Synthetic Platforms for Characterizing and Targeting of SARS-CoV-2 Genome Capping Enzymes

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ABSTRACT: Essential viral enzymes have been successfully targeted to combat the diseases caused by emerging pathogenic RNA viruses (e.g., viral RNA-dependent RNA polymerase). Because of the conserved nature of such viral enzymes, therapeutics targeting these enzymes have the potential to be repurposed to combat emerging diseases, e.g., remdesivir, which was initially developed as a potential Ebola treatment, then was repurposed for COVID-19. Our efforts described in this study target another essential and highly conserved, but relatively less explored, step in RNA virus translation and replication, i.e., capping of the viral RNA genome. The viral genome cap structure disguises the genome of most RNA viruses to resemble the mRNA cap structure of their host and is essential for viral translation, propagation, and immune evasion. Here, we developed a synthetic, phenotypic yeast-based complementation platform (YeRC0M) for molecular characterization and targeting of SARS-CoV-2 genome-encoded RNA cap-0 (guanine-N7)-methyltransferase (N7-MTase) enzyme (nsp14). In YeRC0M, the lack of yeast mRNA capping N7-MTase in yeast, which is an essential gene in yeast, is complemented by the expression of functional viral N7-MTase or its variants. Using YeRC0M, we first identified important protein domains and amino acid residues that are essential for SARS-CoV-2 nsp14 N7-MTase activity. We also expanded YeRC0M to include key nsp14 variants observed in emerging variants of SARS-CoV-2 (e.g., delta variant of SARS-CoV-2 encodes nsp14 A394V and nsp14 P46L). We also combined YeRC0M with directed evolution to identify attenuation mutations in SARS-CoV-2 nsp14. Because of the high sequence similarity of nsp14 in emerging coronaviruses, these observations could have implications on live attenuated vaccine development strategies. These data taken together reveal key domains in SARS-CoV-2 nsp14 that can be targeted for therapeutic strategies. We also anticipate that these readily tractable phenotypic platforms can also be used for the identification of inhibitors of viral RNA capping enzymes as antivirals.

KEYWORDS: SARS-CoV-2, viral RNA capping enzymes, phenotypic platforms, yeast, variants, attenuation

INTRODUCTION

Pathogenic RNA viruses are a great threat to human health and world economy. This is abundantly clear from the current COVID-19 pandemic, which has resulted in more than 470 million infections so far and more than 6 million deaths worldwide in a relatively short period of time.\(^1\) While the COVID-19 pandemic is the most significant RNA virus-related outbreak in the recent past, several other pathogenic RNA virus-related outbreaks have constantly emerged in the last 20 years, e.g., SARS-CoV in 2003, MERS-CoV from 2012 to the present, Ebola virus outbreak from 2013 to 2016, and so on.\(^\text{5−8}\) This constant and sudden emergence of new deadly pathogenic RNA viruses,\(^\text{9−11}\) including pathogenic coronavirus, accentuates the need to develop novel broad-spectrum antivirals and vaccines.\(^\text{12−14}\) Particularly, the broad-spectrum antiviral development strategies have targeted mechanisms that are highly conserved in RNA viruses, e.g., viral replication;\(^\text{10−12}\) importantly, these small molecules have now been repurposed for COVID-19 and have shown significant potency for targeting SARS-CoV-2. One such example is remdesivir, a broad-spectrum antiviral that targets RNA-dependent RNA polymerases.\(^\text{15−17}\) Remdesivir was developed by Gilead Sciences as a part of their program to treat Ebola, hepatitis C, and other viral infections;\(^\text{16−17}\) and was then repurposed to combat the current COVID-19 pandemic. Similarly, there is a significant interest in developing antivirals that target other conserved mechanisms involved in RNA virus replication like viral genome-encoded RNA capping enzymes\(^\text{18}\) and viral proteases.\(^\text{19,20}\) Similarly, essential viral enzymes are also targeted to develop attenuated strains of viruses as potential live attenuated vaccine strains.\(^\text{21,22}\) The studies described here
are our first efforts to develop readily tractable and modular synthetic biology platforms for characterization and targeting of viral genome-encoded RNA capping enzymes from RNA viruses.

SARS-CoV-2 is a strain of coronavirus that causes coronavirus disease 2019 (COVID-19). The relatively large RNA genome of coronaviruses (∼30 kb) encodes several nonstructural proteins (nsp) like RNA polymerase (nsp12), helicase (nsp13), proof-reading exonucleases (nsp14), RNA capping enzymes (e.g., nsp10, nsp14), and proteases (M(pro)), which are essential for high-fidelity replication, translation, and packaging of the viral genomes, as well as evading innate immune responses.23−29 A key feature of the coronavirus replication mechanism is the replication of the viral genome catalyzed by viral RdRP and the incorporation of the RNA cap structure at the 5′-position of the viral RNA genome, which is catalyzed by a viral genome-encoded RNA capping enzyme.30 These viral RNA capping enzymes are essential components of the coronavirus genome replication and translation and are necessary for immune evasion.25,31 The final viral RNA cap-0 structure of the coronavirus genome is identical to that of eukaryotic host mRNAs, i.e., it consists of a 5′-end cap structure that consists of 7-methylguanosine (m7G) linked to the first nucleotide of the transcript mRNA via the 5′−5′ triphosphate bridge.32 Conventional RNA cap maturation process involves three key steps: (i) RNA triphosphatase, which catalyzes the cleavage of the B-phosphate and γ-phosphate, (ii) guanyltransferase that catalyzes the linkage of guanosine (G) linked to the first nucleotide of the transcript mRNA via the 5′−5′ triphosphate bridge, and (iii) methyltransferase that catalyzes the methylation at the N7 position of guanine at cap-0 and 2′-O methylation at cap-1 position using S-adenosylmethionine (AdoMet) as a cofactor. In the case of eukaryotic cells, the mRNA cap structure is necessary for the efficient recognition of the mRNA by the eukaryotic translation initiation factor 4E (eIF4E) for translation initiation.33 The RNA molecules lacking 5′-end cap structure are rapidly degraded in cytoplasmic P-bodies.34

