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# LRMP inhibits cAMP potentiation of HCN4 channels by disrupting intramolecular signal transduction

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## Abstract

Lymphoid restricted membrane protein (LRMP) is a specific regulator of the hyperpolarization-activated cyclic nucleotide-sensitive isoform 4 (HCN4) channel. LRMP prevents cAMP-dependent potentiation of HCN4 but the interaction domains, mechanisms of action, and basis for isoform-specificity remain unknown. Here we identify the domains of LRMP essential for regulation. We show that LRMP acts by disrupting the intramolecular signal transduction between cyclic nucleotide binding and gating. And we demonstrate that multiple unique regions in HCN4 are required for LRMP isoform-specificity. Using patch clamp electrophysiology and Förster resonance energy transfer (FRET), we showed that the initial 227 residues of LRMP and the N-terminus of HCN4 are necessary for LRMP to interact with HCN4. We found that the HCN4 N-terminus and HCN4-specific residues in the C-linker are necessary for regulation of HCN4 by LRMP. And we demonstrate that LRMP-regulation can be conferred to HCN2 by addition of the HCN4 N-terminus along with mutation of 5 residues in the S5 region and C-linker to the cognate HCN4 residues. Taken together, these results suggest that LRMP inhibits HCN4 through an isoform-specific interaction involving the N-terminals of both proteins that prevents the transduction of cAMP binding into a change in channel gating via an HCN4-specific orientation of the N-terminus, C-linker, and S4-S5 linker.

### eLife assessment

This study identifies the molecular determinants of LRMP co-regulation of HCN 4 activity. The evidence supporting the conclusions, which is **compelling**, is backed by rigorous electrophysiological and spectroscopic analysis. The work is **important** because it greatly enhances our understanding of the mechanisms of HCN channel regulation in a tissue-specific manner and highlights a functional role for more disordered regions that have yet to be structurally resolved.

## Introduction

Hyperpolarization-activated, cyclic nucleotide-sensitive (HCN) ion channels are biophysical anomalies. Despite being structurally related to voltage-gated $K^+$ channels — which are activated by membrane depolarization and are highly selective for $K^+$ over $Na^+$ — HCN channels activate in response to membrane hyperpolarization and pass a mixed $Na^+/K^+$ current. In addition, binding of cyclic nucleotides, particularly cAMP, to a conserved C-terminal cyclic nucleotide binding domain (CNBD) potentiates HCN channels by shifting the voltage-dependence of activation to more depolarized potentials, speeding activation, and slowing deactivation (DiFrancesco and Tortora, 1991; Wainger et al., 2001).

While the details of intramolecular transduction between cAMP binding and channel gating have yet to be fully elucidated, some key aspects are known: 1) the unbound CNBD is inhibitory — truncation of the CNBD potentiates channel activation, similar to cAMP binding to the intact CNBD (Wainger et al., 2001); 2) the slowing of channel deactivation in response to cAMP binding occurs through a separate mechanism from the shift in activation voltage dependence, and cannot be replicated by truncation of the CNBD (Wicks et al., 2011; Sunkara et al., 2018); and 3) transduction of the signal for the cAMP-dependent shift in channel activation, but not deactivation, requires interactions of a "a cAMP transduction centre" (Porro et al., 2019; Wang et al., 2020b) comprised of portions of the C-linker (which connects the CNBD to the transmembrane domain), the N-terminal HCN domain (HCND), and the S4-S5 linker.

The inositol 1,4,5-triphosphate receptor-associated proteins, IRAG1 and LRMP/IRAG2 (lymphoid restricted membrane protein), are a family of endoplasmic reticulum (ER) transmembrane proteins that are isoform-specific regulators of HCN4 (Peters et al., 2020, 2022). IRAG and LRMP act by modulating the cAMP sensitivity of HCN4. However, they have opposing effects: IRAG1 causes a gain-of-function by shifting HCN4 activation to more depolarized membrane potentials in the absence of cAMP. In contrast, LRMP causes a loss-of-function by inhibiting cAMP-dependent potentiation of HCN4 activation (Peters et al., 2020). IRAG and LRMP share some sequence homology, particularly in their coiled-coil motifs, and both have been found to regulate $IP_3$ receptor calcium release channels (Schlossmann et al., 2000; Geiselhöringer et al., 2004; Prüschenk et al., 2021). However, the interaction domains, mechanisms of action, and basis for isoform-specificity for their regulation of HCN4 remain unknown.

In this study, we focused on LRMP and investigated the interaction domains and mechanism by which it inhibits the cAMP-dependent shifts in HCN4 activation. Our previous study showed that LRMP acts differently from TRIP8b, a neuronal protein that also prevents cAMP-dependent shifts in HCN channel activation. TRIP8b acts by directly antagonizing cAMP binding (Santoro et al., 2004; Zolles et al., 2009; Bankston et al., 2017; Saponaro et al., 2018). In contrast, LRMP doesn't inhibit cAMP binding to the CNBD, as indicated by the preserved cAMP-dependent slowing of deactivation in the presence of LRMP (Peters et al., 2020). Furthermore, TRIP8b regulates all HCN channel isoforms (Zolles et al., 2009; Santoro et al., 2011), whereas LRMP is specific for the HCN4 isoform (Peters et al., 2020). These observations suggest that LRMP regulates HCN4 by interfering with an isoform-specific step in the signal transduction pathway that links cAMP binding to the shift in activation voltage dependence.

We tested this hypothesis using a combination of patch clamp electrophysiology and FRET. We found that the initial N-terminal 227 residues of LRMP associate with the N-terminus of HCN4 and that the intact HCN4 N-terminus is required for channel regulation by LRMP. Furthermore, we show that two HCN4-specific residues in the C-linker, P545 and T547, are critical for isoform-specific regulation of HCN4 by LRMP. Finally, we found that addition of the HCN4 N-terminus along with mutations in the C-linker and S5 transmembrane segment are sufficient to confer LRMP

regulation to HCN2. These results are consistent with a model in which LRMP inhibits HCN4 via the cAMP transduction centre (Weißgraeber et al., 2017; Porro et al., 2019; Wang et al., 2020b; Saponaro et al., 2021; Kondapuram et al., 2022).

# Materials and Methods

## DNA Constructs

The mouse LRMP construct in PCMV6-Kan/Neo (GenBank AAH52909.1; Cat. #MC228229, Origene, Rockville, MD; **Supplementary** Figure 1), HCN1 in pcDNA3 (generously provided by Dr. Eric Accili), HCN4-2 in pcDNA3.1, HCN4-S719X in pCDNA3.1, and HCN4 Δ1-25 in pcDNA6 (also known as HCN4s, generously provided by Dr. Richard Aldrich) have been described previously (Proenza et al., 2002; Liao et al., 2012; Liu and Aldrich, 2011; Peters et al., 2020). HCN2 was subcloned from pcDNA4 into pcDNA3.1 for this study. Other constructs were synthesized by Twist Biosciences (South San Francisco, CA) or using site-directed mutagenesis either in-house or by Applied Biological Materials (Richmond, Canada). The HCN4 Δ1-62, HCN4 Δ1-130, and HCN4 Δ1-185 deletion clones were made using a site-directed mutagenesis kit (New England Biolabs, Ipswich, MA) and a codon-optimized HCN4 plasmid in the pTwist-CMV-WPRE-Neo vector synthesized by Twist Biosciences.

For FRET experiments, recombinant fusions of mHCN4 and mLRMP were constructed by introducing Cerulean (CER) or Citrine (CIT) fluorescent proteins using PCR-based cloning. The C-termini of HCN4 constructs were tagged with CER, while the C-termini of LRMP constructs were tagged with CIT. Because there are no structures of LRMP or the or the N-terminus of HCN4, and because much of the experimental design was carried out prior to Alphafold's structural predictions (Jumper et al., 2021; Varadi et al., 2022), the specific cut sites were determined empirically. We tried a number of fragments and the ones used in this study were of similar sizes and expressed well in our system. The sites we chose relative to the predicted coiled-coil on LRMP can be seen in **Figure S1**.

All clones used in this study are the murine sequences of LRMP and HCN4. All new constructs were confirmed by DNA sequencing (Barbara Davis Center BioResource Core at the University of Colorado Anschutz Medical Campus; ACGT, Wheeling, IL; or Plasmidsaurus, Eugene, OR). Detailed information about constructs can be found in **supplemental table S1**.

## Cell Lines

HEK 293 cells (ATCC, Manassas, VA) were grown in a humidified incubator at 37°C and 5% $CO_2$ in high glucose DMEM with L-glutamine supplemented with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were transfected 48 hours prior to experiments and were plated on either protamine-coated glass coverslips (for patch clamp experiments) or poly-d-lysine coated glass-bottom dishes (for FRET experiments).

Patch clamp experiments were performed in either transiently transfected HEK293 cells, an HCN4 stable line in HEK293 cells (Zong et al., 2012), or eight new stable cell lines in HEK293 cells: HCN2, HCN4, HCN4 Δ1-62, HCN4 Δ1-130, HCN4 Δ1-185, HCN4 Δ1-200, HCN2 A467P/F469T (HCN2 AF/PT), and HCN4 P545A/T547F (HCN4 PT/AF). Stable cell lines were made by transfecting HEK293 cells with the respective plasmids using Lipofectamine 2000 (Invitrogen, Waltham, MA) according to the manufacturer's instructions. 48 hours post-transfection, 200 ug/mL of G418 disulfate (Alfa Aesar, Haverhill, MA) or Hygromycin B (InvivoGen, San Diego, CA) was added to the cell culture media in place of pen-strep to select for stably transfected cells. Single cell clones were tested using whole-cell patch clamp and the clonal lines that exhibited the largest and most consistent currents

were grown into stable cell lines. Control experiments of HCN4 in the absence of LRMP were conducted alongside recordings in the presence of LRMP to ensure that stably expressed channels had consistent properties over the time course of the study.

Transient transfection of HCN4 constructs and/or LRMP was performed using Fugene6 (Promega, Madison, WI) according to the manufacturer's instructions. Transfections of all constructs that did not include fluorescent tags were performed with the addition of eGFP (at a LRMP to GFP ratio of 4:1) as a co-transfection marker. All data were collected from a minimum of 3 transfections per condition.

## Patch Clamp Electrophysiology

Cells were plated on sterile protamine-coated glass coverslips 24-48 hours prior to experiments. Cells on coverslip shards were transferred to the recording chamber and perfused (~0.5 to 1 mL/min) with extracellular solution containing (in mM): 30 KCl, 115 NaCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, and 5 HEPES. Transiently transfected cells were identified by green fluorescence.

Patch pipettes were pulled from borosilicate glass to a resistance of 1.0-3.0 MOhm when filled with intracellular solution containing (in mM): 130 K-Aspartate, 10 NaCl, 1 EGTA, 0.5 $MgCl_2$, 5 HEPES, and 2 Mg-ATP. 1 mM cAMP was added to the intracellular solution as indicated. All recordings were performed at room temperature in the whole-cell configuration. Data were acquired at 5 KHz, and low-pass filtered at 10 KHz using an Axopatch 200B amplifier (Molecular Devices, San Jose, CA), Digidata 1440A A/D converter and Clampex software (Molecular Devices). Pipette capacitance was compensated in all recordings. Membrane capacitance and series resistance (Rs) were estimated in whole-cell experiments using 5-mV test pulses. Only cells with a stable Rs of <10 MOhm were analyzed. Data were analyzed in Clampfit 10.7 (Molecular Devices).

Channel activation was determined from peak tail current amplitudes at -50 mV following 3-s hyperpolarizing pulses to membrane potentials between -50 mV and -170 mV from a holding potential of 0 mV. Normalized tail current-voltage relationships were fit by single Boltzmann equations to yield values for the midpoint activation voltage ($V_{1/2}$) and slope factor (k). Deactivation time constants were determined using a single exponential fit to tail currents recorded at -50 mV following a pulse to -150 mV. All reported voltages are corrected for a calculated +14 mV liquid junction potential between the extracellular and intracellular solutions.

