NANO LETTERS

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Letter

Lipid Nanoparticle RBD-hFc mRNA Vaccine Protects hACE2 Transgenic Mice against a Lethal SARS-CoV-2 Infection

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Cite This: *Nano Lett.* 2021, 21, 4774–4779

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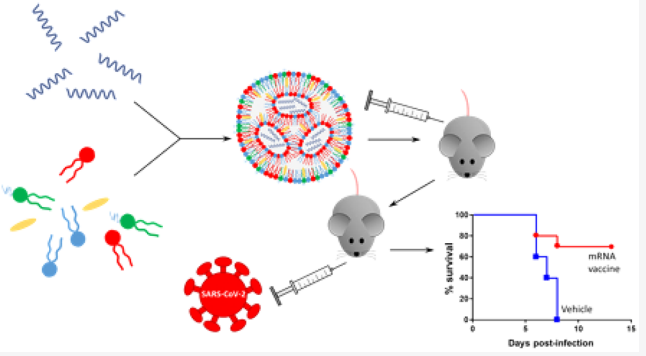
ABSTRACT: The COVID-19 pandemic led to development of mRNA vaccines, which became a leading anti-SARS-CoV-2 immunization platform. Preclinical studies are limited to infection-prone animals such as hamsters and monkeys in which protective efficacy of vaccines cannot be fully appreciated. We recently reported a SARS-CoV-2 human Fc-conjugated receptor-binding domain (RBD-hFc) mRNA vaccine delivered via lipid nanoparticles (LNPs). BALB/c mice demonstrated specific immunologic responses following RBD-hFc mRNA vaccination. Now, we evaluated the protective effect of this RBD-hFc mRNA vaccine by employing the K18 human angiotensin-converting enzyme 2 (K18-hACE2) mouse model. Administration of an RBD-hFc mRNA vaccine to K18-hACE2 mice resulted in robust humoral responses comprising binding and neutralizing antibodies. In correlation with this response, 70% of vaccinated mice withstood a lethal SARS-CoV-2 dose, while all control animals succumbed to infection. To the best of our knowledge, this is the first nonreplicating mRNA vaccine study reporting protection of K18-hACE2 against a lethal SARS-CoV-2 infection.



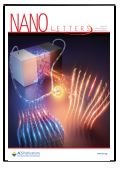
KEYWORDS: *SARS-CoV-2, COVID-19, mRNA vaccine, lipid nanoparticles, ionizable lipids*

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), identified as the causative agent of coronavirus disease 2019 (COVID-19), developed in the last year into a global pandemic, causing (as of March 21, 2021) over 120 million cases and 2.7 million deaths worldwide.[1] Unprecedented international collaboration has led to record time development of several vaccine candidates, of which the mRNA vaccine platform has proven to be highly efficacious in phase 3 clinical studies.[2,3] Consequently, mRNA-based vaccines became the first prophylactic therapeutics to be approved by the UK's Medicine and Healthcare products Regulatory Agency (MHRA) and the US Food and Drug Administration (FDA), authorizing the Pfizer-BioNTech BNT162b2 vaccine, followed by approval of Moderna's mRNA-1273 candidate for global emergency mass use.

As a vaccine platform, mRNA holds several advantages over other vaccine approaches: in contrast to DNA vaccines, mRNA does not require delivery into the nucleus, as it is translated in the cytoplasm. Unlike plasmid DNA and viral vectors, mRNA does not integrate into the genome, therefore presenting higher safety profile. Additionally, mRNA is a noninfectious particle and is unable to cause disease, as theoretically possible with attenuated pathogen-based vaccines. Moreover, mRNA is only transiently active and is degraded by physiological metabolic processes.[4−7] Importantly, mRNA vaccine production is relatively rapid, simple, and inexpensive compared to other vaccine platforms, as was demonstrated recently with the development of SARS-CoV-2 mRNA vaccines by both Moderna and Pfizer.[2,3]

A drawback of recently developed mRNA vaccines is the relatively poor stability during storage. While most vaccines can be stored at 2−8 °C, currently used mRNA vaccines must be kept frozen, at temperatures between −15 and −25 °C (mRNA-1273 by Moderna) or −60 and −90 °C (BNT162B2 by Pfizer). Since these low temperature requirements translate into logistic challenges in vaccine storage and distribution, efforts are currently focused on optimization of LNP-mRNA