During the course of viral/host adaptation and evolution, a viral RNA cap structure that is identical to the host mRNAs has been selected to ensure efficient viral RNA translation and propagation. Essentially, the virus disguises its RNA genome to resemble the host mRNA by incorporating a 5′-end cap structure that is identical to the host mRNA 5′-end cap structure. RNA viruses typically encode their own RNA capping enzymes that are involved in the maturation of the 5′-end RNA cap structure. One of the key reasons why RNA viruses, including coronaviruses, encode their own capping enzymes is because they replicate in the host cytoplasm, whereas the host RNA capping enzymes are localized in the host nucleus.32 As compared to the conserved enzymatic steps involved in the eukaryotic mRNA capping reactions, the
mechanistic details of the enzymatic steps involved in viral RNA capping processes can be highly diverse.\(^{35}\) In addition to the differences in enzyme structure and reaction mechanisms, the viral RNA capping is also diverse in terms of genetic components and protein domain organization. Typically, most RNA viruses encode RNA capping enzymes that first incorporate a guanosine linked to the first nucleotide of the transcript mRNA via the 5′–5′ triphosphate bridge followed by a series of methylation reactions at cap-0 and cap-1 positions (Figure 1D).\(^{35}\) Certain RNA viruses like influenza viruses incorporate a 5′-end cap by a mechanism called the cap-snatching mechanism, where viral enzymes essentially “snatch” and excise the 5′-end of host mRNAs and append it to the viral RNA genome (Figure 1B).\(^{36}\) Certain viruses encode a complex RNA secondary structure, which facilitates cap-independent translation of the viral mRNA sequence.\(^{37}\) SARS-CoV-2 genome encodes several RNA capping enzymes. Previous studies on RNA viruses, like coronavirus (e.g., SARS-CoV-1, which was the cause of SARS, 2002), have demonstrated that nsp14 is one of the key components involved in maintaining replication fidelity and therefore pathogenicity.\(^{38,39}\) Studies with pathogenic replicons of SARS-CoV-1 suggested that nsp14 was essential for high-fidelity replication and efficient viral propagation. Inactivation mutations in the methyltransferase domain of nsp14 of SARS-CoV-1 resulted in severely impaired replication/translational of the virus. Further, these functionally inactive point mutations in the methyltransferase resulted in attenuation in pathogenesis in RNA viruses.\(^{40-42}\) Typically, in vitro enzyme assays and/or pathogenic replicons of viruses have been used to screen for inhibitors of RNA capping enzymes.\(^{41,42}\) however, such platforms are not suitable for high-throughput screening methodologies that are often used for translational efforts. Developing platforms that are compatible with high-throughput screening approaches in standard laboratory settings is crucial for screening potential antivirals and identifying attenuation mutations.

Here, we developed a phenotypic yeast-based complementation platform that can be used in a standard laboratory setting for functional characterization and targeting of SARS-CoV-2 N7-MTase, nsp14 (Yeast platform for RNA Cap-0 N7-Methyltransferase—YerCOM). Through functional characterization of SARS-CoV-2 nsp14, we identified key domains and residues in nsp14 that are crucial for N7-MTase activity. We expanded YerCOM to include key nsp14 variants observed in emerging variants of SARS-CoV-2 (e.g., delta variant of SARS-CoV-2 encodes nsp14 A394V and nsp14 P46L). We also combined YerCOM with directed evolution to identify attenuation mutations in SARS-CoV-2 nsp14; Because of high sequence similarity of nsp14 in emerging coronaviruses, these observations could have implications on live attenuated vaccine development strategies. In addition, all our platforms can be readily used in combination with high-throughput screening and medicinal chemistry approaches to rapidly develop inhibitors of SARS-CoV-2 methyltransferase as potential antivirals.

**RESULTS**

Designing YerCOM and Engineering Yeast Strains for Functional Screening of RNA Cap-0 Methyltransferase Activity. Yeast has been widely used as a model system for the functional screening of various eukaryotic enzymes, and yeast-based platforms are routinely used for phenotypic screening to understand the mechanisms of various diseased states (like cancer) and develop therapeutics.\(^{43,44}\) In addition to this, yeast has also been used as a model organism to characterize the functional activities of a variety of viral enzymes.\(^{39,45-48}\) We first designed a yeast-based platform that could functionally characterize and screen the methyltransferase activity of SARS-CoV-2 nsp14. The literature suggests that coronavirus nsp14 usually has dual activity, i.e., proof-reading exonuclease activity and mRNA cap guanine-N7-methyltransferase activity.\(^{24,30}\) To design an in vivo yeast-based complementation platform for the characterization of the SARS-CoV-2 nsp14 N7-MTase activity, our strategy was to engineer Saccharomyces cerevisiae strains that contained a chromosomal deficiency in an essential gene corresponding to S. cerevisiae mRNA cap-0 N7-MTase and then test if we could complement this yeast deficiency by the plasmid-based expression of SARS-CoV-2 nsp14. The S. cerevisiae mutant strains were expected to survive only if the nsp14 was able to catalyze the mRNA cap-0 N7-MTase reaction; on the other hand, S. cerevisiae strains would be inviable in the absence of such activity. Designing such a phenotypic platform would allow us to use simple yeast phenotypic screening to characterize the N7-MTase activity of SARS-CoV-2 nsp14, identify a methyltransferase domain of SARS-CoV-2 nsp14, identify important catalytic residues, and even screen inhibitors of SARS-CoV-2 nsp14 methyltransferase activity.

