles (36–39), B) CASP-RNA (32), C) Test set obtained from RNASolo (42) and D) Test set from RNA3DB (41). The DI (56), lDDT (54), TM-score (50) and MCQ (57) are included for each structure. The predictions from AlphaFold 3 (in blue) are aligned with the native ones (in orange) using US-align (59).

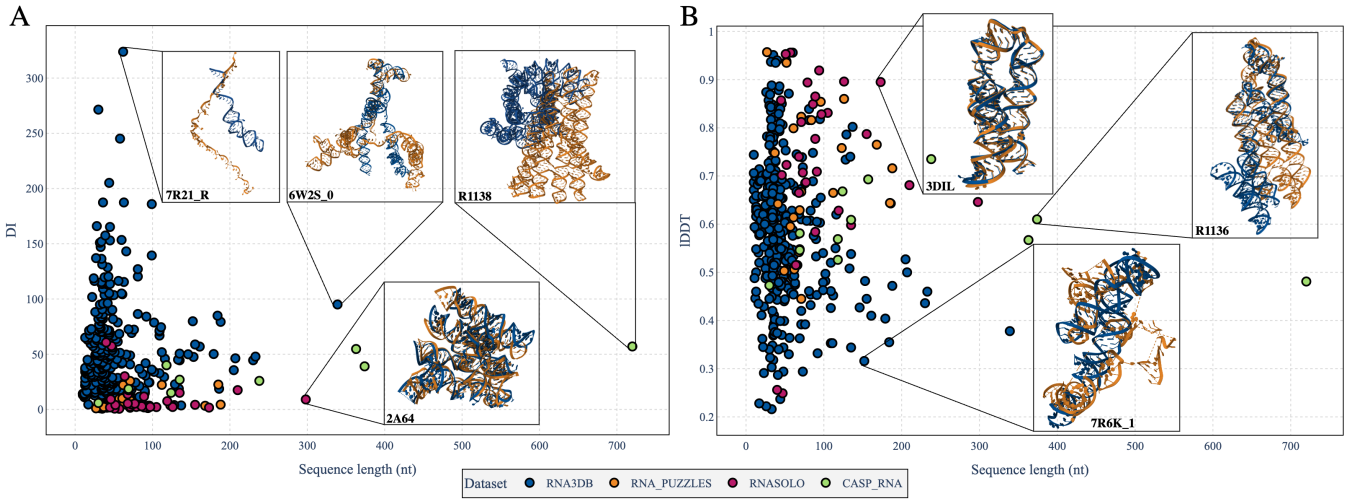

Figure 5. Dependence of two metrics with the sequence length on the prediction of AlphaFold 3 (47) on the four test sets. Some structures are represented, with the native one (in orange) and the aligned prediction by AlphaFold 3 (in blue). A) Results of the DI (56) depending on RNA sequence length. Higher values of DI mean bad predictions, while low values mean accurate predictions. B) Results of IDDt (54) depending on RNA sequence length. Higher values of lDDT mean good predictions, while low values mean bad predictions.

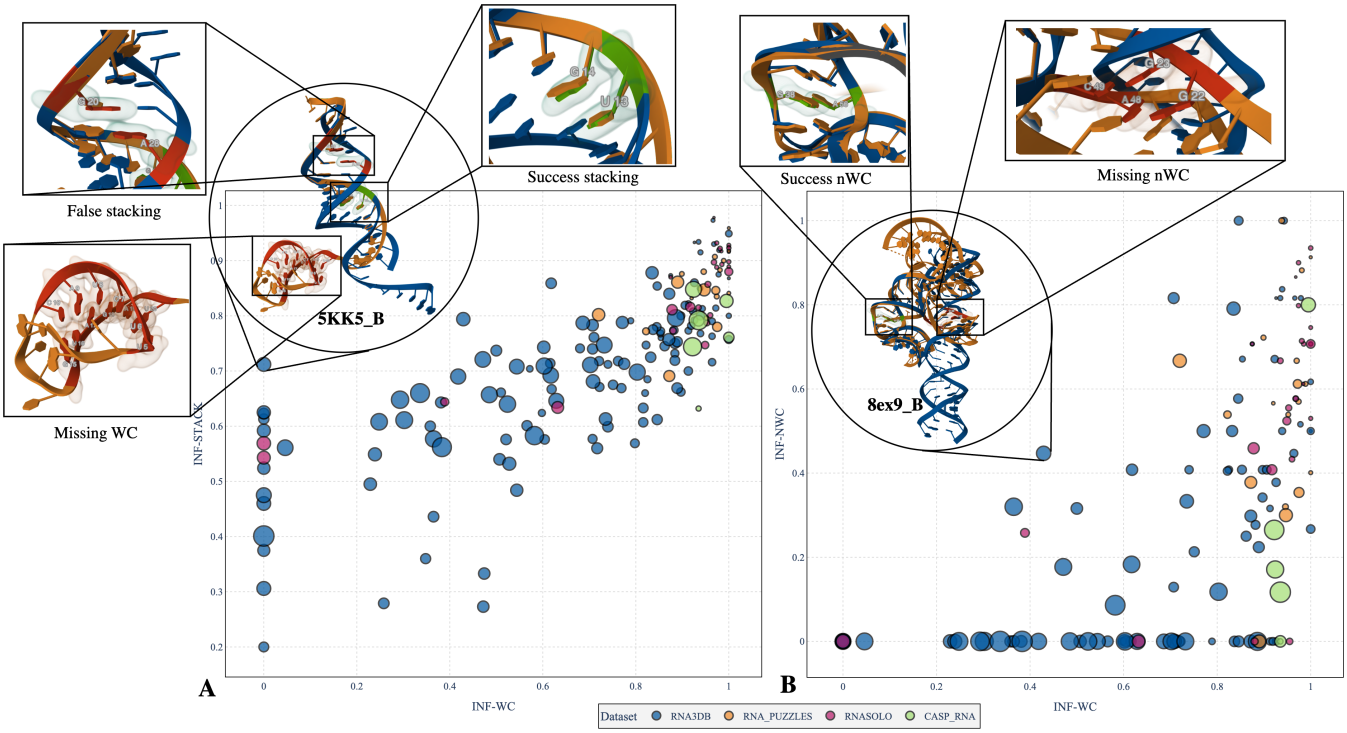

Figure 6. Link between Watson-Crick (WC), non-Watson-Crick (nWC) and stacking (STACK) interactions for the predictions of AlphaFold 3 for the four test sets. The size of each point is proportional to the RMSD: the lower, the better. Only structures with at least one non-Watson-Crick interaction are shown. An INF (56) value of 1 means accurate reproduction of key RNA interactions, while a value near 0 means the structure does not reproduce the interactions. A) INF stacking (INF-STACK) depending on INF Watson-Crick (INF-WC) interactions. B) INF non-Watson-Crick (INF-nWC) depending on INF Watson-Crick (INF-WC)interactions.

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