## FRET Hybridization Assays

HEK293 cells expressing HCN4-CER and LRMP-CIT fusion proteins were examined 48 h after transfection using a Zeiss LSM 710 confocal laser scanning microscope. An area of 500– 2,500 $\mu m^2$ was selected from the overall field of view. Images were taken through a 40× water objective. CER and CIT were excited with separate sweeps of the 458- and 514-nm laser lines of an argon laser directed at the cell with a 458/514-nm dual dichroic mirror. Relative to full power, the excitation power for the imaging sweeps was attenuated to 1% for CER and 0.5% for Citrine. Bleaching was performed by using multiple (20–60) sweeps of the CIT laser at full power. Bleaching was usually complete within 1-2 m. Emitted light was collected between 449 and 488 nm for CER and 525 and 600 nm for CIT. With this setup, there was no contamination of the relevant CER signal from the CIT. For each experiment, the photomultiplier tube gain was adjusted to ensure that the maximum pixel intensity was not >70% saturated. Fluorescence intensity was then measured by drawing regions of interest (ROIs) around the cytoplasmic portion of the cell in ImageJ (Schneider et al., 2012). Masks were occasionally used to eliminate bright fluorescent puncta within the cell (this was a rare occurrence in the CER signal). Percent FRET (E) was calculated as:

$$E = [I_{CERpost} - I_{CERpre}] / I_{CERpost} * 100,$$

where $I_{CERpost}$ is the CER intensity after bleaching and $I_{CERpre}$ is the CER intensity before bleaching.

### Statistical Analysis

All statistical analysis was performed using JMP 15 software (SAS Institute, Cary, NC). Normality was tested using the Shapiro-Wilk test. The log of the deactivation time constant at - 50 mV was used for statistical analysis to ensure the data were normally distributed. To prevent biasing of the results, all data were included except for cells showing large changes in leak or access resistance during the recording, or those for which the access resistance was > 10 MΩ at any point during recording. Tests for differences in the average midpoint of activation for a given HCN channel construct in the presence of LRMP and/or 1 mM cAMP were performed with a 2-way ANOVA. The main independent variables were the absence or presence of LRMP and the absence or presence of 1 mM cAMP in the pipette solution. Differences in the effects of cAMP in the absence or presence of LRMP were analyzed using an interaction term between the main independent variables. For FRET experiments, the recordings of Cerulean tagged HCN4 1-125 and HCN4 125-260 co-expressed with free Citrine were pooled as a control group. $P < 0.05$ was used as the cut-off for a significant effect. All comparisons meeting this criteria are indicated in figures by an asterisks, with exact P-values given in the manuscript text or **Tables 1**-**3**.

## Results

### The N-terminus of LRMP is necessary and sufficient to regulate HCN4

We previously showed that LRMP significantly reduces the cAMP-dependent depolarizing shift in HCN4 activation without any effect on the voltage dependence in the absence of cAMP, and that it does not regulate HCN1 or HCN2 (Peters et al., 2020). We next sought to identify a subdomain within LRMP that is responsible for this regulation. We began with a truncated LRMP construct with a Citrine fluorescent protein replacing the C-terminal ER transmembrane and lumenal domains (LRMP 1-479Cit; **Figs. 1A**-**1D**; **Table 1**). We found that LRMP 1-479Cit inhibited the cAMP sensitivity of HCN4 to an even greater degree than the full-length LRMP (**Table 2**). This more pronounced effect may be due to improved expression of this tagged construct compared to the untagged full-length LRMP, which was detected by co-transfection with GFP, or that removal of the ER-transmembrane segment increased the proximity between LRMP and HCN4 by allowing LRMP to diffuse freely in the cytosol. A key feature of LRMP in our original study is that it does not prevent binding of cAMP to the CNBD of HCN4. This was also the case for LRMP 1-479Cit, as indicated by the significant slowing of deactivation by cAMP even in the presence of LRMP 1-479Cit ($P = 0.0310$; **Fig. 1D**). These results indicate that the ER transmembrane and luminal domains of LRMP are not required for regulation of HCN4 and they support the idea that LRMP limits cAMP potentiation of HCN4 by interfering with a downstream step in the cAMP signal transduction pathway.

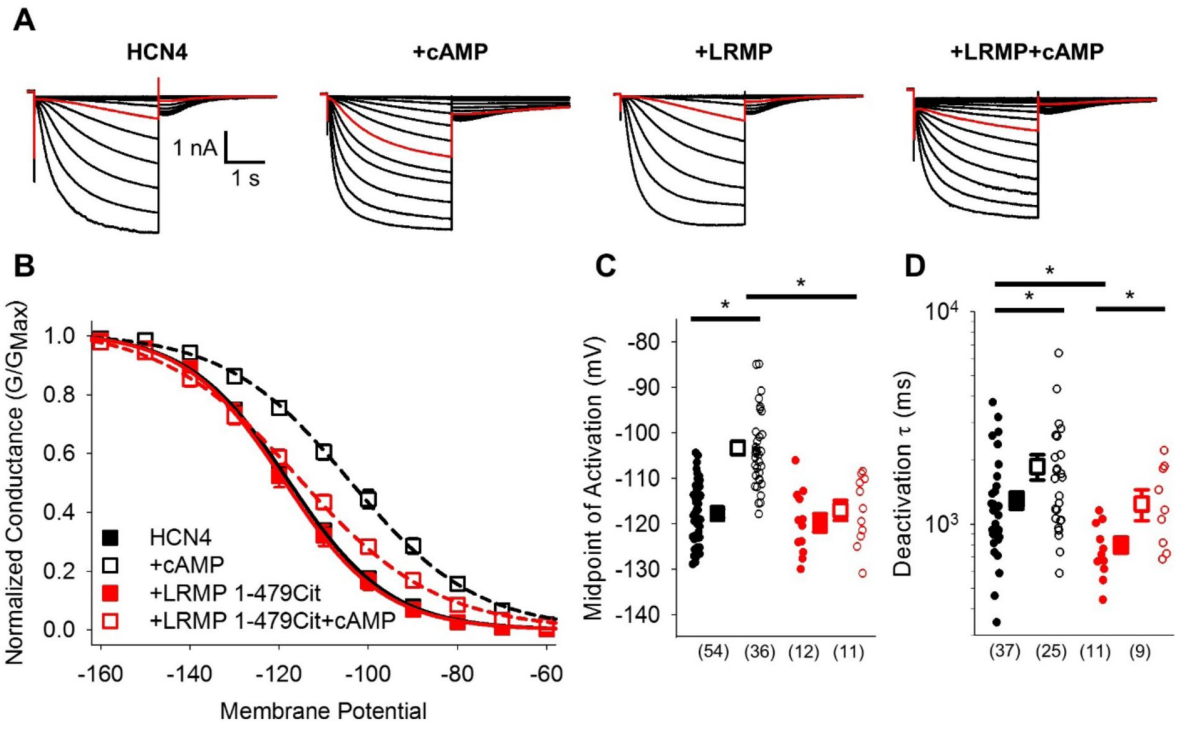
To further resolve which regions of LRMP are required to regulate HCN4, we tested a series of additional truncated LRMP constructs (shown schematically in **Fig. 2A**) for their ability to prevent cAMP-dependent shifts in HCN4 activation. We first split LRMP into two fragments: the LRMP 1-227 construct contains the N-terminus of LRMP with a cut-site near the N-terminus of the predicted coiled-coil sequence, while LRMP 228-539 contains the remainder of the protein. We found that LRMP 1-227 recapitulated the effects of full-length LRMP, while LRMP 228-539 had no effect on HCN4 gating (**Figs. 2B**, **2C**, and **2F**; **Table 1**). However, when we further split the N-terminal domain of LRMP into two fragments tagged with C-terminal Citrines, neither LRMP 1-

| | Control (mV) | cAMP (1mM) (mV) | ΔV½ in cAMP | P-Value Control vs. cAMP |
|---|---|---|---|---|
| HCN4 Control | -117.8 ± 0.9 (54) | -103.4 ± 1.5 (36) | 14.4 mV | **P < 0.0001** |
| HCN4 LRMP 1-479Cit | -119.8 ± 2.0 (12) P = 0.3847 | -117.1 ± 2.2 (11) **P < 0.0001** | 2.7 mV | P = 0.3710 |
| HCN4 LRMP 1-227 | -117.9 ± 1.9 (13) P = 0.9399 | -118.1 ± 1.4 (11) **P < 0.0001** | -0.2 mV | P = 0.9642 |
| HCN4 LRMP 228-539 | -116.1 ± 2.6 (9) P = 0.5265 | -106.3 ± 2.0 (8) P = 0.3100 | 9.8 mV | **P = 0.0069** |
| HCN4 LRMP 1-108Cit | -123.1 ± 1.8 (7) P = 0.0747 | -103.0 ± 2.5 (8) P = 0.8890 | 20.1 mV | **P < 0.0001** |
| HCN4 LRMP 110-230Cit | -118.0 ± 4.0 (9) P = 0.9423 | -106.4 ± 1.3 (12) P = 0.2118 | 11.6 mV | **P = 0.0005** |

Average midpoint of activation (mV) ± standard error of the mean (Number of independent cells). ΔV½ values reflect the difference in population midpoints for whole-cell experiments in the presence vs. absence of cAMP.

**Table 1**

**Midpoints of Activation in HCN4 in the Presence of LRMP Fragments**




## Figure 1

### The cytosolic region of LRMP regulates HCN4 but does not antagonize cAMP binding.

**A:** Exemplar current recordings from HCN4 in the absence or presence of 1 mM cAMP and/or LRMP 1-479Cit. Currents recorded with a -110 mV activating pulse are shown in *red*. **B:** Voltage dependence of activation for HCN4 alone (*black*) or co-transfected with LRMP 1-479Cit (*red*) in the presence or absence of 1 mM intracellular cAMP (*open symbols*). **C:** Average (± standard error of the mean) midpoints of activation for HCN4 in the absence or presence of LRMP 1-479Cit and/or 1 mM cAMP using the same colour scheme as **B**. **D:** Average (± standard error of the mean) time constants of deactivation for HCN4 in the absence or presence of LRMP 1-479Cit and/or 1mM cAMP using the same colour scheme as **B**. Small circles in **C** and **D** represent individual cells and values in parentheses are the number of independent recordings for each condition. * indicates a significant ($P < 0.05$) difference. All means, standard errors, and exact P-values are in **Table 1**.