To engineer this complementation platform, we used a commercially available S. cerevisiae strain (ATCC # 4023376)\(^{30}\) as starting yeast strains. This is a diploid strain of S. cerevisiae where the abd1 gene on one of the copies of chromosome II is replaced by a G418 resistance marker (referred to as the S. cerevisiae abd1::kanMX4 diploid). The gene product corresponding to abd1 is the yeast mRNA cap-0 (guanine-N7)-methyltransferase, which is an essential enzyme for the viability of S. cerevisiae. Our first goal was to isolate haploid strains of yeast with a single copy of chromosome II containing abd1 gene deletion. Because abd1 is an essential gene,\(^{49}\) we have to complement abd1 deficiency by the plasmid-based expression of abd1. To accomplish this, we constructed a curable plasmid pMO1, which contains abd1 expression cassette under a pTP11 promoter and ura3 gene as the selection marker and transformed the S. cerevisiae abd1::kanMX4 diploid strain with pMO1 plasmid (Table 1). These strains were then subjected to sporulation for 5 days and recovered on selection medium lacking uracil but containing G418. This resulted in the growth of two possible clonal populations (Figure 2A): (i) S. cerevisiae abd1::kanMX4 diploid strain containing plasmid pMO1 and (ii) S. cerevisiae abd1::kanMX4 haploid strain containing plasmid pMO1. As per the YerCOM design, we needed to isolate the haploid strain as the starting host strain. To this end, we screened surviving colonies to identify S. cerevisiae abd1::kanMX4 haploid strain containing plasmid pMO1. We isolated the total genomic DNA from a subset of individual colonies and analyzed it using primers specific to the S. cerevisiae mating allele (MAT) to identify if the strains contained both MATa and MATa (indicating diploid strains) or just one of them (indicating haploid strain). Through this screening, we identified S. cerevisiae abd1::kanMX4 haploid strain containing plasmid pMO1 (Figure S2). The resulting haploid strains had a replacement of abd1 gene by the G418 resistance cassette on chromosome II (Figure S2). In these strains, the chromosomal deficiency of the essential S. cerevisiae gene, abd1, was rescued.
Table 1. Details of Plasmids Used in This Study

| plasmid       | P_2Pl-driven expression cassette | plasmid marker | plasmid backbone |
|--------------|---------------------------------|---------------|-----------------|
| pMO1         | abd1                            | URA3          | pRS416          |
| pMO2         | nsp14-mce1                      | LEU2          | pRS425          |
| pMO3         | mce1                            | LEU2          | pRS425          |
| pMO4         | nsp14 (D331A)-mce1              | LEU2          | pRS425          |
| pROS1        | nsp14 (Y512–Q528 deletion)-mce1 | LEU2          | pRS425          |
| pROS2        | nsp14 MTase domain (D292–Q528)  | LEU2          | pRS425          |
| pROS9        | nsp14 (D243A)-mce1              | LEU2          | pRS425          |
| pROS8        | nsp14 ExoN domain (A2-V291)     | LEU2          | pRS425          |
| pROS12       | nsp14 (A2-G249 deletion)-mce1   | LEU2          | pRS425          |
| pROS13       | nsp14 (A2-L186 deletion)-mce1   | LEU2          | pRS425          |
| pROS14       | nsp14 (C485–Q528 deletion)-mce1 | LEU2          | pRS425          |
| pMO15        | nsp14 (V381L)-mce1              | LEU2          | pRS425          |
| pMO16        | nsp14 (A394V)-mce1              | LEU2          | pRS425          |
| pMO17        | nsp14 (P46L)–mce1               | LEU2          | pRS425          |
| pMO18        | nsp14 (P412H)-mce1              | LEU2          | pRS425          |
| pMO32        | nsp14                            | LEU2          | pRS425          |
| pMO27        | nsp14 (W293F)-mce1              | LEU2          | pRS425          |
| pMO28        | nsp14 (F368N)-mce1              | LEU2          | pRS425          |
| pMO33        | nsp14 (I42V)-mce1               | LEU2          | pRS425          |

by the pMO1 expressing functional ABD1 (strain name: S. cerevisiae abd1::kanMX4 pMO1 haploid). Since pMO1 has a Ura3 marker, it can be cured in the presence of 5-fluoro-orotic acid (5-FOA), which is converted to 5-fluorouracil, which is a toxic molecule. So, in the case of S. cerevisiae abd1::kanMX4 pMO1 haploid strains, the addition of 5-FOA would be expected to result in the loss of cell viability due to curing of pMO1 resulting in the complete lack of nuclear- and plasmid-based abd1 expressions, which is lethal to S. cerevisiae strains. We confirmed that S. cerevisiae abd1::kanMX4 pMO1 haploid strains are not viable when cultured in synthetically defined medium containing 5-FOA. This can be seen from Figure 2B,C; no cell growth is observed in the presence of S-FOA, indicating that the lack of ABD1 expression is lethal to the cells. On the other hand, growth is observed in the control strains S. cerevisiae abd1::kanMX4 diploid pMO1 in the presence of 5-FOA as one of the copies of chromosomes II in the diploid strain still possesses native abd1 gene. Taken together, these data suggest that the S. cerevisiae abd1::kanMX4 pMO1 haploid strain can be used for the functional screening of N7-MTase activity. This haploid strain is used for developing subsequent YeRC0M platforms described in this study (Table 1 shows a list of all plasmids constructed and used in this study; Figure S1 and Table S4 show plasmid map details, and Table S5 shows a list of all of the strains engineered and used in this study).

