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Prestin (Slc26a5) is a motor protein previously considered to be expressed exclusively in outer hair cells (OHCs) of the inner ear. However, we recently identified the functional expression of prestin in the heart. Nonlinear capacitance (NLC) measurement in OHCs is used to evaluate the signature function of prestin, which exhibits membrane potential-dependent conformational changes. Here, we describe detailed recording techniques and quantification methods for NLC to evaluate the prestin function in mouse ventricular myocytes.

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Highlights

Prestin is functionally characterized by nonlinear capacitance (NLC) in the cell

Prestin is expressed in cardiomyocytes

Prestin accounts partly for the NLC of cardiomyocytes

We describe recording techniques and quantification methods for NLC in cardiomyocytes

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Protocol to assess two distinct components of the nonlinear capacitance in mouse cardiomyocytes

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SUMMARY
Prestin (Slc26a5) is a motor protein previously considered to be expressed exclusively in outer hair cells (OHCs) of the inner ear. However, we recently identified the functional expression of prestin in the heart. Nonlinear capacitance (NLC) measurement in OHCs is used to evaluate the signature function of prestin, which exhibits membrane potential-dependent conformational changes. Here, we describe detailed recording techniques and quantification methods for NLC to evaluate the prestin function in mouse ventricular myocytes.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2021).

BEFORE YOU BEGIN
The protocol below describes the specific steps for nonlinear capacitance (NLC) recordings in mouse ventricular myocytes. However, the NLC recording protocol can also be applied to cardiomyocytes from other species, as well as other cell types including Chinese hamster ovary (CHO) cells with heteroexpression of prestin (Slc26a5). Additionally, the protocol can be used to study other determinants of membrane capacitance.

Prestin was previously considered to be exclusively expressed in the outer hair cells of inner ear, functioning as a cochlear motor protein and cochlear amplifier, characterized by NLC. However, prestin was recently identified to be functionally expressed in cardiomyocytes to amplify actin-myosin force generation accounting partly for the NLC of cardiomyocytes. Therefore, prestin serves a broader cellular motor function (Zhang et al., 2021). The function of prestin can be directly assessed by NLC recordings and analyses.

All animal care and procedures were performed in accordance with the protocols approved by Institutional Animal Care and Use Committee of the University of California, Davis and in accordance with National Institutes of Health guidelines. The experiments described in the protocol were conducted in a blinded fashion. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), except specifically indicated. Ventricular myocytes were isolated from wild-type (WT) and Slc26a5−/− (Prestin knockout) mice.

Genotyping
Timing: 1–2 days
The Slc26a5<sup>−/−</sup> mouse model was generated previously (Liberman et al., 2002; Yamashita et al., 2015) and maintained in C57Bl/6J and SV-129 mixed background. Genotyping analyses were performed using RT-PCR of genomic DNA from WT and Slc26a5<sup>−/−</sup> mice. The primers used are as follow:

WT (Forward): 5’-GCTTGATGATTGGAGGTGTG-3’;

WT (Reverse): 5’-CTGAATGATTCCTGAAAGTAAGG-3’;

Slc26a5<sup>−/−</sup> (Forward): 5’-CTGGTGATCCGAGTTGACCTGACCC-3’

Slc26a5<sup>−/−</sup> (Reverse): 5’-GATCGCTATCAGGACATAGCG-3’

WT band is 175 bp, and Slc26a5<sup>−/−</sup> band is 500 bp.

### PCR cycling conditions

| Steps             | Temperature | Time  | Cycles |
|-------------------|-------------|-------|--------|
| Initial Denaturation | 95°C        | 10 min | 1      |
| Denaturation      | 95°C        | 30 s   | 30–40 cycles |
| Annealing         | 55°C        | 30 s   |        |
| Extension         | 72°C        | 30 s   |        |
| Final extension   | 72°C        | 10 min | 1      |
| Hold              | 4°C         |        | Forever |