| | Control (mV) | +LRMP (mV) | LRMP vs. Control | Control ΔV½ in cAMP (mV) | LRMP ΔV½ in cAMP (mV) |
|---|---|---|---|---|---|
| **HCN4** | -117.8 ± 0.9 (54)[#] | -120.1 ± 2.2 (14)[#] | P = 0.3530 | 14.4 mV | 5.4 mV |
| **+cAMP** | -103.4 ± 1.5 (36) | -114.7 ± 2.6 (16) | **P < 0.0001** | | |
| | **P < 0.0001** | P = 0.0724 | | | |
| **HCN2** | -109.3 ± 1.5 (8) | -114.4 ± 1.9 (8) | P = 0.1101 | 19.0 mV | 26.5 mV |
| **+cAMP** | -90.3 ± 3.2 (8) | -87.9 ± 1.6 (8) | P = 0.4293 | | |
| | **P < 0.0001** | **P < 0.0001** | | | |
| **HCN4 Δ1-25** | -118.1 ± 2.2 (19) | -121.0 ± 2.7 (10) | P = 0.3859 | 17.0 mV | 4.5 mV |
| **+cAMP** | -101.1 ± 2.6 (13) | -116.5 ± 1.5 (12) | **P = 0.0001** | | |
| | **P < 0.0001** | P = 0.2236 | | | |
| **HCN4 Δ1-62** | -116.5 ± 1.7 (8) | -118.8 ± 1.9 (10) | P = 0.4089 | 17.4 mV | 10.9 mV |
| **+cAMP** | -99.1 ± 2.2 (10) | -107.9 ± 1.4 (8) | **P = 0.0027** | | |
| | **P < 0.0001** | **P = 0.0003** | | | |
| **HCN4 Δ1-130** | -115.2 ± 2.2 (11) | -117.4 ± 1.3 (6) | P = 0.5651 | 13.9 mV | 10.5 mV |
| **+cAMP** | -101.3 ± 2.3 (8) | -106.9 ± 3.7 (7) | P = 0.1481 | | |
| | **P = 0.0003** | **P = 0.0152** | | | |
| **HCN4 Δ1-185** | -117.1 ± 2.1 (12) | -125.5 ± 2.3 (8) | P = 0.0500 | 14.0 mV | 21.9 mV |
| **+cAMP** | -103.1 ± 3.3 (13) | -103.6 ± 3.1 (8) | P = 0.8913 | | |
| | **P < 0.0001** | **P < 0.0001** | | | |
| **HCN4 V604X** | -101.7 ± 2.0 (12) | -102.0 ± 1.9 (6) | P = 0.9407 | -3.0 mV | — |
| **+cAMP** | -104.7 ± 4.9 (6) | — | — | | |
| | P = 0.4698 | | | | |
| **HCN4 S719X** | -124.9 ± 1.3 (17) | -121.4 ± 1.6 (20) | P = 0.1217 | 18.4 mV | 7.3 mV |
| **+cAMP** | -106.5 ± 1.6 (22) | -114.1 ± 1.6 (18) | **P = 0.0010** | | |
| | **P < 0.0001** | **P = 0.0018** | | | |
| **HCN4 PT/AF** | -127.6 ± 1.6 (21) | -127.8 ± 1.9 (15) | P = 0.9311 | 10.5 mV | 15.7 mV |
| **+cAMP** | -117.1 ± 2.2 (15) | -112.1 ± 2.1 (15) | P = 0.0785 | | |
| | **P = 0.0002** | **P < 0.0001** | | | |
| **HCN2 AF/PT** | -106.5 ± 1.9 (15) | -107.7 ± 1.5 (11) | P = 0.5858 | 18.3 mV | 21.3 mV |
| **+cAMP** | -88.2 ± 0.7 (16) | -86.4 ± 1.6 (13) | P = 0.3987 | | |
| | **P < 0.0001** | **P < 0.0001** | | | |
| **HCN4-2** | -112.7 ± 2.8 (11) | -111.9 ± 2.2 (14) | P = 0.8290 | 17.9 mV | 9.4 mV |
| **+cAMP** | -94.8 ± 3.1 (15) | -102.5 ± 1.9 (16) | **P = 0.0284** | | |
| | **P < 0.0001** | **P = 0.0092** | | | |
| **HCN2 VVGPT** | -103.4 ± 2.1 (11) | -105.6 ± 2.8 (10) | P = 0.5305 | 14.5 mV | 12.3 mV |
| **+cAMP** | -88.9 ± 2.2 (10) | -93.3 ± 3.0 (9) | P = 0.2278 | | |
| | **P = 0.0002** | **P = 0.0018** | | | |
| **HCN2-4N VVGPT** | -104.6 ± 2.1 (16) | -100.9 ± 2.3 (14) | P = 0.2183 | 15.0 mV | 1.0 mV |

**Table 2**

**Midpoints of Activation for HCN Channel Constructs**

| | | | | | |
|---|---|---|---|---|---|
| **+cAMP** | -89.6 ± 2.3 (16)<br>**P < 0.0001** | -99.9 ± 1.2 (11)<br>P = 0.7574 | **P = 0.0019** | | |

Average midpoint of activation ± standard error of the mean (Number of independent cells). ΔV½ values reflect the difference in population midpoints for whole-cell experiments in the presence vs. absence of cAMP.

[#] HCN4 control and cAMP data in the absence of LRMP is the same as in Table 1.

**Table 2** (continued)

108Cit nor LRMP 110-230Cit regulated HCN4 (**Figs. 2D-F**; **Table 1**). Thus, the first 227 residues of LRMP are sufficient to regulate HCN4 and it seems likely that residues in both halves of the LRMP N-terminus participate in this regulation.

## The N-terminus of HCN4 is required for regulation by LRMP

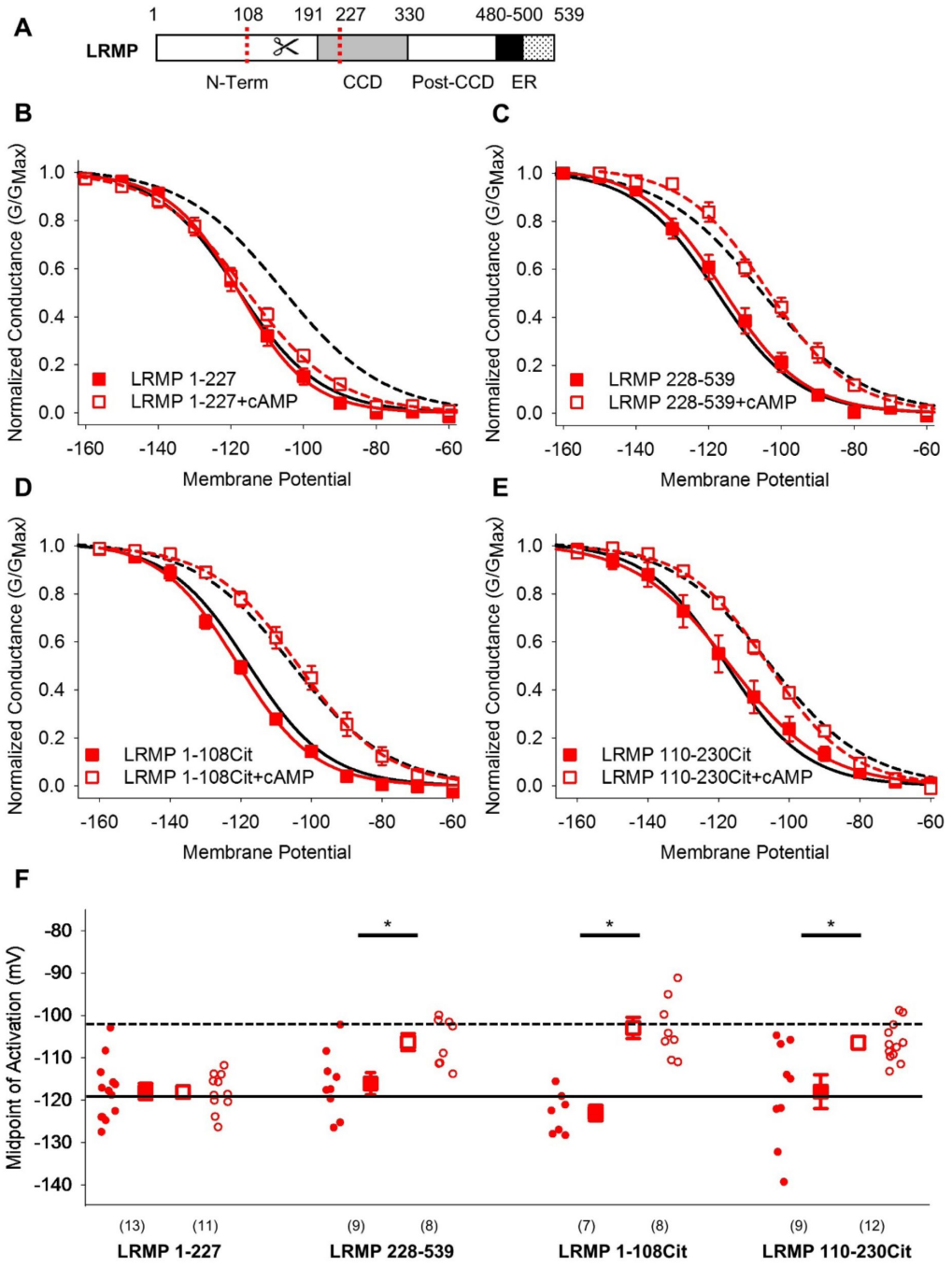
We next examined the domains of the HCN4 channel that are necessary for regulation by LRMP. Since LRMP regulates only the HCN4 isoform, we focused on the large non-conserved regions in the distal N- and C-terminals as potential sites for LRMP regulation. We first examined the N-terminus by testing the ability of LRMP to regulate a series of HCN4 channels with progressively larger truncations (Δ1-25, Δ1-62, Δ1-130, Δ1-185, and Δ1-200; shown schematically in **Fig. 3A**). The four smaller deletions all produced functional channels with normal cAMP-dependent shifts in activation, albeit with smaller current amplitudes in HCN4 Δ1-130 and Δ1-185 (**Figs. 3D and 3E** ***insets***). The HCN4 Δ1-200 construct produced insufficient current amplitude for analysis.

When the first 25 residues in the HCN4 N-terminus were truncated, LRMP still prevented cAMP from shifting HCN4 activation, just as in the WT HCN4 channel (**Figs. 3B** and **3F**; **Table 2**). Truncation of residues 1-62 led to a partial LRMP effect where cAMP caused a significant depolarizing shift in the presence of LRMP, but the activation in the presence of LRMP and cAMP was hyperpolarized compared to cAMP alone (**Figs. 3C** and **3F**; **Table 2**). In the HCN4 Δ1-130 construct, cAMP caused a significant depolarizing shift in the presence of LRMP; however, the midpoint of activation in the presence of LRMP and cAMP showed a non-significant trend towards hyperpolarization compared to cAMP alone (**Figs. 3D** and **3F**; **Table 2**). Finally, truncation of the first 185 residues, which removes most of the non-conserved region of the HCN4 N-terminus, completely abolished LRMP regulation of the channel (**Figs. 3E** and **3F**; **Table 2**); when LRMP was present, cAMP caused a significant depolarizing shift in the HCN4 Δ1-185 activation, and the midpoint of activation in the presence of both LRMP and cAMP was not significantly different from the midpoint in the presence of cAMP alone. These results suggest that the multiple subdomains within the non-conserved N-terminus of HCN4 are necessary for functional regulation by LRMP.

We also investigated LRMP regulation of two C-terminal truncations in HCN4: HCN4 S719X, which removes the C-terminus distal to the CNBD (Liao et al., 2012), and HCN4 V604X, which additionally removes the CNBD (shown schematically in **Fig. 4A**). We found that truncation of the distal C-terminus (HCN4-S719X) reduced but did not eliminate LRMP regulation of HCN4. In the presence of both LRMP and cAMP, the activation of HCN4-S719X was still significantly hyperpolarized compared to the presence of cAMP alone (**Figs. 4B** and **4C**; **Table 2**). While cAMP caused a significant (~7 mV) shift in HCN4-S719X activation in the presence of LRMP, this was less than half the shift in the absence of LRMP (~18 mV). HCN4-V604X, which truncates the channel between the C-linker and CNBD, shifts channel activation to more depolarized potentials and completely prevents cAMP-dependent regulation (**Figs. 4D** and **4E**; **Table 2**), similar to the effects of the homologous HCN2-V526X mutant (Wainger et al., 2001). LRMP did not alter the gating of HCN4-V604X in the absence of cAMP, and the lack of cAMP binding obviously prevented the investigation of any LRMP inhibition of cAMP-dependent potentiation (**Figs. 4D** and **4E**). While these results do not preclude a contribution of the C-terminus to modulation of HCN4 by LRMP, the persistent regulation when the distal C-terminus is truncated indicates that this region is not required.