**Building YeRC0M-nsp14 for SARS-CoV-2 nsp14 N7-MTase Activity.** Next, we investigated if we could use the S. cerevisiae abd1::kanMX4 pMO1 haploid-based platform to functionally characterize SARS-CoV-2 nsp14 N7-MTase activity. Particularly, we investigated if the S. cerevisiae abd1::kanMX4 haploid abd1 deficiency can be rescued by the expression of SARS-CoV-2 RNA cap-0 N7-MTase, nsp14.

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**Figure 2.** Engineering YeRC0M for functional screening of RNA cap-0 methyltransferase activity. (A) Scheme of workflow to generate S. cerevisiae abd1::kanMX4 pMO1 haploid. S. cerevisiae abd1::kanMX4 pMO1 diploid was sporulated and dissected, which led to two possible populations of strains surviving on selection media: S. cerevisiae abd1::kanMX4 pMO1 diploid and S. cerevisiae abd1::kanMX4 pMO1 haploid. S. cerevisiae abd1::kanMX4 pMO1 diploid can grow in the presence of 5-FOA because abd1 is expressed from the genome. S. cerevisiae abd1::kanMX4 pMO1 haploid cannot grow in the presence of 5-FOA because growth is dependent on the plasmid-based expression of abd1; curing pMO1 results in no yeast growth. (B) Both S. cerevisiae abd1::kanMX4 pMO1 diploid and S. cerevisiae abd1::kanMX4 pMO1 haploid grow in the absence of S-FOA, but only S. cerevisiae abd1::kanMX4 pMO1 diploid grows in the presence of 5-FOA due to the chromosomal expression of abd1. Data represent three biological replicates. (C) Total growth after 48 h of S. cerevisiae abd1::kanMX4 pMO1 haploid (haploid) and S. cerevisiae abd1::kanMX4 pMO1 diploid (diploid) in the presence of S-FOA. Each circle represents an individual replicate. Bars represent the average of three replicates, and error bars represent mean ± SD. (D) Enzymatic scheme demonstrating S′ capping of yeast mRNAs using native yeast enzymes (Ceg1, Cet1, ABD1).
To screen for this possibility, we generated a fusion between SARS-CoV-2 nsp14 sequence and human mce1 gene, which was expected to direct the viral nsp14 to RNA polymerase II complex and incorporated it into a plasmid with P_TPI promoter and leu2 marker (plasmid name: pMO2). We also constructed a control plasmid lacking nsp14 and expressing only mce1 (plasmid pMO3). We transformed pMO2 and pMO3 into S. cerevisiae abd1::kanMX4 haploid to generate S. cerevisiae abd1::kanMX4 pMO1 haploid and S. cerevisiae abd1::kanMX4 pMO1 pMO2 haploid and S. cerevisiae abd1::kanMX4 pMO1 pMO3 pMO4 haploid, respectively. If the SARS-CoV-2 nsp14-MEC1 fusion catalyzes the RNA cap-0 N7-methylation of the native yeast mRNAs, then S. cerevisiae abd1::kanMX4 pMO1 pMO2 haploid will survive in the presence of 5-FOA where the abd1 expressing plasmid pMO1 is cured. This would indicate that the SARS-CoV-2 nsp14-MEC1 fusion was able to functionally rescue abd1 deficiency in S. cerevisiae abd1::kanMX4 haploid strains. On the other hand, if no growth was observed for S. cerevisiae abd1::kanMX4 pMO1 pMO2 pMO3 pMO4 haploid strains in the presence of 5-FOA, this would indicate that the SARS-CoV-2 nsp14-MEC1 fusion was unable to functionally rescue abd1 deficiency in S. cerevisiae abd1::kanMX4 haploid strains. As demonstrated in Figure 3B,C, the S. cerevisiae abd1::kanMX4 pMO1 pMO2 pMO3 pMO4 haploid was able to survive in the presence of 5-FOA, indicating that the SARS-CoV-2 nsp14-MEC1 fusion was able to rescue a complete lack of nuclear and plasmid expression of mce1.

Figure 3. Using YeRC0M for functional characterization of SARS-CoV-2 nsp14 activity. (A) SARS-CoV-2 nsp14 plasmid expression is able to recover S. cerevisiae abd1::kanMX4 haploid growth in the presence of 5-FOA. (B) The ability of WT nsp14 and a series of mutants and truncations of nsp14 (see Table 1 for details) to catalyze the N7-methylation of yeast mRNAs was studied by transforming their respective plasmid construct in S. cerevisiae abd1::kanMX4 pMO1 haploid and monitoring their ability to recover growth in the presence of 5-FOA. Data represent three biological replicates. (C) Representative strains listed in panel (B) were grown in a liquid selection medium in the presence of 5-FOA, and the total growth after 48 h was measured (OD_{600}). Each circle represents an individual replicate. Bars represent the average of four replicates, and error bars represent mean ± SD. Note: in the figure legend of the bar charts, pMO2 corresponds to S. cerevisiae abd1::kanMX4 pMO1 pMO2 haploid, pMO3 corresponds to S. cerevisiae abd1::kanMX4 pMO1 pMO3 pMO4 haploid, and so on. (D) Enzymatic scheme demonstrating 5’ capping of yeast mRNAs using yeast enzymes (Ceg1, Cet1) and SARS-CoV-2 nsp14.
On the other hand, the control plasmid lacking nsp14 was unable to rescue the growth of *S. cerevisiae abd1::kanMX4* haploid in the presence of 5-FOA, indicating that human mce1 alone was unable to rescue the *abd1* deficiency in *S. cerevisiae abd1::kanMX4* haploid strains. Taken together, these data suggested that SARS-CoV-2 nsp14 was active (which is consistent with the *in vitro* enzyme assays) and was able to catalyze the N7-methylation of the RNA cap-0 of *S. cerevisiae* mRNAs resulting in the survival of *S. cerevisiae abd1::kanMX4* in the absence of *abd1*. We also investigated if instead of using nsp14-mce1 fusion, we could use nsp14 by itself (plasmid: pMO32) for the functional rescue of *S. cerevisiae abd1::kanMX4* haploid strain in the presence of 5-FOA. Therefore, all future analysis was performed with variants of nsp14 fused to mce1 targeting domain. These sets of experiments suggested that this engineered phenotypic platform could be used for the functional screening of SARS-CoV-2 nsp14 N7-MTase activity.