Received: March 30, 2021
Revised: May 20, 2021
Published: May 25, 2021



https://doi.org/10.1021/acs.nanolett.1c01284
Nano Lett. 2021, 21, 4774–4779

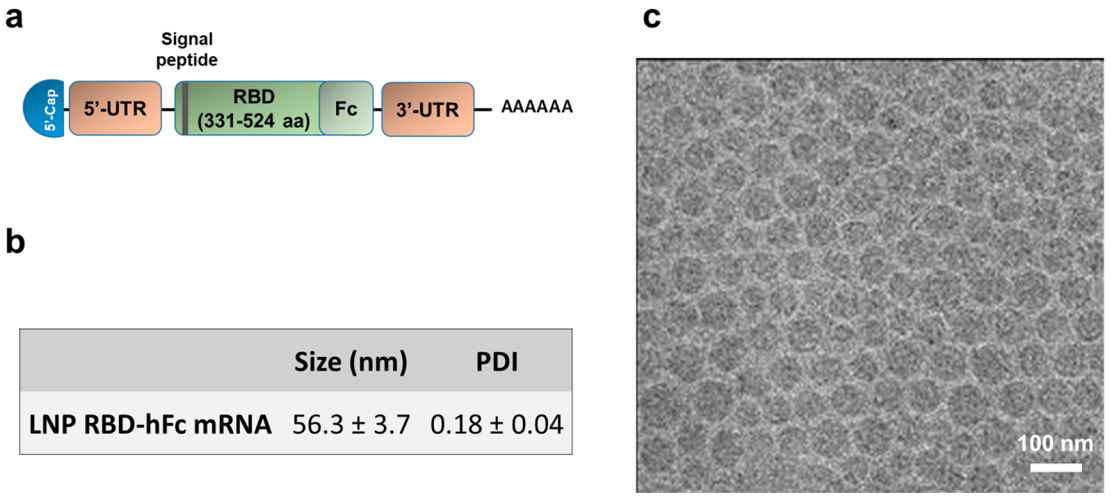


Figure 1. Design of LNP RBD-hFc mRNA and physicochemical characterization. (a) Schematic representation of the RBD-hFc mRNA construct. (b) Size distribution and polydispersity index (PDI) of LNPs measured by dynamic light scattering. (c) A representative Cryo-EM image of LNP-encapsulated RBD-hFc mRNA. Scale bar 100 nm.

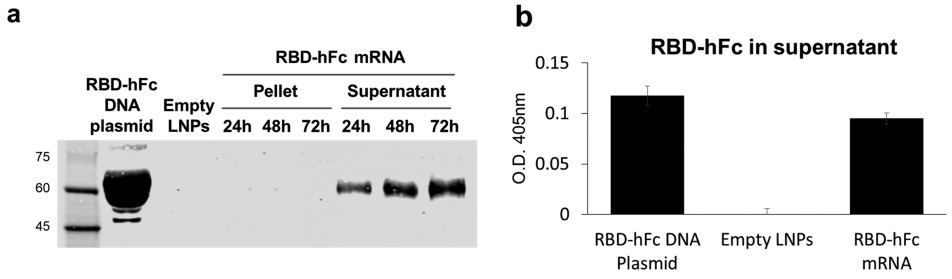


Figure 2. *In vitro* characterization of RBD-hFc expression. (a) Western blot analysis of HEK293 cells transfected with RBD-hFc DNA plasmid (1.5 μg/mL, 72 h), empty LNPs (72 h), or LNP RBD-hFc mRNA (1 μg/mL, 24−72 h). (b) Anti-hACE2 ELISA for the evaluation of hACE2 binding by hFc-RBD. Plates were coated with hACE2 (2 μg/mL), and supernatant fractions of RBD-hFc DNA plasmid-, empty LNPs-, or LNP RBD-hFc mRNA-transfected cells (72 h) were analyzed.

formulations toward increased stability that will allow non-frozen storage.[8]

The high efficacy of mRNA vaccines in eliciting a robust and effective immunologic response, together with a high safety profile and a simple, rapid manufacturing process, makes this platform extremely attractive for the prevention of infectious diseases.