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** |        |            |
| HEPES               | Sigma-Aldrich, USA | H4034 |
| K-Glutamate         | Sigma-Aldrich, USA | G1501 |
| EGTA                | Sigma-Aldrich, USA | E4378 |
| TEA-Cl              | Sigma-Aldrich, USA | T2265 |
| TEA-OH              | Sigma-Aldrich, USA | 331635 |
| SYLGARD™ 184        | Dow Corning | 025052 |
| 2,3-Butanedione monoxide (BDM) | Sigma-Aldrich, USA | B0753 |
| Blebbistatin        | Abcam, USA | Ab120425 |
| Salicylic acid      | Sigma-Aldrich, USA | W398500 |
| Collagenase type II | Worthington, USA | LS004176 |
| Ketamine hydrochloride injection (100 mg/mL) | Vedco, Inc., USA | NDC 50989-161-06 |
| Xylazine Sterile Solution (20 mg/mL) | Akorn Inc., USA | NDC59399-110-20 |
| Buprenorphone hydrochloride injection (0.3 mg/mL) | Par Pharmaceutical, USA | NDC42023-179-05 |
| Heparin (1000 USP units/mL) | Fresenius Kabi USA, LLC | NDC63323-540-57 |
| **Software and algorithms** |        |            |
| GraphPad Prism      | GraphPad Software | https://www.graphpad.com |
| Origin 6.1 software | OriginLab Corp., Northampton, MA | https://originlab.com |
| BioRender           | BioRender | https://biorender.com |
| pClamp10 software   | Molecular Devices, LLC, Sunnyvale, CA | https://mdc.custhelp.com/app/ |

(Continued on next page)
MATERIALS AND EQUIPMENT

© Timing: 2–4 h

Solution preparations:

Note: Prepare all solutions using the water from ELGA Veolia water purification system (Purelab Chorus PC1LSCXM2) with a resistivity of 18.2 MΩ·cm.

Note: Prepare at room temperature (21°C–22°C).

Note: Filtration with a 0.22 μm filter.

Note: Store solutions at 4°C, and maximum time for storage is 7 days.

Note: Before use, warm the solution to room temperature.

- Blebbistatin stock solution: 100 mM blebbistatin stock solution is made by adding 855 μL of dimethyl sulfoxide (DMSO) to 25 mg of blebbistatin.

Refer to the key resources table and materials and equipment sections for the list of materials and equipment.
• Ca^{2+}-free Tyrode’s solution (osmolarity: 292 ± 6 mOsm/kg, n=7, i.e., seven independent measurements)

| Reagent       | Final concentration (mM) | Amount / 1 L |
|---------------|--------------------------|--------------|
| NaCl          | 140                      | 8.18 g       |
| KCl           | 5.4                      | 0.40 g       |
| MgCl₂·6H₂O    | 1                        | 0.20 g       |
| D-Glucose     | 10                       | 1.80 g       |
| HEPES         | 10                       | 2.38 g       |

pH 7.4 with NaOH

• Ca^{2+}-free Tyrode’s solution containing collagenase type II:

Add 40 mg collagenase type II to 50 mL Ca^{2+}-free Tyrode’s solution to a final concentration of 0.8 mg/mL. The collagenase activity for digestion solution is ~224 U/mL based on the collagenase activity of ~280 U/mg.

• High potassium solution (osmolarity: 278 ± 7 mOsm/kg, n=6)

| Reagent       | Final concentration (mM) | Amount / 1 L |
|---------------|--------------------------|--------------|
| K-Glutamate   | 120                      | 24.38 g      |
| KCl           | 20                       | 1.49 g       |
| MgCl₂·6H₂O    | 1                        | 0.20 g       |
| D-Glucose     | 10                       | 1.80 g       |
| HEPES         | 10                       | 2.38 g       |
| EGTA          | 0.3                      | 0.11 g       |

pH 7.4 with KOH

• Bath and pipette solutions for NLC recordings (osmolarity: 265 ± 9 mOsm/kg, n=6)

Note: The same bath and pipette solutions will maintain equal osmolarity across the cell membrane, and the TEA-containing solutions on both sides will minimize ionic currents across the cell membrane. This will minimize contaminations by ionic currents, and avoid the asymmetrical osmolarity challenges to the cell membrane during NLC recordings, to improve the quality of NLC recordings.