Using YeRC0M-nsp14 Platform to Determine Key Domains Necessary for SARS-CoV-2 nsp14 N7-MTase Activity. Next, we used the YeRC0M-nsp14 platform to determine the key domains and amino acid residues in SARS-CoV-2 nsp14 that are crucial for the N7-MTase activity. To demonstrate the functional relevance of the putative
methyltransferase domain of nsp14 to its N7-MTase activity, we generated the truncation variant of SARS-CoV-2 nsp14 that lacked the N7-MTase domain (deletions from amino acids 292–528, plasmid pRO2 containing nsp14 Δ292–528). Next, S. cerevisiae abd1::kanMX4 pMO1 haploid was transformed with pRO2 to generate S. cerevisiae abd1::kanMX4 pMO1 pRO2 haploid strain.

To determine if nsp14 Δ292–528 truncation possessed N7-MTase activity, S. cerevisiae abd1::kanMX4 pMO1 pRO2 haploid strain was treated with 5-FOA, as shown in Figure 3B,C; these strains were unable to survive upon 5-FOA treatment, indicating that nsp14 Δ292–528 truncation was catalytically inactive. As expected, this suggested that the MTase domain of nsp14 was essential for N7-MTase activity. We next investigated that the relevance of exonuclease domain was for the N7-MTase activity of nsp14. We constructed a plasmid pRO3 containing a variant of nsp14 that lacked the ExoN domain (amino acids 2–292, nsp14 Δ2–292 truncation). We then transformed S. cerevisiae abd1::kanMX4 pMO1 haploid strain with pRO8 to generate S. cerevisiae abd1::kanMX4 pMO1 pRO8 haploid strain. As before, we performed 5-FOA treatment, and as shown in Figure 3B,C, surprisingly, the S. cerevisiae abd1::kanMX4 pMO1 pRO8 haploid was unable to survive in the presence of 5-FOA, indicating that the fusion of methyltransferase domain of nsp14 alone to mce1 was not sufficient for efficient RNA cap-0 N7-MTase activity. We hypothesized that the ExoN domain of nsp14 likely plays a structural role in the efficient N7-MTase activity and predicted that the functionally inactive exonuclease domain along with the MTase domain of nsp14 fused to mce1 would be able to complement abd1 deficiency. To test this hypothesis, we generated a catalytically inactive variant of ExoN34 (nsp14 D243A, plasmid pRO9), transformed it in host cells to generate S. cerevisiae abd1::kanMX4 pMO1 pRO4 haploid, and showed that S. cerevisiae abd1::kanMX4 pMO1 pRO9 haploid was able to survive in the presence of 5-FOA (Figure 3B,C). This observation was consistent with our hypothesis that while it may not be necessary to have a catalytically active ExoN domain, the structural features of the ExoN domain of nsp14 could play a role in the efficient catalysis of the nsp14 N7-MTase activity.

Next, we investigated the roles of several zinc finger domains in SARS-CoV-2 nsp14. Nsp14 has two zinc finger domains near the N-terminus and one zinc finger domain near the C-terminus, which could play a role in binding of the RNA substrate. To investigate the importance of these domains for N7-MTase activity, we made four truncation variants of nsp14: (i) C-terminal truncation (nsp14 Δ485–528, plasmid pRO14), (ii) C-terminal 17 amino acid truncation (nsp14 Δ512–528, plasmid pRO1), (iii) nsp14 Δ2–186 containing a deletion of one zinc finger domain (plasmid pRO12), and (iv) nsp14 Δ2–249 containing a deletion of two zinc finger domains (plasmid pRO13). Plasmids corresponding to each of these variants were transformed into S. cerevisiae abd1::kanMX4 pMO1 haploid to generate corresponding haploid strains. As shown in Figure 3B,C, none of the haploid strains were able to survive in the presence of 5-FOA, indicating the importance of zinc finger domains to nsp14 activity.

Expanding YeRC0M-nsp14 Platforms for nsp14 Mutations Observed in Variants of SARS-CoV-2 nsp14 Activity. Since COVID-19 was declared a pandemic, remarkable efforts are ongoing to track various variants of SARS-CoV-2. Some of the notable recent variants of SARS-CoV-2 are B.1.1.529 (omicron), B.1.617.2 (delta), AZ.5, C.1.2, among others. Significant efforts have been focused on understanding the mutations observed in the S gene and their correlation to the escape from a vaccine-induced immune response. We noted that several of the SARS-CoV-2 variants of concern also have mutations in the gene encoding nsp14. The B.1.1.529 (omicron) variant encodes for nsp14 I42V, B.1.617.2 (delta) variant encodes for two different variants of nsp14 (A394V and P46L), AT.1 variant encodes for nsp14 V381L, B.1.616 1 variant encodes for nsp14 L157F, and R.1 variant encodes for P412H. Importantly, along with variations in the ExoN domain of nsp14, variations are also observed in the N7-MTase domain of nsp14. To understand the effect of these mutations on nsp14 N7-MTase activity, we constructed plasmids corresponding to each of these variants of nsp14 and transformed them into our starting yeast strains, S. cerevisiae abd1::kanMX4 pMO1 haploid. Upon treatment with 5-FOA, we observed similar growth of the corresponding yeast strains as compared to the yeast strains expressing wild-type nsp14 (i.e., S. cerevisiae abd1::kanMX4 pMO1 pMO2 haploid; Figure 4B,C). As before, we also generated S. cerevisiae abd1::kanMX4 pMO15 haploid, S. cerevisiae abd1::kanMX4 pMO16 haploid, S. cerevisiae abd1::kanMX4 pMO17 haploid, S. cerevisiae abd1::kanMX4 pMO18 haploid, and S. cerevisiae abd1::kanMX4 pMO33 haploid strains by two rounds of growth in the presence of 5-FOA, which resulted in the complete curing of pMO1. These strains can be readily used for phenotypic screening purposes for evaluating the activities of inhibitors targeting key nsp14 variants. We characterized the growth rates of each of these strains in comparison to S. cerevisiae abd1::kanMX4 pMO2 haploid (Figure S4).