The main obstacle in the *in vivo* administration of mRNA is its efficient delivery to target cells. In recent years, a wide range of different delivery approaches was developed, mainly in the context of *in vivo* siRNA delivery, some of which have been implemented also for the delivery of mRNA. Lipid nanoparticles (LNPs) represent the most advanced and frequently used carrier platform for mRNA delivery and are currently the standard method being used to introduce mRNA vaccines to humans participating in clinical trials worldwide. LNPs are generally comprised of a combination of four elements: cholesterol, a phospholipid, polyethylene glycol (PEG)-linked lipid, and an ionizable lipid. Of these components, the ionizable lipid has been shown to play a central role in the effective delivery and subsequent translation of the mRNA and encoded-protein expression.[6,9] In the current study, we directly examined the *in vivo* efficacy of an LNP-encapsulated human Fc-conjugated SARS-CoV-2 receptor binding domain (RBD-hFc)-based mRNA vaccine encompassing an ionizable lipid (lipid #14) previously demonstrated to be highly potent in eliciting a robust immune response in vaccinated BALB/c mice.[10] In order to evaluate the efficacy of the vaccine against viral infection, the K18-hACE2 transgenic mice model was employed, a model previously shown to recapitulate the susceptibility to SARS-CoV-2 infection. Prime-boost intramuscular immunization of K18-hACE2 mice with 5 μg of LNP RBD-hFc mRNA led to 70% protection against a lethal 3×10^3 plaque forming units (PFU) SARS-CoV-2 intranasal infection which resulted in the death of all untreated animals. To the best of our knowledge, this is the first report demonstrating high efficacy of a conventional (nonreplicating) mRNA vaccine against a lethal SARS-CoV-2 infection in an hACE2 mice model.

An LNP RBD-hFc mRNA vaccine was designed, based on a previous lipid formulation screen that we recently reported.[10] The results of the screen led to the selection of lipid #14, which was highly potent in eliciting strong humoral and cellular responses in BALB/c mice immunization studies. A schematic representation of the hFc-fused RBD mRNA construct is shown in Figure 1a. Following LNP preparation, samples were analyzed for size and uniformity by dynamic light scattering (DLS). As can be seen in Figure 1b, particles were small (~55 nm) and uniformly distributed, as evidenced by an average polydispersity index (PDI) of <0.2. Cryogenic electron microscopy (Cryo-EM) analysis supported the DLS data, showing small and uniform particles (Figure 1c).

In order to evaluate the translation of RBD-hFc mRNA into a functional protein, we performed *in vitro* transfection of