## The N-terminus of LRMP associates with the N-terminus of HCN4

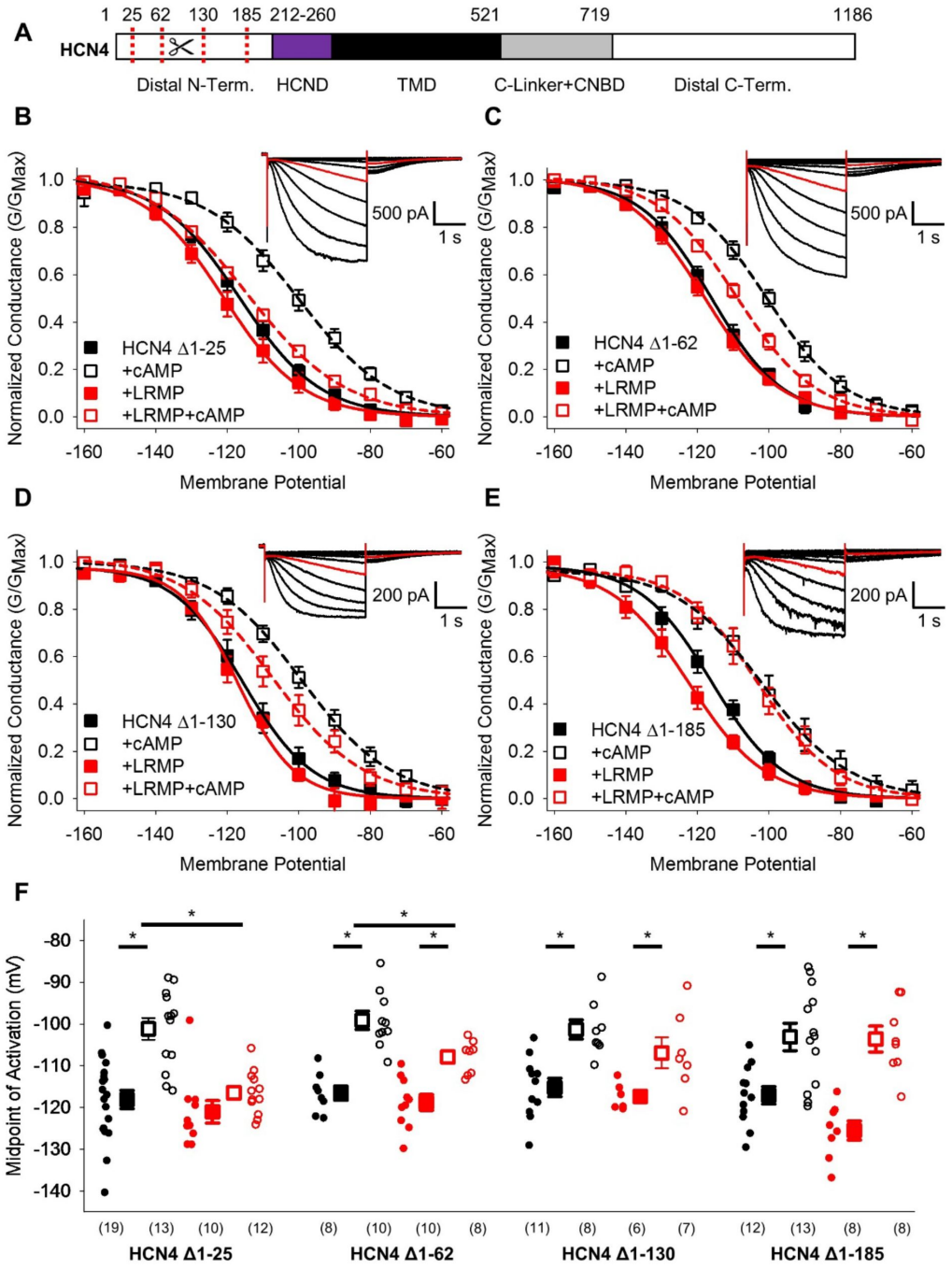
To test for physical association between different regions on LRMP and HCN4, we used a FRET-based hybridization assay called FRET two-hybrid that measures fluorescent energy transfer between fluorescent protein-tagged fragments of proteins expressed in cells. A similar approach has been used to define interactions between the N- and C-termini of EAG channels as well as




## Figure 2

### The pre-coiled-coil region of the LRMP N-terminus is necessary and sufficient to regulate HCN4.

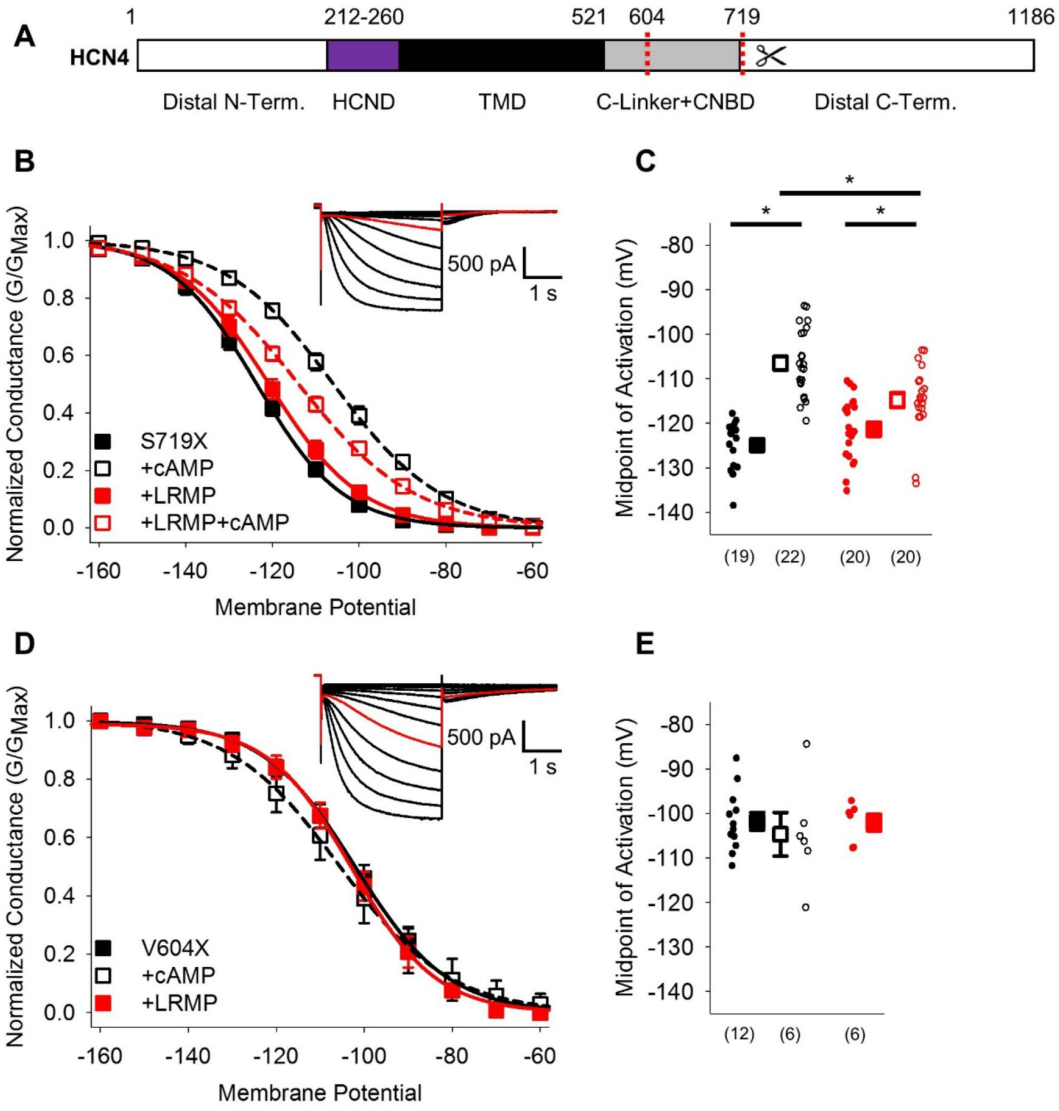
**A:** Schematic of LRMP showing the coiled-coil domain (CCD) and ER-transmembrane and luminal domains (ER) as predicted by Alphafold (Q60664). The locations of cut sites in the LRMP coiled-coil and N-terminal domains are indicated (*red dotted lines*). **B-E:** Voltage-dependence of activation for HCN4 in the absence (*black*) or presence (*red*) of LRMP 1-227 (**B**), LRMP 228-539 (**C**), LRMP 1-108 (**D**), or LRMP 110-230 (**E**), and/or 1mM intracellular cAMP (*open symbols*). The midpoints of activation for HCN4 with (*dotted line*) or without (*solid line*) 1 mM cAMP in the absence of LRMP are shown. **F:** Average (± standard error of the mean) midpoints of activation for HCN4 in the absence or presence of LRMP constructs and/or 1mM cAMP using the same colour scheme as **B-E**. Small circles represent individual recordings and values in parentheses are the number of independent recordings for each condition. * indicates a significant ($P < 0.05$) difference. All means, standard errors, and exact P-values are in **Table 1**.




## Figure 3

### The distal HCN4 N-terminus is required for functional regulation by LRMP.

**A:** Schematic representation of HCN4 showing truncation sites (*red dotted lines*) in the non-conserved distal N-terminus (TMD: Transmembrane domain). **B-E:** Voltage-dependence of activation for HCN4 Δ1-25 (**B**), HCN4 Δ1-62 (**C**), HCN4 Δ1-130 (**D**), and HCN4 Δ1-185 (**E**) in the absence (*black*) or presence of LRMP (*red*) and/or 1mM intracellular cAMP (*open symbols*). **B-E *insets*:** Exemplar current recordings for HCN4 Δ1-25 (**B**), HCN4 Δ1-62 (**C**), HCN4 Δ1-130 (**D**), and HCN4 Δ1-185 (**E**) in the absence of LRMP and cAMP. Currents recorded with a -110 mV activating pulse are shown in *red*. **F:** Average (± standard error of the mean) midpoints of activation for HCN4 Δ1-25, HCN4 Δ1-62, HCN4 Δ1-130, and HCN4 Δ1-185 in the absence or presence of LRMP and/or 1mM cAMP using the same colour scheme as **B-E**. Small circles represent individual recordings and values in parentheses are the number of independent recordings for each condition. * indicates a significant ($P < 0.05$) difference. All means, standard errors, and exact P-values are in **Table 2**.