Using YeRC0M-nsp14 to Identify Inactivation and Attenuation Variants of SARS-CoV-2 nsp14. Essential viral enzymes are often targeted to develop attenuated strains of viruses as potential live attenuated vaccine strains.21,22 Our next goal was to identify inactivation and attenuation mutations in SARS-CoV-2 nsp14. Based on the available crystal structure,52,53 literature precedence,19 and nsp14 sequence analysis from diverse coronaviruses (Figure S5),55 we performed proof-of-concept experiments to demonstrate the importance of D331 residue nsp14 to N7-MTase activity.52,53 We constructed a plasmid pMO4, derived from pMO2, where we replaced residue D331 with alanine. We transformed pMO4 into S. cerevisiae abd1::kanMX4 pMO4 haploid and as expected, observed that the corresponding strain was unable to survive in the presence of 5-FOA, indicating that this point mutation resulted in a functionally inactive variant of nsp14. Since S-adenosylmethionine (AdoMet) is a necessary coenzyme for N7-MTase activity, we targeted AdoMet binding pocket to identify variants of nsp14 that could have attenuated activity. Because our approaches are compatible with high-throughput phenotypic screening, we used site-saturated mutagenesis/screening approach to determine inactivating mutations in SARS-CoV-2 nsp14. Such identification would facilitate a precise understanding of the functional relevance of highly conserved (Figure S5) noncatalytic residues to the activity of nsp14. To this end, we used YeRC0M-nsp14 platform for the directed evolution of SARS-CoV-2 nsp14.36 First, we generated a randomized site saturation mutagenesis library at residues A354, C388, and Y369. We then transformed this library into S. cerevisiae abd1::kanMX4 pMO1 haploid, and through replica
plating in the presence and absence of 5-FOA, we were able to identify the key residue changes in the AdoMet binding pocket that result in the inactivation of nsp14 (Figure S5). Notably, every inactivated mutant we identified had position C388 mutated (Figure S5). The list of inactivating mutations that we identified using this approach is shown in Figure S5. Our next goal was to identify attenuated variants of SARS-CoV-2 nsp14. Because this library had a very inactivation rate, to identify attenuated variants of nsp14, we constructed a randomized site saturation mutagenesis library at residues W293 and F368 in the AdoMet binding pocket. Our sequence alignment suggests that while W293 is a highly conserved residue (Figures S5 and 6A,B), F368 is not as highly conserved. We transformed S. cerevisiae abd1::kanMX4 pMO1 haploid strains with W293X-F368X nsp14 library and screened ∼130 colonies through replica plating in the presence of 5-FOA. Through this screening approach, we were able to identify an attenuated mutant of nsp14 (Figure S5A), which resulted in the significantly slower growth of S. cerevisiae abd1::kanMX4 haploid strains in the presence of complementation plasmid expressing nsp14 library variant. We then sequenced this nsp14 variant and observed that this variant had the following changes: W293F and F368N. To elucidate the relevance of each of individual alterations, we generated and transformed plasmids expressing nsp14-W293F (pMO27) and nsp14-F368N (pMO28) individually into S. cerevisiae abd1::kanMX4 haploid strains. We then monitored the growth of the corresponding strains over 48 h (Figure 5D) in the presence of 5-FOA. We observed that W293F was responsible for most of the attenuated activity (Figure 5D). Upon further screening of the W293X/F368X and D353X libraries, we identified several additional attenuation mutations of nsp14: F368L, D353T, and D353A (Figure 5E).

To understand the biochemical basis of the identified attenuation double mutant, we used SWISS-MODEL and AlphaFold to predict the structure of both WT nsp14 and attenuated nsp14 to compare it to the crystal structure of nsp14 (PDB 5C8T). W293F opens up the AdoMet binding region, the active site, which may have altered the binding of coenzyme AdoMet to nsp14. Both
models predict a slight shift in the positions of C388 and Y369, further opening up the AdoMet binding pocket in nsp14.

**DISCUSSION**

To combat emerging pathogenic RNA viruses, there is an immediate need to develop broadly applicable platforms that can be rapidly and rationally modulated based on emerging pathogens. For example, to combat the current COVID-19 pandemic, using modular vaccine or antiviral development platforms, remarkable progress has been made in relatively short time. These therapeutic approaches have targeted highly conserved, essential viral enzymes and proteins involved in viral infection and replication. Inspired by these observations, we sought to develop readily tractable and modular synthetic biology platforms targeting another essential and highly conserved, but relatively less explored, step in coronavirus translation and replication, i.e., capping of the viral RNA genome.

As mentioned earlier, typically, in vitro assays or in vivo studies with viral replicons of pathogenic human RNA viruses have been used for studying and targeting RNA capping mechanisms. Such platforms using viral replicons of pathogenic human RNA viruses are often not compatible for use in standard biosafety level 2 setting and can also be significantly challenging to genetically manipulate, thereby making them intractable for basic molecular virology studies and high-throughput translational efforts targeting RNA capping enzymes. Here, we developed a synthetic phenotypic yeast platform for molecular characterization and targeting of SARS-CoV-2 genome-encoded enzymes involved in capping of the viral RNA genome.