| Reagent       | Final concentration (mM) | Amount / 1 L |
|---------------|--------------------------|--------------|
| TEA-Cl        | 145                      | 24.03 g      |
| MgCl₂·6H₂O    | 2                        | 0.41 g       |
| D-Glucose     | 5                        | 0.90 g       |
| HEPES         | 10                       | 2.38 g       |
| EGTA          | 1                        | 0.38 g       |

pH 7.4 with TEA-OH

• Bath solutions containing 10 mM salicylate for NLC recordings (osmolarity: 277 ± 7 mOsm/kg, n=6)
**Step-by-Step Method Details**

**Mouse Ventricular Myocyte Isolation and Preparation**

© Timing: 1–2 h

**Note:** Preheat the water bath (Model 9105, Fisher Scientific, USA) of the Langendorff apparatus to 37°C.

1. **Mouse Anesthesia.** Inject 80 mg/kg of ketamine and 5 mg/kg of xylazine to adult mice (both male and female, 12–16 weeks) intraperitoneally. Assess level of anesthesia by firm toe pinch.
2. **Inject 300 USP unit sodium heparin intraperitoneally.**
3. **Perform a midline thoracotomy and heart excision.**
4. **Transfer the heart to a petri dish containing ice-cold Ca²⁺-free Tyrode’s solution.**
5. **Trim the connective tissues using a fine scissor.**
6. **Cannulate the heart through aorta onto a Langendorff apparatus prefilled with Ca²⁺-free Tyrode’s solution, gassed with 100% O₂.** Retrogradely perfuse the heart with the Ca²⁺-free Tyrode’s solution until stable baseline is achieved.

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### Bath Solutions Details

| Reagent               | Final concentration (mM) | Amount / L |
|-----------------------|--------------------------|------------|
| TEA-Cl                | 145                      | 24.03 g    |
| MgCl₂·6H₂O            | 2                        | 0.41 g     |
| D-Glucose             | 5                        | 0.90 g     |
| HEPES                 | 10                       | 2.38 g     |
| EGTA                  | 1                        | 0.38 g     |
| 2,3-Butanedione Monoxime | 10                     | 1.01 g     |
| pH 7.4 with TEA-OH    |                          |            |

- Bath solutions containing 10 mM BDM for NLC recordings (osmolarity: 270 ± 6 mOsmol/kg, n=6)

| Reagent               | Final concentration (mM) | Amount / L |
|-----------------------|--------------------------|------------|
| TEA-Cl                | 145                      | 24.03 g    |
| MgCl₂·6H₂O            | 2                        | 0.41 g     |
| D-Glucose             | 5                        | 0.90 g     |
| HEPES                 | 10                       | 2.38 g     |
| EGTA                  | 1                        | 0.38 g     |
| Salicylic acid        | 10                       | 1.38 g     |
| pH 7.4 with TEA-OH    |                          |            |

- Bath solutions containing 25 μM blebbistatin for NLC recordings (osmolarity: 265 ± 8 mOsmol/kg, n=6)
Tyrode’s solution at 37°C, circulated by a perfusion pump (MasterFlex, Cole Parmer Instrument Co., USA).

7. Washout the blood by perfusion for 3 min, and adjust the perfusion speed at a flow rate of 4–6 mL/min and control the initial pressure at ≈75 mmHg using a pressure monitor (BP-1, World Precision Instruments, USA) connected to the Langendorff apparatus. Switched the perfusion solution to Ca²⁺-free Tyrode’s solution containing collagenase type II (0.8 mg/mL) for digestion of the tissue.

8. Continuous monitoring of the declining perfusion pressure for 15–20 min during tissue digestion by collagenase type II, until the pressure is below 30 mmHg. Check the color of the heart surface, and the softness of heart tissue to determine the digestion level.

9. Perfuse the heart with high potassium solution for 3 min. The high potassium solution depolarizes the membrane potentials of cardiomyocytes to allow cardiomyocytes and cardiac tissues to become less excitable.

10. Remove the heart from the perfusion apparatus and transfer to a petri dish containing high potassium solution. Mince the left ventricle and mechanically dissociate the tissue to obtain the cardiomyocytes by pipetting.

11. Harvest single isolated cardiomyocytes by filtering through nylon mesh cell strainer (100 μm) and store the cell in high potassium solution at room temperature for recording. The cardiomyocytes in high potassium solution will be quiescent and can be stored for 6–8 h.