## Figure 4

### The HCN4 C-terminus is not the primary site for functional regulation by LRMP.

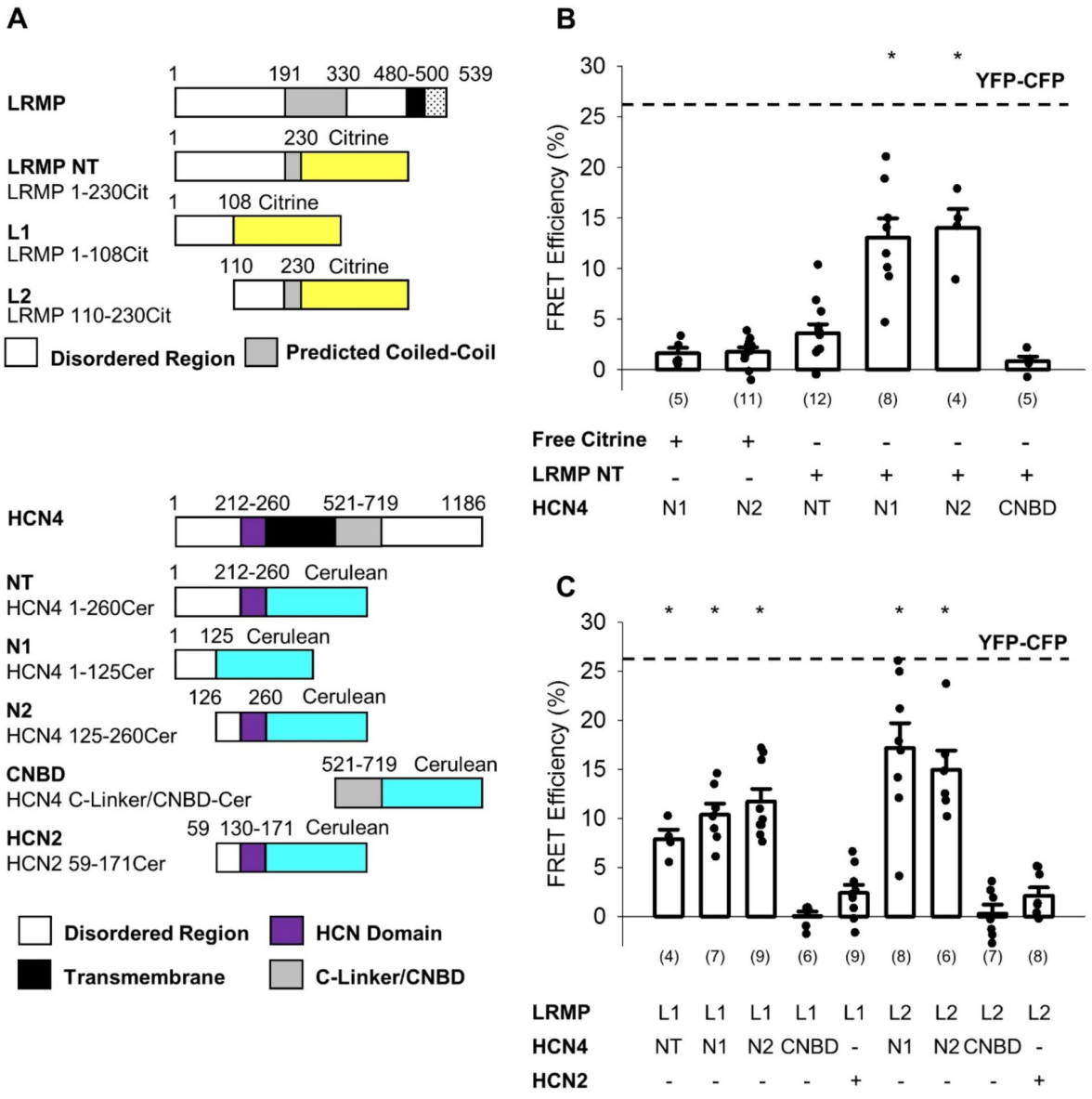
**A:** Schematic representation of HCN4 showing truncation sites (*red dotted lines*) of the distal C-terminus and CNBD (TMD: Transmembrane domain). **B:** Voltage-dependence of activation for HCN4 S719X in the absence (*black*) or presence of LRMP (*red*) and/or 1mM intracellular cAMP (*open symbols*). **C:** Average (± standard error of the mean) midpoints of activation for HCN4 S719X in the absence or presence of LRMP and/or 1mM cAMP using the same colour scheme as **B**. **D:** Voltage-dependence of activation for HCN4 V604X in the absence or presence of LRMP or 1mM intracellular cAMP using the same colour scheme as **B**. **E:** Average (± standard error of the mean) midpoints of activation for HCN4 V604X in the absence or presence of LRMP or 1mM cAMP using the same colour scheme as **B**. Small circles represent individual recordings in **C** and **E** and values in parentheses are the number of independent recordings for each condition. **B and D *insets*:** Exemplar current recordings for HCN4 S719X (**B**) and HCN4 V604X (**D**) in the absence of LRMP and cAMP. Currents recorded with a -110 mV activating pulse are shown in *red*. * indicates a significant ($P < 0.05$) difference. All means, standard errors, and exact P-values are in **Table 2**.

between Calmodulin and $Ca_V1.2$ (Gianulis et al., 2013; Erickson et al., 2003). FRET two-hybrid has a number of advantages. First, detection of association between domains occurs in the native cellular environment. In addition, this approach decreases the false-negative rate by using short protein fragments to reduce distances between the fluorophores, thus reducing the potential for missed protein interaction. Fragments of LRMP that were tagged on the C-terminus with Citrine were co-expressed in HEK293 cells with fragments of HCN4 tagged on the C-terminus with Cerulean (**Fig. 5A**). We then measured FRET using the acceptor photobleaching method (Bastiaens and Jovin, 1996; Wouters et al., 1998; Klipp et al., 2020).

A Citrine-tagged construct corresponding to the functionally active domain of the LRMP N-terminus (LRMP NT, LRMP residues 1-230) did not significantly FRET with the full-length HCN4 N-terminus (NT, HCN4 residues 1-260; **Fig. 5B**; **Table 3**). However, these fragments are large and likely unstructured, thus the fluorophores could be positioned at a distance greater than the range FRET can measure, which is ~20-80Å. Indeed, when we expressed LRMP NT with halves of the HCN4 N-terminus — HCN4 N1 (residues 1-125) and HCN4 N2 (residues 126-260) — we measured significant FRET compared to control cells co-transfected with Cer-HCN4 fragments and Citrine alone (i.e., without any LRMP sequence; **Fig. 5B**; **Table 3**). Halves of the LRMP N-terminus — LRMP L1 (residues 1-108) and LRMP L2 (residues 110-230) — also exhibited significant FRET with the whole HCN4 N-terminus and with HCN4 N-terminal fragments (**Fig. 5C**; **Table 3**). No significant FRET was observed between LRMP fragments and a fragment of the HCN2 N-terminus that contains the conserved HCND and is analogous to HCN4 N2 (**Fig. 5C**; **Table 3**). And none of the LRMP fragments tested exhibited significant FRET with an HCN4 C-Linker/CNBD construct compared to control experiments (**Figs. 5B** and **5C**; **Table 3**). Cerulean-tagged fragments of the distal C-terminus showed insufficient expression for FRET experiments. Ultimately these data suggest that the N-terminus of LRMP interacts with regions of the non-conserved distal N-terminus of HCN4.

## Mutants in the HCN4 C-linker disrupt LRMP's functional effects

Prior work has shown that transduction of cAMP-binding to shifts in channel activation require a tripartite interaction of a transduction centre comprised of the N-terminal HCND, the C-linker, and the S4-S5 linker (Porro et al., 2019; Wang et al., 2020b; Kondapuram et al., 2022). Since LRMP interacts with the HCN4 N-terminus and disrupts cAMP-dependent potentiation downstream of the cAMP binding site, we hypothesized that it may act via this cAMP transduction centre. Although the sequence of the transduction centre is highly conserved among HCN channel isoforms, the overall conformation of the region differs subtly between the known HCN channel structures of HCN1 and HCN4 (Lee and MacKinnon, 2017; Saponaro et al., 2021). We identified two HCN4-specific residues in the C-linker, P545 and T547, that could contribute to the HCN4-specific conformation, signal transduction, and LRMP regulation (**Fig. 6A**). Mutation of these two residues to the cognate HCN2 amino acids, rendered the HCN4-P545A/T547F channel completely insensitive to LRMP, although it responded to cAMP with an ~10 mV shift in activation voltage (**Figs. 6B** and **6C**; **Table 2**), indicating that the unique residues in the HCN4 C-linker are important for LRMP regulation. However, the impact of these two HCN4 residues appears to depend on the overall context of the C-linker and C-terminus. A chimera containing the HCN4 N-terminus and transmembrane domains (residues 1-518) with the HCN2 C-linker, CNBD and C-terminus (442-863), termed HCN4-2 (Liao et al., 2012) was still partially regulated by LRMP (**Figs. 6D** and **6E**; **Table 2**). This result is satisfyingly consistent with our finding that deletion of the distal C-terminus in the HCN4-S719x construct reduces the overall effect of LRMP (**Fig. 4B**). Together these data suggest that unique residues in the HCN4 C-linker are important for regulation by LRMP but that the distal C-terminus may also contribute to the regulation, potentially via allosteric effects on the orientation of the transduction centre.




## Figure 5

### The N-terminus of LRMP FRETs with the N-terminus of HCN4

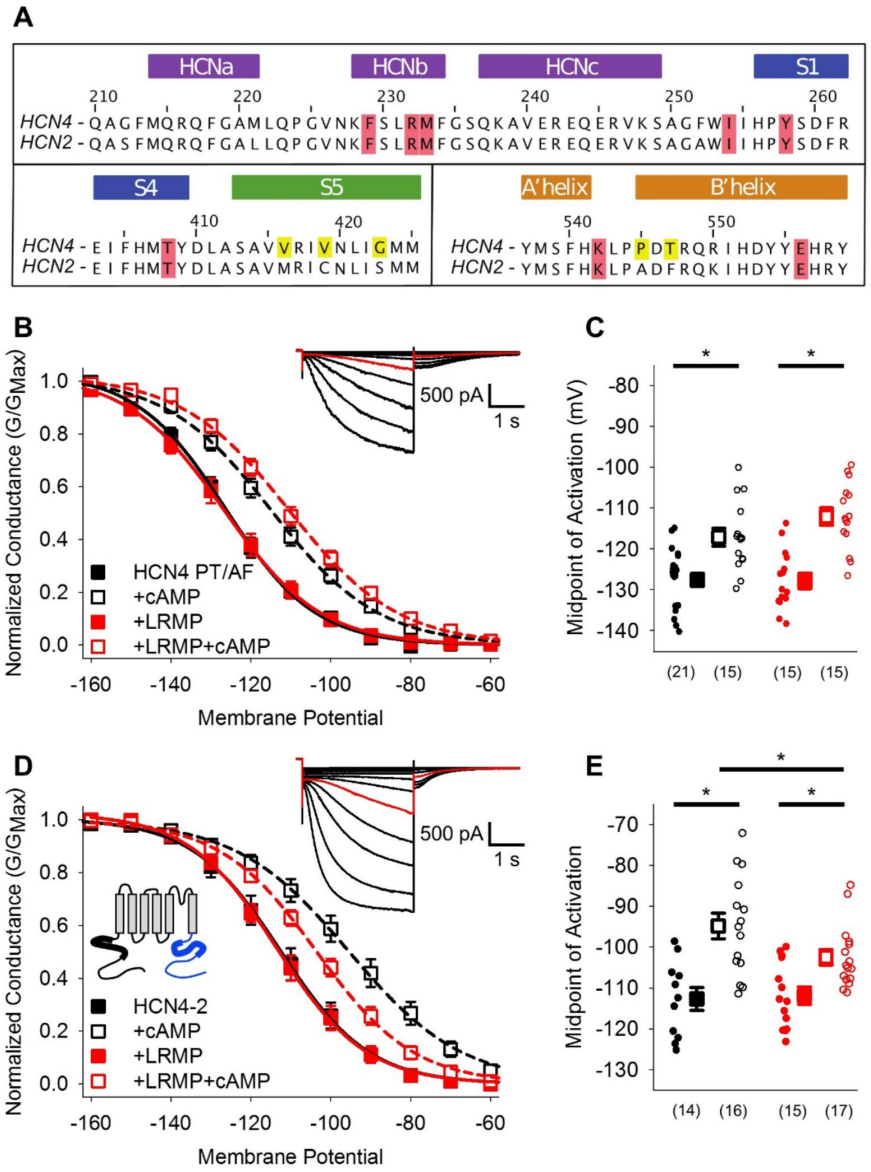
**A:** Schematic representations of the Citrine-tagged LRMP fragments and Cerulean-tagged HCN4 and HCN2 fragments used in FRET experiments. **B:** Average (± standard error of the mean) acceptor photobleaching FRET efficiency between free Citrine or the Citrine-tagged N-terminal region of the LRMP (LRMP NT) and the Cerulean-tagged HCN4 N-terminus (NT), halves of the HCN4 N-terminus (N1 and N2), or the HCN4 C-Linker/CNBD. The dotted line is the average FRET in YFP-CFP concatemers from a prior study (Wang et al., 2020a). **C:** Average (± standard error of the mean) acceptor photobleaching FRET efficiency between Citrine-tagged fragments of the LRMP N-terminus (L1 and L2) and Cerulean-tagged fragments of HCN4 or HCN2. Small circles in **B** and **C** represent individual recordings and values in parentheses are the number of independent recordings for each condition. * indicates a significant ($P < 0.05$) difference compared to control FRET in cells co-transfected with free Citrine and with Cerulean-tagged HCN4 N-terminal fragments. All means, standard errors, and exact P-values are in **Table 3**.