https://doi.org/10.1021/acs.nanolett.1c01284
Nano Lett. 2021, 21, 4774−4779

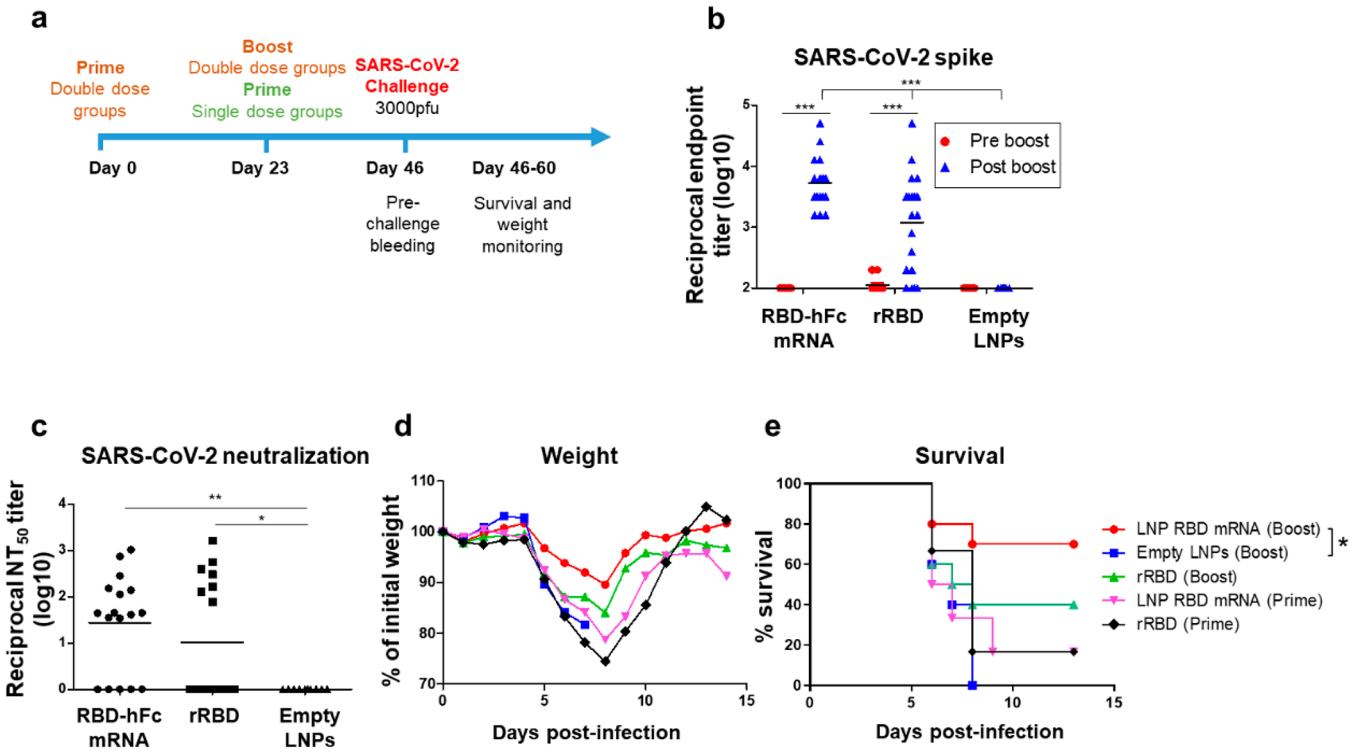


Figure 3. LNP RBD-hFc mRNA vaccine protects hACE2 trangenic mice against a lethal SARS-CoV-2 challenge. (a) Schematic diagram of immunization regimens and serum samples collection. K18-hACE2 mice were intramuscularly administered with LNP RBD-hFc mRNA (5 μg) or rRBD (10 μg) at a prime-only (n = 6) or prime-boost (n = 10) vaccination regimen. Control animals were administered with empty LNPs (prime-boost) (n = 5). Serum samples were collected 23 ("preboost") and 46 ("postboost") days after priming and were assayed for SARS-CoV-2 spike-specific IgG antibodies (b) and neutralizing antibodies against SARS-CoV-2 (c) as described in the Materials and Methods section of the Supporting Information. Animals were then intranasally infected with 3×10^3 PFU of SARS-CoV-2 and monitored for weight loss (d) and survival (e). Statistical analysis was performed using a two-way ANOVA with Bonferroni's multiple comparisons test (for ELISA data), one-way ANOVA followed by post hoc Newman−Keuls test (for neutralizing antibodies data), or log-rank (Mantel−Cox) test (for survival data) (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

HEK293 cells with LNP RBD-hFc mRNA and determined protein expression and target binding. Western blot analysis established expression of RBD-hFc in the supernatant but not in the cell pellets, confirming the extracellular secretion of the translated RBD-hFc exhibiting the expected molecular size (Figure 2a). To confirm the functionality of the translated RBD-hFc, we applied ELISA against hACE2. Binding was evaluated for cell supernatant samples collected 72 h following transfection with LNP-encapsulated RBD-hFc mRNA, empty LNPs, or RBD-hFc-expression plasmid. As demonstrated (Figure 2b), comparable binding was observed for RBD-hFc expressed by both mRNA and control DNA plasmid but not for the empty LNPs.