Whole-cell patch-clamp recordings

@ Timing: 4–8 h

12. Recording glass patch pipette preparation. Pull borosilicate glass capillaries (World Precision Instruments, TW150F-4) to make glass patch pipette with a P-97 micropipette puller (Sutter Instruments, Novato, CA), and polish the pipette tip with a microforge (Narishige MF-830, Tokyo, Japan). The resistance of the electrodes is 1.5–3.0 MΩ when filled with the pipette solution.

△ CRITICAL: To minimize pipette capacitance, pipettes should be filled with a small amount of internal solution and relatively thicker wall glass capillaries should be used. Coating the pipette tip with a layer of SYLARD™ 184 (Dow Corning) will also reduce the pipette capacitance.

13. Set the voltage-clamp protocol. In Clampex 10 software, set the stair voltage protocol ranging from −140 to +100 mV with 10-mV increment as shown in Figure 1A.

14. Patch-clamp recordings. Seed cardiomyocytes in a recording chamber with a volume size of 0.5 mL connected to a gravity driven solution perfusion system. The perfusion speed is kept at 1–2 mL/min.

△ CRITICAL: To minimize pipette capacitance, the solution level is kept low to reduce the depth of the pipette immersion.

a. Connect the recording chamber with the silver/silver chloride ground electrode by a 3 M KCl agar bridge.

b. Five minutes after seeding the cells, healthy cardiomyocytes will attach to the cover glass of the recording chamber, and the perfusion of the solution will wash away the unhealthy and dead cells.

c. Use a square 5 mV test pulse with a holding potential of 0 mV for seal test, and form a seal with a resistance larger than ten giga-ohm.
d. After giga-ohm seal formation, compensate the pipette capacitance in cell-attached mode by adjusting “Fast” and “Slow” pipette capacitance compensation knobs of the amplifier to minimize the pipette capacitive currents.

e. After rupturing the membrane in whole-cell configuration with a seal resistance of at least one giga-ohm, hold the membrane potential at 0 mV.

f. With continuous perfusion, perform whole-cell recordings at room temperature (21°C–22°C) to record the capacitive currents using the voltage stair protocol and an Axopatch 200A amplifier, Digidata 1440 digitizer, and pClamp10 software (Molecular Devices, LLC., Sunnyvale, CA). The recordings are digitally filtered at 1 kHz and digitized at 2 kHz.

15. Pharmacological interventions. To separate the NLC component resulting from prestin function, use bath solution containing 10 mM salicylate to perfuse the cells for 3 min, and then record the salicylate-sensitive capacitive currents during perfusion.
**Note:** 10 mM salicylate is used for effective NLC inhibition in outer hair cells with a half-maximal concentration of 1.6 mM (Kakehata and Santos-Sacchi, 1996; Zheng et al., 2000). The salicylate inhibitory effect on NLC is shown in Figure 1C, left panel.

**Note:** During the capacitive current recordings, we also add two different excitation-contraction (EC) uncouplers, 25 μM blebbistatin (BLEB) or 10 mM 2,3-butanedione monoxime (BDM) to the bath solution for 3 min to inhibit the myosin ATPase to disengage cellular excitation from contraction (Bond et al., 2013), and then record the capacitive currents during perfusion.

### Analyses of NLC using biophysical and mathematical methods

**Timing:** 1–2 weeks

16. Use pClamp 10, Microsoft Excel, Origin 6.1, and GraphPad Prism software to analyze the capacitive currents.

17. Determine total membrane capacitance \(C_m\) by analyzing the capacitive currents and measuring the input resistance \(R_{in}\) at each step voltage \(V_c\). \(C_m = (R_{in}/R_m)Q/V_c\), where \(R_m\) \((R_m = R_{in} + R_s)\) is the input resistance which can be determined by the steady state current and \(V_{Ci}\), \(R_m\) is membrane resistance, \(R_s\) is the series resistance which can be calculated by the time constant \(\tau\) of the capacitor discharge current, the charge movement \(Q\), step voltage and \(R_{in}\) \((R_s = \tau V_{Ci} R_{in}/(Q R_{in} + \tau V_{Ci}))\).

**Note:** As part of \(R_s\), the pipette resistance has been considered and accounted for in determining the \(C_m\) from the experimental data, and will not affect the quantification of NLC due to pipette resistance changes in different recordings.