| Citrine | Cerulean | FRET Efficiency (%) | P-Value vs. Control |
|---|---|---|---|
| Free Citrine | HCN4 1-125 or 125-260 | 1.7 ± 0.3 | |
| | HCN4 1-125 | 1.6 ± 0.6 (5) | |
| | HCN4 125-260 | 1.8 ± 0.4 (11) | |
| LRMP 1-230 | HCN4 1-260 | 3.6 ± 0.9 (12) | P = 0.8471 |
| | HCN4 1-125 | 13.1 ± 1.9 (8) | **P < 0.0001** |
| | HCN4 125-260 | 14.0 ± 1.9 (4) | **P < 0.0001** |
| | HCN4 C-Linker/CNBD | 0.8 ± 0.5 (5) | P = 1.0000 |
| LRMP 1-108 | HCN4 1-260 | 7.9 ± 1.0 (4) | **P = 0.0265** |
| | HCN4 1-125 | 10.4 ± 1.1 (7) | **P < 0.0001** |
| | HCN4 125-260 | 11.7 ± 1.3 (9) | **P < 0.0001** |
| | HCN4 C-Linker/CNBD | 0.0 ± 0.5 (6) | P = 0.9869 |
| | HCN2 N-Term | 2.4 ± 0.8 (9) | P = 1.0000 |
| LRMP 110-230 | HCN4 1-125 | 17.2 ± 2.5 (8) | **P < 0.0001** |
| | HCN4 125-260 | 15.0 ± 2.0 (6) | **P < 0.0001** |
| | HCN4 C-Linker/CNBD | 0.3 ± 0.9 (7) | P = 0.9949 |
| | HCN2 N-Term | 2.1 ± 0.8 (8) | P = 1.0000 |

Average midpoint of activation ± standard error of the mean (Number of independent cells)

**Table 3**

**Acceptor Photobleaching FRET Between LRMP and HCN Channel Fragments**




## Figure 6

### Mutants in the HCN4 C-linker disrupt LRMP's functional effects.

**A:** Sequence alignments of the HCN channel HCND (*purple*), voltage-sensor (*blue*), pore (*green*), and C-linker regions (*orange*) known to regulate cAMP-transduction. Non-conserved HCN4 residues in the S5 and C-linker regions are highlighted in yellow, and some of the residues believed to participate in cAMP-transduction are highlighted in red. **B:** Voltage-dependence of activation for HCN4 P545A/T547F (PT/AF) in the absence (*black*) or presence of LRMP (*red*) and/or 1mM intracellular cAMP (*open symbols*). **C:** Average (± standard error of the mean) midpoints of activation for HCN4 PT/AF in the absence or presence of LRMP and/or 1mM cAMP using the same colour scheme as **B**. **D:** Voltage-dependence of activation for HCN4-2 (HCN4 1-518 + HCN2 442-863) in the absence or presence of LRMP and/or 1mM intracellular cAMP using the same colour scheme as **B**. ***Schematic Inset*:** Schematic of the chimeric HCN4-2 channel with HCN4 sequence shown in black and HCN2 in blue. The HCN and cyclic-nucleotide binding domains are indicated as thicker line segments. **E:** Average (± standard error of the mean) midpoints of activation for HCN4-2 in the absence or presence of LRMP and/or 1mM cAMP using the same colour scheme as **B**. **B and D *insets*:** Exemplar current recordings for HCN PT/AF (**B**) and HCN4-2 (**D**) in the absence of LRMP and cAMP. Currents recorded with a -110 mV activating pulse are shown in *red*. Small circles represent individual recordings in **C** and **E** and values in parentheses are the number of independent recordings for each condition. * indicates a significant ($P < 0.05$) difference. All means, standard errors, and exact P-values are in **Table 2**.

### The HCN4 N-Terminus and cAMP transduction centre confer LRMP regulation to HCN2

Given that LRMP regulation involves HCN4-specific sequences in the C-linker region and distal N-terminus, we next investigated whether we could confer LRMP sensitivity to HCN2 by manipulating these regions. Mutation of the two non-conserved residues in the C-linker (HCN2 A467P/F469T) alone were not sufficient to confer regulation by LRMP onto HCN2 (**Figs. 7A** and **7B**; **Table 2**). In addition to the changes in the C-linker, comparison of the HCN1 and HCN4 structures shows different orientations of the S4-S5 region between HCN1 and HCN4 that may be responsible for differences in regulation of cAMP-sensitivity between channel isoforms (Saponaro et al., 2021). The S4-S5 linkers are fully conserved across HCN isoforms, however, 3 residues near the intracellular side of S5 differ between HCN2 and HCN4 (**Fig. 6A**). We generated an HCN2 construct with all five non-conserved S5 and C-linker residues mutated to the corresponding HCN4 amino acids (HCN2 M338V/C341V/S345G/A467P/F469T). These mutations did not confer LRMP regulation to HCN2 (**Figs. 7C** and **7D**; **Table 2**), consistent with our data showing that the HCN4 N-terminus is required for LRMP regulation of channel gating (**Fig. 3**) and may confer partial sensitivity to LRMP in HCN2 (**Fig. 6**).

Finally, we made a chimeric HCN2 channel that contains the distal HCN4 N-terminus (residues 1-212, prior to the HCN domain) and the 5 non-conserved residues of the HCN4 S5 segment and C-linker elbow. The resulting HCN2-4N VVGPT channel has a similar voltage-dependence of activation as HCN2 and a normal response to cAMP in the absence of LRMP (**Figs. 7E** and **7F**; **Table 2**). However, the HCN2-4N VVGPT channel was fully regulated by LRMP — it became insensitive to cAMP in the presence of LRMP (**Figs. 7E** and **7F**; **Table 2**). Thus, the HCN4 N-terminus and a small number of HCN4-specific residues near the cAMP-transduction centre residues are sufficient to confer LRMP regulation to HCN2.

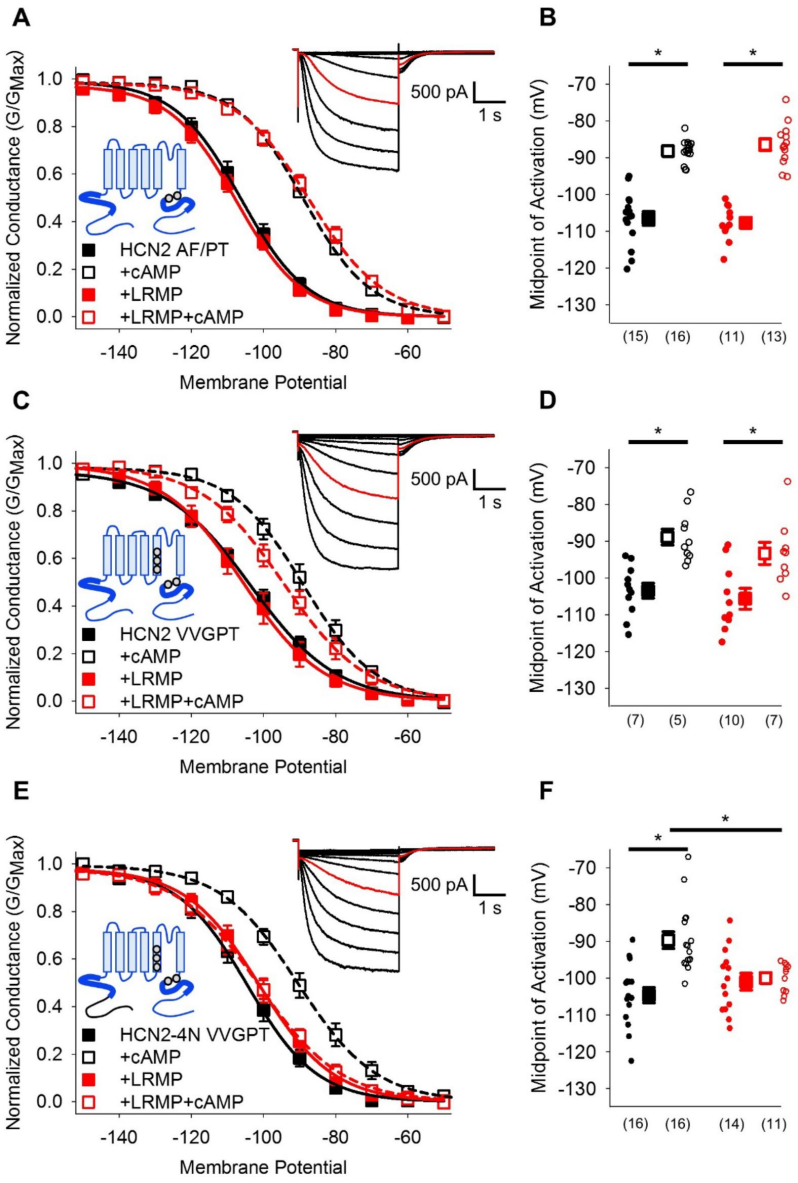
## Discussion

Ion channels families, such as HCN channels, are conveniently described by their shared properties. However, differences between isoforms underlie nuanced physiological functions of ion channels in tissues and present the opportunity for the design of drugs with higher specificity. Our present results reveal how subtle — and seemingly inconsequential — differences between isoforms in the same channel family can confer important differences in regulation. In the specific case of LRMP regulation of HCN4 channels, our data identify unique features of HCN4 that render its cAMP sensitivity particularly malleable, and thus could contribute to its unique function in the sinoatrial node of the heart.

Although LRMP prevents the cAMP-dependent shift in HCN4 activation, LRMP does not act by preventing cAMP from binding to the channel. Instead, we show here that the N-terminal domains of LRMP and HCN4 are required for both physical interaction and regulation. Our data further show that LRMP acts by disrupting transduction between cAMP binding and the shift in voltage-dependence in a manner that depends on HCN4-specific residues in multiple domains, including the C-linker, S5, and C-terminus.

### An intramolecular transduction centre between the C-Linker, HCND, and S4-S5 linker links cAMP binding to shifts in activation

Recent studies indicate that binding of cAMP to the C-terminal CNBD is transduced to a shift in HCN channel activation via an intramolecular cAMP transduction centre formed by interactions between the C-linker, N-terminal HCND, and S4-S5 linker (Weißgraeber et al., 2017; Porro et al.,




## Figure 7

### HCN4-specific residues and the HCN4 N-terminus confer LRMP regulation on HCN2

**A:** Voltage-dependence of activation for HCN2 A467P/F469T (AF/PT) in the absence (*black*) or presence of LRMP (*red*) and/or 1mM intracellular cAMP (*open symbols*). **B:** Average (± standard error of the mean) midpoints of activation for HCN2 AF/PT in the absence or presence of LRMP and/or 1mM cAMP using the same colour scheme as **A**. **C:** Voltage-dependence of activation for HCN2 VVGPT (M338V/C341V/S345G/A467P/F469T) in the absence or presence of LRMP and/or 1mM intracellular cAMP using the same colour scheme as **A**. **D:** Average (± standard error of the mean) midpoints of activation for HCN2 VVGPT in the absence or presence of LRMP and/or 1mM cAMP using the same colour scheme as **A**. **E:** Voltage-dependence of activation for HCN2-4N VVGPT (HCN4 1-212 + HCN2 135-863 M338V/C341V/S345G/A467P/F469T) in the absence or presence of LRMP and/or 1mM intracellular cAMP using the same colour scheme as **A**. **F:** Average (± standard error of the mean) midpoints of activation for HCN2-4N VVGPT in the absence or presence of LRMP and/or 1mM cAMP using the same colour scheme as **A**. ***Sample current insets*:** Exemplar current recordings for HCN2 AF/PT (**A**), HCN2 VVGPT (**C**), and HCN2-4N VVGPT (**E**) in the absence of LRMP and cAMP. Currents recorded with a -110 mV activating pulse are shown in *red*. ***Schematic Insets*:** Schematics of the chimeric channels with HCN4 sequence shown in black and HCN2 in blue. The HCN and cyclic-nucleotide binding domains are indicated as thicker line segments. Small circles represent individual recordings in **B, D** and **F** and values in parentheses are the number of independent recordings for each condition. * indicates a significant ($P < 0.05$) difference. All means, standard errors, and exact P-values are in **Table 2**.