ACE2 receptor is the main mediator of SARS-CoV-2 binding and entry into infected cells. However, mouse ACE2 does not support binding of SARS-CoV-2, and therefore conventional laboratory mice strains are not suitable for infection studies.[11] In order to evaluate the *in vivo* efficacy of the LNP RBD-hFc mRNA vaccine against a lethal SARS-CoV-2 challenge, we employed a human ACE2 (K18-hACE2) transgenic mice model, in which hACE2 expression is driven by the epithelial cell cytokeratin-18 (K18) promoter. This model has been shown to enable efficient SARS-CoV-2 infection, leading to severe disease and lethality, and has been the model of choice for several SARS-CoV-2 immunization and therapy studies.[12−14] Groups of 6−8-week-old K18-hACE2 mice were intramuscularly administered with 5 μg of LNP RBD-hFc mRNA. Control experimental groups were immunized with empty LNPs or recombinant RBD-hFc (rRBD) protein (10 μg, subcutaneously). Prime-only or prime-boost vaccination regimens were employed, with the animals being primed at day 0 and boosted 23 days later (see vaccination outline in Figure 3a). To characterize the humoral response of the vaccinated animals, blood samples were collected prior to the challenge.

As shown in Figure 3b, a single intramuscular immunization ("preboost") with either LNP RBD-hFc mRNA (n = 10) or rRBD (n = 11) did not elicit a significant humoral response, with most animals exhibiting antispike titers of <100. Conversely, a robust and statistically significant antibody response was observed in both LNP RBD-hFc mRNA (n = 16) and rRBD (n = 19) prime-boost immunization groups ("postboost") compared with the empty LNP control group (n = 8). Within the immunization groups, the recorded antispike titers were significantly higher in mice administered with LNP RBD-hFc mRNA (mean $\log_{10}$ titer = 3.7) compared with rRBD-administered mice (mean $\log_{10}$ titer = 3.0). We next evaluated the postboost *in vitro* neutralizing antibody response using a SARS-CoV-2 plaque reduction neutralization test (PRNT). In line with the binding antibody titers, a substantial neutralizing antibody response was detected in both immunization groups, with the LNP RBD-hFc mRNA group exhibiting a stronger response compared with the rRBD group (although not statistically significant) (Figure 3c). Twenty-

https://doi.org/10.1021/acs.nanolett.1c01284
Nano Lett. 2021, 21, 4774−4779

three days following the last administered dose, all mice were challenged with 3×10^3 PFU of SARS-CoV-2 via the intranasal route and monitored daily for weight loss and survival. As shown in Figure 3d, weight loss was recorded in all animal groups, starting at day 5 and reaching a maximal loss at day 8 postchallenge. Thereafter, surviving animals began to steadily recover and return to initial weight approximately at day 11−12 postchallenge. Mice receiving empty LNPs, or a single administration of either LNP RBD-hFc mRNA or rRBD, showed a significant drop in weight (∼20−25% weight loss). In contrast, mice administered with a booster dose exhibited a less pronounced weight loss of 10% and 15% for LNP RBD-hFc mRNA and rRBD immunization groups, respectively (Figure 3d). Most importantly, while the 3×10^3 PFU SARS-CoV-2 challenge was 100% (5/5) fatal to empty LNP-administered control animals, prime-boost vaccination of mice with LNP RBD-hFc mRNA resulted in a 70% (7/10) survival rate. In accordance with the recorded humoral response, which was more robust in the LNP RBD-hFc mRNA group, prime-boost vaccination with rRBD led to a lower survival rate of 40% (4/10). Lastly, supported by the low antibody titers recorded preboost, only 16% (1/6) of mice receiving prime-only vaccinations survived (Figure 3e).

mRNA vaccines against SARS-CoV-2 have been studied extensively in the past year, both in preclinical and clinical studies.[15] Most SARS-CoV-2 mRNA vaccine preclinical studies begin with evaluation of the immunologic response elicited by the vaccine in animal models which are not susceptible to SARS-CoV-2 infection (i.e., BALB/c, C57/BL6 mice). In order to examine the efficacy of the vaccine, SARS-CoV-2 infection-prone animal models are frequently used (e.g., hamsters and nonhuman primates). In many studies, these animals are vaccinated and then challenged with virulent SARS-CoV-2, leading to symptomatic disease, weight loss, and extensive viral replication in different tissues but not death. Therefore, the protective effect of the vaccine is commonly evaluated by its ability to diminish viral replication, elicit a robust immune response, and reduce weight loss.[16−18]