18. Determine the NLC. \(C_m = C_{lin} + C_v\), where \(C_{lin}\) is the linear capacitance, and \(C_v\) is the nonlinear capacitance. Normalized NLC can be expressed as \([C_m - C_{lin}]/C_{lin}\). \(C_v\) is described as a function of voltage and fitted with the first derivative of a Boltzmann function describing the nonlinear charge’s movement (Santos-Sacchi, 1991). Specifically, we plot \(C_v\) as a function of voltage and fitted with the first derivative of Boltzmann function:

\[
C_v = (Q_{max}ze/kT) \times \exp(-ze(V-V_h)/kT)/(1 + \exp(-ze(V-V_h)/kT))^2,
\]

where \(V\) is the membrane potential, \(V_h\) is the voltage at half-maximal nonlinear charge transfer, \(e\) is the electron charge, \(k\) is Boltzmann’s constant, \(T\) is the absolute temperature, \(z\) is the valence, and \(Q_{max}\) is maximum nonlinear charge transfer. The original recording traces of the total capacity currents are shown in Figure 1B. The normalized NLC is shown in Figure 1C. The total NLC has two components, one is generated from the voltage-gated ion channel gating charge movement, and another one results from prestin activities.

### EXPECTED OUTCOMES

We record and compare the NLC of cardiomyocytes from WT and Slc26a5\(^{−/−}\) mice (Zhang et al., 2021). Ablation of Slc26a5 results in a significant reduction and depolarization shift of NLC \(V_h = −37 \pm 2\) mV, \(n = 11\) and \(−18 \pm 3\) mV, \(n = 14\) for WT and Slc26a5\(^{−/−}\), respectively; \(p<0.05\) as shown in Figure 1C, left panel; there are no significant changes of \(C_{lin}\) \((167 \pm 15\) pF for WT, and \(166 \pm 12\) pF for Slc26a5\(^{−/−}\), \(n=14\); \(Q_{max}/C_{lin}\) for WT is significantly larger than that of Slc26a5\(^{−/−}\) \((10.81 \pm 0.99\) v.s. \(7.74 \pm 1.03\) nC/\(\mu\)F, \(p<0.05, n=11\) for WT and \(n=14\) for Slc26a5\(^{−/−}\)). In the presence of 10 mM salicylate, \(Q_{max}/C_{lin}\) for WT cardiomyocytes is significantly reduced \((7.21 \pm 1.33\) nC/\(\mu\)F, \(n=6, p<0.05\)), while \(Q_{max}/C_{lin}\) for Slc26a5\(^{−/−}\) cardiomyocytes is not significantly altered \((7.56 \pm 1.41\) nC/\(\mu\)F, \(n=5\)). The voltage sensitivity, \(K (kT/ze)\), can be calculated based on the \(z\) values with \(K = 17.16 \pm 1.15\) mV \((n=11\) for WT\) and \(15.20 \pm 1.31\) mV \((n=14\) for Slc26a5\(^{−/−}\)), \(p=NS\).

The excitation-contraction uncouplers, BLEB and BDM significantly restrict the dynamic range of NLC, and the amplitude is markedly reduced in Slc26a5\(^{−/−}\) cardiomyocytes as shown in Figure 1C,
middle and right panels. $V_h$ for Slc26a5−/− ventricular myocytes showed a depolarization shift in the presence of BLEB and BDM, similar to that in the absence of BLEB and BDM. $V_h$ for WT and Slc26a5−/− ventricular myocytes after BLEB are $-37 \pm 5$ (n = 4) and $-25 \pm 3$ (n = 5) mV, respectively. $V_h$ for WT and Slc26a5−/− ventricular myocytes after BDM are $-43 \pm 4$ (n = 3) and $-32 \pm 3$ (n = 7) mV, respectively. NLC for WT and Slc25a5−/− at baseline are shown in dotted lines in the middle and right panels. The NLC of Slc26a5−/− myocytes at −20, −10, 0, 10, 20, 30, and 40 mV in the presence of BLEB or BDM is significantly smaller than that in the absence of the myosin inhibitors (p<0.05). The effects of BLEB and BDM on NLC suggest the functional coupling of myofilaments with prestin, and possibly the regulation of ion channel gating properties by prestin in cardiomyocytes.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

We estimate a sample size of 4 per experiment to detect at least 15% difference between WT and Slc26a5−/− groups with alpha = 0.05 for a two-tailed test to achieve a power of 95%, assuming the standard deviation of the differences to be 5% (SigmaStat, Systat Software Inc.). No data were excluded. Data are presented as mean ± SEM. Statistical comparisons were analyzed by t test (two-tailed). Statistical significance was considered to be achieved when p < 0.05.