2019; Wang et al., 2020b; Saponaro et al., 2021; Kondapuram et al., 2022). There are multiple individual interactions within the transduction centre which have been described in detail (Porro et al., 2019; Kondapuram et al., 2022; Elbahnsi et al., 2023; Wang et al., 2020b). While most of these interactions are conserved among HCN channel isoforms, there are some isoform-specific differences that likely contribute to the unique sensitivity of HCN4 to LRMP and to other regulators that act to modify the cAMP response. Most notably, the S4-S5 linker of HCN4 adopts a different conformation compared to HCN1. Since the residues in the S4-S5 linker are completely conserved across HCN channel isoforms, we speculate that subtle differences in the sequence of nearby areas of the C-linker and S5 of HCN4 underlie the unique S4-S5 conformation of HCN4.

Despite often being grouped as the two cAMP-sensitive HCN isoforms, it is clear that cAMP signal transduction in HCN4 and HCN2 differs in several regards. The cAMP-dependent shift in HCN4 (~14 mV) is smaller than in HCN2 (~20 mV; **Table 2**). Transduction of cAMP binding is sensitive to divalent cations in HCN4 but not HCN2 (Saponaro et al., 2021; Peters et al., 2023). And, the cAMP-dependent shift in activation can be disrupted in HCN4 by regulatory factors such as cyclic-dinucleotides (Lolicato et al., 2014) as well as LRMP and IRAG (Peters et al., 2020). While there is not yet a structure available for HCN2, it is possible that differences in the orientation of the S4-S5 linker and the transduction centre makes HCN4 channels more sensitive to these perturbations in its cAMP signal transduction compared to HCN2.

## Proposed model: LRMP disrupts cAMP regulation of HCN4 activation at the cAMP transduction centre

In our proposed model for how LRMP disrupts the cAMP-dependent shift in HCN4 activation, LRMP is tethered to HCN4 via an interaction between the N-terminals of the two proteins. Within the HCN4, the interaction with LRMP occurs via the distal N-terminus, which is not resolved in channel structures (Lee and MacKinnon, 2017; Saponaro et al., 2021) and is completely divergent between HCN channel isoforms. In contrast, the conserved HCND does not appear to participate in binding of LRMP, however it is known to be an important component of the cAMP transduction centre (Porro et al., 2019; Kondapuram et al., 2022). It is also worth noting that the N-terminus of HCN4 is 260 amino acids long, compared to 140 and 209 in HCN1 and HCN2. Given the evolutionary and metabolic costs of maintaining long unique domains in these highly conserved proteins, it is possible that they serve other isoform-specific regulatory roles that await future discovery.

We found that truncation of the HCN4 N-terminus abolishes regulation by LRMP without affecting cAMP-dependent regulation. This finding was further corroborated by FRET experiments showing interactions between LRMP and the HCN4 N-terminus, but not the highly conserved CNBD. While these results do not preclude a modest role of the distal HCN4 C-terminus in LRMP regulation — as suggested by the partial LRMP regulation of the HCN4-2 and HCN4-719X channels — they clearly indicate that the N-terminus is critical for LRMP regulation of HCN4. Furthermore, the distal HCN4 C-terminus was not required to confer LRMP sensitivity to HCN2 (**Figs. 7E** and **7F**), suggesting that HCN4-specific residues in this region are not responsible for isoform-specific regulation by LRMP.

Most surprisingly, we were able to confer LRMP sensitivity to HCN2 by introducing only the HCN4 distal N-terminus and mutating 5 residues in the C-linker and S5 regions to the cognate HCN4 residues. It is highly unlikely that LRMP directly contacts the residues in S5, and our FRET experiments did not reveal an interaction with the C-linker/CNBD either (**Fig. 5**). Thus, our model is that LRMP interacts with the N-terminus of the HCN4 and prevents cAMP regulation of the channel allosterically via effects on the unique transduction centre. We propose that the isoform specificity arises both from the unique distal N-terminal interaction site and from the unique orientation of the transduction centre in HCN4.

## Potential physiological implications

The first half of LRMP's cytosolic domain (residues 1-230) that make up the N-terminus of the protein is necessary and sufficient to interact with and regulate HCN4. Because the C-terminus of LRMP is embedded in the ER, the N-terminal region of LRMP would naturally be in closer proximity to HCN4 in the plasma membrane. LRMP also interacts with and regulates $Ca^{2+}$ release through inositol triphosphate ($IP_3$) receptors in the ER membrane, likely via a site in the coiled-coil region (Prüschenk et al., 2021). Together these results suggest the intriguing possibility of coordination between the activity of $IP_3$Rs and HCN4 and the formation of ER-plasma membrane junctions in cells where LRMP and HCN4 are co-expressed. For example, in sinoatrial myocytes HCN4 and SR $Ca^{2+}$ release, including through $IP_3$ receptors, are both known to regulate pacemaking (DiFrancesco, 2010; Peters et al., 2021; Capel et al., 2021). A potential interaction with LRMP (or IRAG1), could serve to coordinate these important processes.

## Limitations

In this study, a FRET hybridization approach was used to identify macro-regions of LRMP and HCN4 that can interact with each other in a cellular context. It is important to acknowledge that this technique cannot resolve the atomic details of the interaction, which would ideally be addressed in the future by a co-structure of the proteins or at least their interaction domains. Other limitations of the approach are that the FRET efficiency measurement depends on the relative expression of each fragment, the affinity of the interaction, the orientation of the fluorophores, and the distance between the two fluorophores. This may explain why longer LRMP (LRMP 1-230Cit) and HCN4 (HCN4 1-260Cer) fragments showed lower FRET efficiency than did smaller fragments within these domains (**Fig. 5**). Also, the approach may miss interactions that involve complex tertiary structures where binding involves multiple regions of the protein. Despite these limitations, our FRET and functional results together are consistent with the model that the N-terminals of HCN4 and LRMP directly interact.

Unfortunately, the available HCN4 structures do not resolve the distal N-terminus (Shintre et al., 2018; Saponaro et al., 2021), and the structure of LRMP has yet to be resolved. This lack of structural information hindered our decisions about specific cut sites for LRMP and HCN4 constructs and restricts our ability to predict the precise residues that are involved in the described interactions. For example, we found that LRMP interacts with isolated fragments representing each half of the HCN4 N-terminus. This could be explained by a diffuse interaction composed of multiple contacts, or our cut site overlapping a contiguous interaction site. The partial disruption of LRMP regulation of the HCN4 Δ1-62 and Δ1-130 deletion constructs suggests that multiple or diffuse interactions are likely. Similarly, we found that LRMP residues 1-108 and 110-230 both interacted with the HCN4 N-terminus in FRET assays, but neither fragment alone was able to regulate the channel. As with the HCN4 N-terminus, this difference could be explained by multiple important regions and/or our cut site overlapping the functionally relevant site. Ultimately, these questions will require a structure of the LRMP-HCN4 interaction interfaces.

## Summary

Overall, these data support a model for LRMP regulation of HCN4 where LRMP interacts with the HCN4 N-terminus to allosterically disrupt cAMP signal transduction between the C-linker, N-terminus, and S4-S5 linker (Porro et al., 2019; Wang et al., 2020b). The specific regulation of only the HCN4 isoform by LRMP is determined by both the non-conserved distal N-terminus and non-conserved residues in the C-linker and S5 of HCN4, which may result in a unique orientation of the cAMP transduction centre in HCN4. While a potential physiological role for LRMP regulation of HCN4 remains unknown, our data show that LRMP is a useful biophysical tool to study the intramolecular signal transduction between cAMP binding and the shift in HCN4 activation.

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The authors declare they have no conflict of interest.

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## Supplementary Figure 1

### Mouse LRMP sequence.

Sequence for the mouse LRMP construct used in this study showing the predicted start of the coiled-coil, ER transmembrane, and ER lumenal domains as well as cut sites used for constructs in this study.

| Construct | Plasmid | Tag | Source |
|---|---|---|---|
| HCN1 | pCDNA3.1 | None | Dr. Eric Accili |
| HCN2 | pCDNA3.1 | None | |
| HCN2 A467P/F469T | pTwist-CMV-WPRE-Neo | None | Twist Biosciences |
| HCN4 Δ1-25 | pCDNA6 | None | Dr. Richard Aldrich |
| HCN4 Δ1-62 | pTwist-CMV-WPRE-Neo | None | |
| HCN4 Δ1-130 | pTwist-CMV-WPRE-Neo | None | |
| HCN4 Δ1-185 | pTwist-CMV-WPRE-Neo | none | |
| HCN4 Δ1-200 | pTwist-CMV-Hygro | None | Twist Biosciences |
| HCN4 P545A/T547F | pCDNA3.1 | None | ABM |
| HCN4 V604X | pCDNA3.1 | None | ABM |
| HCN4 S719X | pCDNA3.1 | None | |
| HCN4-2 | pCDNA3.1 | None | |
| HCN2 VVGPT | pTwist-CMV-BG-WPRE-Neo | None | Twist Biosciences |
| HCN2-4N VVGPT | pTwist-CMV-BG-WPRE-Neo | None | Twist Biosciences |
| LRMP | pCMV6 Kan/Neo | None | Origene |
| Myc-LRMP | pCMV6 Kan/Neo | Myc-Tag | |
| LRMP 1-227 | pTwist-CMV | Myc-Tag | Twist Biosciences |
| LRMP 228-539 | pTwist-CMV | Myc-Tag | Twist Biosciences |
| HCN4 1to125 | pcDNA3.1 | C-term Cerulean | |
| HCN4 125to260 | pcDNA3.1 | C-term Cerulean | |
| HCN4 1to260 | pcDNA3.1 | C-term Cerulean | |
| HCN4 C-Linker/CNBD (521-719) | pcDNA3.1 | C-term Cerulean | |
| LRMP 1to479 | pcMVBG | C-term Citrine | |
| LRMP 1-230 | pcMVBG | C-term Citrine | |
| LRMP 110to230 | pcMVBG | C-term Citrine | |
| LRMP 1to108 | pcMVBG | C-term Citrine | |

## Table S1

**DNA Constructs Used in this Study**

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**Reviewer #1 (Public Review):**

Summary:

The authors use truncations, fragments, and HCN2/4 chimeras to narrow down the interaction and regulatory domains for LRMP inhibition of cAMP-dependent shifts in the voltage dependence of activation of HCN4 channels. They identify the N-terminal domain of HCN4 as a binding domain for LRMP, and highlight two residues in the C-linker as critical for the regulatory effect. Notably, whereas HCN2 is normally insensitive to LRMP, putting the N-terminus and 5 additional C-linker and S5 residues from HCN4 into HCN2 confers LRMP regulation in HCN2.

Strengths:

The work is excellent, the paper well written, and the data convincingly support the conclusions which shed new light on the interaction and mechanism for LRMP regulation of HCN4, as well as identifying critical differences that explain why LRMP does not regulate other isoforms such as HCN2.

https://doi.org/10.7554/eLife.92411.2.sa2

**Reviewer #2 (Public Review):**

Summary:

HCN-4 isoform is found primarily in sino-atrial node where it contributes to the pacemaking activity. LRMP is an accessory subunit which prevents cAMP-dependent potentiation of HCN4 isoform but does not have any effect on HCN2 regulation. In this study, the authors combine

electrophysiology, FRET with standard molecular genetics to determine the molecular mechanism of LRMP action on HCN4 activity. Their study shows parts of N- and C-termini along with specific residues in C-linker and S5 of HCN4 are crucial for mediating LRMP action on these channels. Furthermore, they show that the initial 224 residues of LRMP are sufficient to account for most of the activity. In my view, the highlight of this study is Fig. 7 which recapitulates LRMP modulation on HCN2-HCN4 chimera. Overall, this study is an excellent example of using time-tested methods to probe the molecular mechanisms of regulation of channel function by an accessory subunit.