Figure 6. Sequence and structural analysis of WT and attenuated nsp14 mutant. (A) Sequence alignment of nsp14 protein sequences from a diverse set of α, β, and γ coronaviruses. Complete sequence alignment is shown in Figure S5. Amino acid residues highlighted in red (D331, D243) are highly conserved residues, and their mutation to alanine is known to abolish methyltransferase function (D331A) and exonuclease function (D243A) of nsp14. Amino acid residues highlighted in yellow (W293, A354, F368, Y369, and C388) are highly conserved residues that we targeted for random mutagenesis in this study. Amino acid residues highlighted in green are mutations found in recent SARS-CoV-2 variants and studied for their effects on nsp14 methyltransferase function in this study. Accession numbers for representative nsp14 sequences: SARS-CoV-1 (AY278741), SARS-CoV-2 (MT318827.1), Pangolin CoV (MT121216.1), Bat CoV (MN996532), Mappie-robim-CoV (005352853), Canada Goose CoV (QCB65096), Mink CoV (009019180), and Erineaceus hedgehog (Q6A70691). Sequence alignment was performed using Clustal Omega EMBL-EBI multiple sequence alignment tool. (B) Evolutionary relationships between strains used in sequence alignment analysis predicted using Clustal Omega. (C) Superimposition of the WT nsp14 crystal structure (PDB code: 5C8T) (wheat ribbon and sticks) with predicted WT nsp14 SWISS-MODEL structure (slate blue ribbon and sticks). (D) Superimposition of the WT nsp14 crystal structure (PDB: 5C8T) (wheat ribbon and sticks) with the predicted nsp14 SWISS-MODEL structure (slate blue ribbon and sticks) for attenuated nsp14 mutant (F368N, W293F). Zoomed-in active site images show residues interacting with ligand S-adenosylmethionine (SAM). Residues mutated in this study (C388, A354, F368, Y369, W293) are shown.

We anticipate these platforms to be modular and readily adaptable to a range of emerging
RNA viruses. Further, since these platforms are compatible for use in standard laboratory setting for fundamental molecular virology and translational efforts, we believe that these approaches will fundamentally increase our understanding of the molecular mechanisms involved in capping of the viral RNA genome as well as provide a modular phenotypic screening platform that would accelerate the development of broad-spectrum antiviral and live attenuated vaccine candidates.

**METHODS**

Strains Commercially Purchased. *Saccharomyces cerevisiae* strains are derived from YBR236C BY4743, homozygous diploid MATa/MATα his3delta1/his3delta1 leu2delta0/leu2delta0 lys2delta0/+ met15delta0/+ ura3delta0/ura3delta0 deltaABD1 (ATCC 4033376).

Sporulation and Germination Procedure. To produce haploid *S. cerevisiae* ∆ABD1 pMO1 from diploid *S. cerevisiae* ∆ABD1 pMO1, diploid *S. cerevisiae* ∆ABD1 pMO1 was subject to sporulation conditions (incubation in 0.3% KOAc at 23 °C) for 5 days. The sporulated cells were then treated with zymolyase and plated on synthetic defined media lacking uracil. Several colonies were screened for their ploidy using oligonucleotides AM965/AM966/AM967 amplifying the *S. cerevisiae* MATa allele.

Growth Media Conditions. All *S. cerevisiae* cultures were shaken aerobically at 30 °C and 250 rpm in synthetic defined medium containing 0.67% of nitrogen base without amino acids, 2% of glucose, 0.02 mg/mL of lysine, 0.02 mg/mL of methionine, 0.02 mg/mL of histidine, and 0.1 mg/mL of carbeneccillin. Leucine of 0.012 mg/mL was added when *S. cerevisiae* ∆abd1 pMO1 haploid was cultured.

Plasmid Curing Procedure. Plasmids containing ura3 marker were cured by growing cultures in synthetically defined medium containing 1 mg/mL of 5-fluoroorotic acid (5-FOA) and 0.02 mg/mL of uracil and incubating at 30 °C for 48 h.

Construction of Plasmids. Plasmid maps are included in Figure S1. Plasmid mapBenchmark links are provided in Table S4. All single-stranded and double-stranded DNA oligonucleotide fragments were purchased from Integrated DNA Technologies (IDT). Single-stranded oligonucleotide sequences (gblocks) used for Gibson assembly are listed in Table S1. Double-stranded oligonucleotide sequences (gblocks) used for Gibson assembly are listed in Table S2. Genomic DNA fragments used in the construction of plasmids are listed in Supporting Table S3. When noted, coding sequences were codon-optimized for *S. cerevisiae* expression using IDT codon optimization software (https://www.idtdna.com/CodonOpt).

pMO1: the promoter sequence, pTP11, was amplified from the gDNA of *S. cerevisiae* YPH500 using the oligonucleotides MO41/MO42. The gene corresponding to ABD1 was amplified from the gDNA of *S. cerevisiae* YPH500 using the oligonucleotides MO45/MO46. pRS416 ura3 was linearized using the oligonucleotides MO44/MO43. pTP11 and aab1 gene fragments were inserted into linearized pRS416 ura3 by Gibson assembly to afford pMO1.

pMO2: double-stranded nsp14 and mce1 gblocks codon-optimized for *S. cerevisiae* were purchased from Integrated DNA Technologies (IDT). Mce1 gBlock was amplified using oligonucleotides JC136/MO4. Nsp14 gBlock was amplified using oligonucleotides MO57/MO75. pRS425 leu2 pTP11 was linearized using the oligonucleotides MO58/JC135. Nsp14 and mce1 fragments were inserted into linearized pRS425 leu2 by Gibson assembly to afford pMO2.