**LIMITATIONS**

NLC recorded by the voltage protocol includes not only the component from prestin activities but also NLC component resulting from voltage-gated ion channel gating charge movement. This is a limitation for the quantification of NLC in WT mouse cardiomyocytes. However, we took advantage of the Slc26a5−/− cardiomyocytes and specific prestin inhibitor to dissect the NLC component resulting from prestin.

**TROUBLESHOOTING**

**Problem 1**
Cardiomyocyte quality control is critical for the patch-clamp recordings. The isolation procedures of mouse cardiomyocytes need to be optimized during the experiments. (Steps 1–11)

**Potential solution**
Heart excision, trimming and cannulation need to be rapid and precise. Heart excision should be properly performed to ensure the remaining ascending aorta section is long enough for cannulation. The aorta should be securely cannulated to ensure that there is no leak for the maintenance of proper retrograde perfusion. The concentrations of collagenase type II may need to be adjusted and optimized with each new batch of collagenase.

**Problem 2**
Cardiomyocytes may not seed well on the cover glass of the chamber depending on the cell quality, glass type and surface quality. The perfusion may wash away the cells during recordings. (Step 14)

**Potential solution**
Choose cover glass from different manufacturers (for example Corning, VWR) may help. Another solution is to coat the cover glass with 0.1% (w/v) Poly-L-Lysine for 30 min before seeding the cells.

**Problem 3**
Leak currents can affect the capacitive current recordings and make the data analysis difficult and generate errors in NLC analyses. Therefore, it is paramount to obtain a very high and consistent giga-ohm seal throughout the recordings to minimize the leak current in the capacitive current recordings. (Step 14)

**Potential solution**
A very high and consistent giga-ohm seal (at least greater than 1 giga-ohm for the whole-cell configuration) needs to be achieved and maintained during the recordings by altering the pipette-tip
shape and polishing, optimizing the cardiomyocyte isolation protocol through setting the collage-
nase digestion time, perfusion pressure, and improving the recording techniques. Obtaining healthy
and high-quality ventricular myocytes is also essential for successful recordings.

**Problem 4**
Ventricular myocytes are relatively large with electromotility and multiple voltage-gated ion channels
embedded in the cell membrane, and the total NLC represents only ~10% of the total linear capac-
itance. Furthermore, the NLC from prestin activities represents a very small component of the total
NLC. (Steps 15, 17, and 18)

**Potential solution**
The capacity current recordings on cardiomyocytes need to be of very high quality in order to
differentiate between these two distinct NLC components, especially the one originated from
prestin.

**Problem 5**
NLC analysis is time-consuming that includes curve fitting for each step voltage and the fitting of
NLC data by the first derivative of a Boltzmann function to extract the related parameters. The errors
introduced during this step will significantly change the results. (Steps 17 and 18)

**Potential solution**
High-quality capacitive current recordings are critical for precise NLC analysis. At the same time,
each curve fitting needs to be examined to ensure good fitting, even performed by automatic com-
puter analysis. Finally, we need to choose the appropriate software tools for the Boltzmann function
fitting.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be
fulfilled by the lead contact, Xiao-Dong Zhang (xdzhang@ucdavis.edu).

**Materials availability**
This study did not generate new unique reagents or animal models.

**Data and code availability**
The data supporting the current study have not been deposited in a public repository because they
are only used for the publication of this manuscript but are available from the corresponding author
on request.

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**AUTHOR CONTRIBUTIONS**
Conceptualization, X.-D.Z., E.N.Y., and N.C.; experiments and data analyses, X.-D.Z., M.C.P.F., and
V.T.; resources, E.N.Y., N.C., and X.-D.Z.; funding acquisition, X.-D.Z., E.N.Y., and N.C.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.
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