In the current study, we sought to evaluate the efficacy of the vaccine by using the K18-hACE2 mouse model, which has been shown to be highly susceptible to SARS-CoV-2 infection. Prime-boost immunization of K18-hACE2 mice with the LNP RBD-hFc mRNA vaccine, elicited a specific humoral response, as evidenced by the detection of binding as well as neutralizing antibodies. Most notably, immunization enabled 70% survival of animals administrated with a lethal dose of SARS-CoV-2 compared with full mortality in the control group of unvaccinated animals, demonstrating the significant protective efficacy of vaccine-induced neutralizing antibodies. The protective effect of immunization with RBD is based on the fact that SARS-CoV-2 virus recognizes ACE2 as the receptor for host cell entry. SARS-CoV-2 spike (S) protein consists of the S1 and S2 subunits. The S1 includes the RBD, which specifically recognizes the ACE2 receptor and plays a crucial role in mediating viral entry into cells,[19] therefore serving as a target for neutralization by antibodies.

Indeed, the significant role and efficacy of prophylactic and therapeutic neutralizing antibodies against SARS-CoV-2 infection have been demonstrated in several recent publications which addressed the therapeutic efficacy of antibodies targeting various domains of the spike protein.[13,20−22] Thus, this mechanism of viral entry neutralization represents the basis for all prophylactic and postexposure countermeasures against SARS-CoV-2. Yet, only a few studies have evaluated so far the efficacy of SARS-CoV-2 mRNA vaccines in hACE2 transgenic mice. A recent study by the Arcturus and Singapore's Duke-NUS Medical School consortium described full protection of K18-hACE2 mice following a single dose of a self-transcribing and replicating RNA (STARR)-based vaccine candidate.[23] A different study by the Chinese Academy of Sciences also employed a hACE2 mouse model for evaluation of vaccine protection efficacy.[24] The data in that study, however, is based on immune humoral response, weight loss, lung viral titer, and lung immunohistochemistry analysis and not on survival of immunized animals. In summary, to the best of our knowledge, the current study demonstrates for the first time SARS-CoV-2 mRNA vaccine-mediated protection of K18-hACE2 mice against an otherwise lethal viral infection.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.1c01284.

Materials and methods describing following experimental procedures: cell lines and animals used in study, design of mRNA, preparation and characterization of lipid nanoparticles, cryo-EM imaging, *in vitro* transfection procedure, Western blot analysis, production of recombinant SARS-CoV-2 proteins, design of animal vaccination studies, SARS-CoV-2 infection procedure, ELISA analysis, plaque reduction neutralization test, and statistical analysis (PDF)

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https://doi.org/10.1021/acs.nanolett.1c01284
Nano Lett. 2021, 21, 4774−4779

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Notes

The authors declare the following competing financial interest(s): D.P. receives licensing fees (to patents on which he was an inventor) from, invested in, consults (or on scientific advisory boards or boards of directors) for, lectured (and received a fee), or conducts sponsored research at TAU for the following entities: Alnylam Pharmaceuticals Inc., Arix Biosciences Inc., ART Biosciences, BioNtech RNA Pharmaceuticals, Centricus, Diagnostear Ltd., EPM Inc., Earli Inc., lmpetis Biosciences, Kernal Biologics, GPCR Inc., Medison Pharma Ltd., Newphase Ltd., NLC Pharma Ltd., Nanocell Therapeutics, NanoGhosts Ltd., Precision Nanosystems Inc., Paul Hastings Inc., Regulon, Roche, SciCann, Shire Inc., SirTLabs Corporation, VLX Ventures, TATA Cooperation, Teva Pharmaceuticals Inc., and Wize Pharma Ltd. All other authors declare no competing financial interests.

■ ACKNOWLEDGMENTS

This work was supported by the Lewis Trust grant to D.P. We thank Hila Cohen, Liat Bar-On, Shirley Lazar, Tal Noy-Porat, and Yinon Levi for their assistance and support and Shmuel Yitzhaki, Ohad Mazor, and Emanuelle Mamroud for their insightful discussions and encouragement. We also thank Amir Rosner, Tseela David, and Beni Shareabi for animal husbandry. We thank Inbar Freilich for her professional contribution to the Cryo-EM analysis.

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