pMO3: pMO2 was linearized using oligonucleotides MO215/MO132. The linearized fragment was ligated by KLD reaction (NEB #MO548S) to afford pMO3.

pMO4: point mutation D331A was introduced into pMO2 by linearizing pMO2 using the oligonucleotides MO69/MO70. The plasmid was ligated by KLD reaction to afford pMO4.

pROS1: the C-terminal 17 amino acids of nsp14 were removed from pMO2 by amplifying nsp14 using oligonucleotides MO186/MO57. pMO2 was linearized to exclude the C-terminal 17 amino acids of nsp14 using oligonucleotides MO132/MO58. The nsp14 domain fragment was inserted into the linearized pMO2 by Gibson assembly to afford pROS1.

pROS2: the methyltransferase domain of nsp14 was removed from pMO2 by amplifying the exonuclease domain of nsp14 using oligonucleotides MO189/MO57. pMO2 was linearized to exclude the methyltransferase domain of nsp14 using oligonucleotides MO132/MO58. The exonuclease domain fragment was inserted into the linearized pMO2 by Gibson assembly to afford pROS2.

pROS8: the exonuclease domain of nsp14 was removed from pMO2 by amplification of the methyltransferase domain of nsp14 using oligonucleotides ROS17/ROS16. pMO2 was linearized to exclude the exonuclease domain of nsp14 using oligonucleotides ROS7/ROS8. The methyltransferase domain fragment was inserted into the linearized pMO2 by Gibson assembly to afford pROS8.

pROS9: point mutation D243A mutation was introduced into pMO2 by linearizing pMO2 using the oligonucleotides ROS18/ROS19. The plasmid was ligated by KLD reaction to afford pMO4.

pROS12: pMO2 was linearized using oligonucleotides ROS21/ROS30 to delete amino acids A2-G249. The linearized fragment was ligated by KLD reaction to afford pROS12.

pROS13: pMO2 was linearized using oligonucleotides ROS21/ROS31 to delete amino acids A2-L186. The linearized fragment was ligated by KLD reaction to afford pROS13.

pROS14: pMO2 was linearized using oligonucleotides ROS8/ROS32 to delete amino acids C485−Q528. The linearized fragment was ligated by KLD reaction to afford pROS14.

pMO15: point mutation V381L was introduced into nsp14 by linearizing pMO2 using oligonucleotides MO246/MO247. The linearized fragment was ligated by KLD reaction to afford pMO15.

pMO16: point mutation A394V was introduced into nsp14 by linearizing pMO2 using oligonucleotides MO248/MO249. The linearized fragment was ligated by KLD reaction to afford pMO16.

pMO17: point mutation P46L was introduced into nsp14 by linearizing pMO2 using oligonucleotides MO242/MO243. The linearized fragment was ligated by KLD reaction to afford pMO17.

pMO18: point mutation P412H was introduced into nsp14 by linearizing pMO2 using oligonucleotides MO250/MO251. The linearized fragment was ligated by KLD reaction to afford pMO18.

C388X/Y369X/A354X library construction: a hydrophobic amino acid focused library was made by randomizing codons at C-terminal amino acids of nsp14 using oligonucleotides MO57/MO75. pRS425 leu2 pTP11 was linearized using the oligonucleotides MO58/JC135. Nsp14 and mce1 fragments were inserted into linearized pRS425 leu2 by Gibson assembly to afford pMO2.

pMO3: pMO2 was linearized using oligonucleotides MO215/MO132. The linearized fragment was ligated by KLD reaction (NEB #MO548S) to afford pMO3.

pMO4: point mutation D331A was introduced into pMO2 by linearizing pMO2 using the oligonucleotides MO69/MO70. The plasmid was ligated by KLD reaction to afford pMO4.
positions C388, Y369, and A354. This was done by amplifying pMO2 using oligonucleotides MO151/MO156 to create a library insert. A second amplification using oligonucleotides MO151/MO157 was performed on this fragment to extend the insert. The insert was ligated to a linearized backbone fragment that was made by amplifying pMO2 with oligonucleotides MO126/MO127. The insert and backbone fragments were ligated using Gibson assembly to afford the library.

W293X/F368X library construction: a library at positions W293 and F368 was made by amplifying pMO2 with oligonucleotides MO221/MO212 to create a library insert. The insert was ligated to a linearized backbone fragment that was made by amplifying pMO2 with oligonucleotides MO218/MO211. The insert and backbone fragments were ligated using Gibson assembly to afford the library.

pMO32: pMO2 was linearized using oligonucleotides MO264/DS001 to remove mce1. The linearized fragment was made by linearizing pMO2 using oligonucleotides MO258/MO259. The linearized fragment was ligated by KLD reaction to afford pMO32.

pMO27: point mutation W293F was introduced into nsp14 by linearizing pMO2 using oligonucleotides MO258/MO259. The linearized fragment was ligated by KLD reaction to afford pMO27.

pMO28: point mutation F368N was introduced into nsp14 by linearizing pMO2 using oligonucleotides MO260/MO261. The linearized fragment was ligated by KLD reaction to afford pMO28.

pMO33: point mutation I42V was introduced into nsp14 by linearizing pMO2 using oligonucleotides MO265/MO266. The linearized fragment was ligated by KLD reaction to afford pMO33.

D353X library construction: a library at positions D353 made by amplifying pMO2 with oligonucleotides MO262/JC136 to create a library insert. The insert was ligated to a linearized backbone fragment that was made by amplifying pMO2 with oligonucleotides MO263/JC135. The insert and backbone fragments were ligated using Gibson assembly to afford the library.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.2c00359.

Supporting information has additional data figures, plasmid maps, and oligonucleotide sequences used for plasmid construction (PDF).

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Notes
The authors declare no competing financial interest.

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