The authors adequately addressed my earlier concerns.

https://doi.org/10.7554/eLife.92411.2.sa1

**Reviewer #3 (Public Review):**

Summary:

Using patch clamp electrophysiology and Förster resonance energy transfer (FRET), Peters and co-workers showed that the disordered N-terminus of both LRMP and HCN4 are necessary for LRMP to interact with HCN4 and inhibit the cAMP-dependent potentiation of channel opening. Strikingly, they identified two HCN4-specific residues, P545 and T547 in the C-linker of HCN4, that are close in proximity to the cAMP transduction centre (elbow Clinker, S4/S5-linker, HCND) and account for the LRMP effect.

Strengths:

Based on these data, the Authors propose a mechanism in which LRMP specifically binds to HCN4 via its isotype-specific Nterminal sequence and thus prevents the cAMP transduction mechanism by acting at the interface between the elbow Clinker, the S4S5-linker, the HCND.

Weaknesses:

Although the work is interesting, there are some discrepancies between data that need to be addressed.

- I suggest inserting in Table 1 and in the text, the Δ shift values (+cAMP; + LRMP; +cAMP/LRMP). This will help readers.

- Figure 1 is not clear, the distribution of values is anomalously high. For instance, in 1B the distribution of values of V1/2 in the presence of cAMP goes from - 85 to -115. I agree that in the absence of cAMP, HCN4 in HEK293 cells shows some variability in V1/2 values, that nonetheless cannot be so wide (here the variability spans sometimes even 30 mV) and usually disappears with cAMP (here not).
This problem is spread throughout the ms, and the measured mean effects indeed always at the limit of statistical significance. Why so? Is this a problem with the analysis, or with the recordings?
There are several other problems with Figure 1 and in all figures of the ms: the Y scale is very narrow while the mean values are marked with large square boxes. Moreover, the exemplary activation curve of Fig 1A is not representative of the mean values reported in Figure 1B, and the values of 1B are different from those reported in Table 1.
On this ground it is difficult to judge the conclusions and it would also greatly help if exemplary current traces would also be shown.

- "....HCN4-P545A/T547F was insensitive to LRMP (Figs. 6B and 6C; Table 1), indicating that the unique HCN4 C-linker is necessary for regulation by LRMP. Thus, LRMP appears to regulate

HCN4 by altering the interactions between the C-linker, S4-S5 linker, and N-terminus at the cAMP transduction centre."

Although this is an interesting theory, there are no data supporting it. Indeed, P545 and T547 at the tip of the C-linker elbow (fig 6A) are crucial for LRMP effect, but these two residues are not involved in the cAMP transduction centre (interface between HCND, S4S5 linker and Clinker elbow), at least for the data accumulated till now in the literature. Indeed, the hypothesis that LRMP somehow inhibits the cAMP transduction mechanism of HCN4 given the fact that the two necessary residues P545 and T547 are close to the cAMP transduction centre, awaits to be proven.

Moreover, I suggest analysing the putative role of P545 and T547 in the light of the available HCN4 structures. In particular, T547 (elbow) point towards the underlying shoulder of the adjacent subunit and, therefore, it is in a key position for the cAMP transduction mechanism. The presence of bulky hydrophobic residues (very different nature compared to T) in the equivalent position of HCN1 and HCN2 is also favouring this hypothesis. In this light, it will also be interesting to see whether single T547F mutation is sufficient to prevent LRMP effect.

https://doi.org/10.7554/eLife.92411.2.sa0

**Author response:**

The following is the authors' response to the original reviews.

> ***eLife assessment***
>
> *This is a useful study examining the determinants and mechanisms of LRMP inhibi:on of cAMP regula:on of HCN4 channel ga:ng. The evidence provided to support the main conclusions is unfortunately incomplete, with discrepancies in the work that reduce the strength of mechanis:c insights.*

Thank you for the reviews of our manuscript. We have made a number of changes to clarify our hypotheses in the manuscript and addressed all of the poten:al discrepancies by revising some of our interpreta:on. In addi:on, we have provided addi:onal experimental evidence to support our conclusions. Please see below for a detailed response to each reviewer comment.

> ***Public Reviews***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *The authors use truncations, fragments, and HCN2/4 chimeras to narrow down the interaction and regulatory domains for LRMP inhibition of cAMP-dependent shifts in the voltage dependence of activation of HCN4 channels. They identify the N-terminal domain of HCN4 as a binding domain for LRMP, and highlight two residues in the C-linker as critical for the regulatory effect. Notably, whereas HCN2 is normally insensitive to LRMP, putting the N-terminus and 5 additional C-linker and S5 residues from HCN4 into HCN2 confers LRMP regulation in HCN2.*
>
> *Strengths:*
>
> *The work is excellent, the paper well written, and the data convincingly support the conclusions which shed new light on the interaction and mechanism for LRMP regulation of HCN4, as well as identifying critical differences that explain why LRMP does not regulate other isoforms such as HCN2.*

Thank you.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *HCN-4 isoform is found primarily in the sino-atrial node where it contributes to the pacemaking activity. LRMP is an accessory subunit that prevents cAMP-dependent potentiation of HCN4 isoform but does not have any effect on HCN2 regulation. In this study, the authors combine electrophysiology, FRET with standard molecular genetics to determine the molecular mechanism of LRMP action on HCN4 activity. Their study shows that parts of N- and C-termini along with specific residues in C-linker and S5 of HCN4 are crucial for mediating LRMP action on these channels. Furthermore, they show that the initial 224 residues of LRMP are sufficient to account for most of the activity. In my view, the highlight of this study is Fig. 7 which recapitulates LRMP modulation on HCN2-HCN4 chimera. Overall, this study is an excellent example of using time-tested methods to probe the molecular mechanisms of regulation of channel function by an accessory subunit.*
>
> *Weaknesses:*
>
> *(1) Figure 5A- I am a bit confused with this figure and perhaps it needs better labeling. When it states Citrine, does it mean just free Citrine, and "LRMP 1-230" means LRMP fused to Citrine which is an "LF" construct? Why not simply call it "LF"? If there is no Citrine fused to "LRMP 1-230", this figure would not make sense to me.*

We have clarified the labelling of this figure and specifically defined all abbreviations used for HCN4 and LRMP fragments in the results section on page 14.

> *(2) Related to the above point- Why is there very little FRET between NF and LRMP 1-230? The FRET distance range is 2-8 nm which is quite large. To observe baseline FRET for this construct more explanation is required. Even if one assumes that about 100 amino are completely disordered (not extended) polymers, I think you would still expect significant FRET.*

FRET is extremely sensitive to distance (to the 6th power of distance). The difference in contour length (maximum length of a peptide if extended) between our ~260aa fragment and our ~130 aa fragments is on the order of 450Å (45nm), So, even if not extended it is not hard to imagine that the larger fragments show a weaker FRET signal. In fact, we do see a slightly larger FRET than we do in control (not significant) which is consistent with the idea that the larger fragments just do not result in a large FRET.

Moreover, this hybridization assay is sensitive to a number of other factors including the affinity between the two fragments, the expression of each fragment, and the orientation of the fluorophores. Any of these factors could also result in reduced FRET.

We have added a section on the limitations of the FRET 2-hybrid assay in the discussion section on page 20. Our goal with the FRET assay was to provide complimentary evidence that shows some of the regions that are important for direct association and we have edited to the text to make sure we are not over-interpreting our results.

> *(3) Unless I missed this, have all the Cerulean and Citrine constructs been tested for functional activity?*

All citrine-tagged LRMP constructs (or close derivatives) were tested functionally by coexpression with HCN (See Table 1 and pages 10-11). Cerulean-tagged HCN4 fragments are of course intrinsically not-functional as they do not include the ion conducting pore.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *Using patch clamp electrophysiology and Förster resonance energy transfer (FRET), Peters and co-workers showed that the disordered N-terminus of both LRMP and HCN4 are necessary for LRMP to interact with HCN4 and inhibit the cAMP-dependent potentiation of channel opening. Strikingly, they identified two HCN4-specific residues, P545 and T547 in the C-linker of HCN4, that are close in proximity to the cAMP transduction centre (elbow Clinker, S4/S5-linker, HCND) and account for the LRMP effect.*
>
> *Strengths:*
>
> *Based on these data, the authors propose a mechanism in which LRMP specifically binds to HCN4 via its isotype-specific N-terminal sequence and thus prevents the cAMP transduction mechanism by acting at the interface between the elbow Clinker, the S4S5-linker, the HCND.*
>
> *Weaknesses:*
>
> *Although the work is interesting, there are some discrepancies between data that need to be addressed.*
>
> *(1) I suggest inserting in Table 1 and in the text, the Δ shift values (+cAMP; + LRMP; +cAMP/LRMP). This will help readers.*

Thank you, Δ shift values have been added to Tables 1 and 2 as suggested.

> *(2) Figure 1 is not clear, the distribution of values is anomalously high. For instance, in 1B the distribution of values of V1/2 in the presence of cAMP goes from - 85 to -115. I agree that in the absence of cAMP, HCN4 in HEK293 cells shows some variability in V1/2 values, that nonetheless cannot be so wide (here the variability spans sometimes even 30 mV) and usually disappears with cAMP (here not).*

With a large N, this is an expected distribution. In 5 previous reports from 4 different groups of HCN4 with cAMP in HEK 293 (Fenske et al., 2020; Liao et al., 2012; Peters et al., 2020; Saponaro et al., 2021; Schweizer et al., 2010), the average expected range of the data is 26.6 mV and 39.9 mV for 95% (mean ± 2*SD) and 99% (mean ± 3*SD) of the data, respectively. As the reviewer mentions the expected range from these papers is slightly larger in the absence of cAMP. The average SD of HCN4 (with/without cAMP) in papers are 9.9 mV (Schweizer et al., 2010), 4.4 mV (Saponaro et al., 2021), 7.6 mV (Fenske et al., 2020), 10.0 mV (Liao et al., 2012), and 5.9 mV (Peters et al., 2020). Our SD in this paper is roughly in the middle at 7.6 mV. This is likely because we used an inclusive approach to data so as not to bias our results (see the statistics section of the revised manuscript on page 9). We have removed 2 data points that meet the statistical classification as outliers, no measures of statistical significance were altered by this.

> *This problem is spread throughout the manuscript, and the measured mean effects are indeed always at the limit of statistical significance. Why so? Is this a problem with the analysis, or with the recordings?*

The exact P-values are NOT typically at the limit of statistical significance, about 2/3rds would meet the stringent P < 0.0001 cut-off. We have clarified in the statistics section (page 10) that any comparison meeting our significance threshold (P < 0.05) or a stricter criterion is treated equally in the figure labelling. Exact P-values are provided in Tables 1-3.

> *There are several other problems with Figure 1 and in all figures of the manuscript: the Y scale is very narrow while the mean values are marked with large square boxes. Moreover, the exemplary activation curve of Figure 1A is not representative of the mean values reported in Figure 1B, and the values of 1B are different from those reported in Table 1.*

Y-axis values for mean plots were picked such that all data points are included and are consistent across all figures. They have been expanded slightly (-75 to -145 mV for all HCN4 channels and -65 to -135 mV for all HCN2 channels). The size of the mean value marker has been reduced slightly. Exact midpoints for all data are also found in Tables 1-3.

The GV curves in Figure 1B (previously Fig. 1A) are averages with the ±SEM error bars smaller than the symbols in many cases owing to relatively high n's for these datasets. These curves match the midpoints in panel 1C (previously 1B). Eg. the midpoint of the average curve for HCN4 control in panel A is -117.9 mV, the same as the -117.8 mV average for the individual fits in panel B.

We made an error in the text based on a previous manuscript version about the ordering of the tables that has now been fixed so these values should now be aligned.

> *On this ground, it is difficult to judge the conclusions and it would also greatly help if exemplary current traces would be also shown.*

Exemplary current traces have been added to all figures in the revised manuscript.

> *(3) "....HCN4-P545A/T547F was insensitive to LRMP (Figs. 6B and 6C; Table 1), indicating that the unique HCN4 C-linker is necessary for regulation by LRMP. Thus, LRMP appears to regulate HCN4 by altering the interactions between the C-linker, S4-S5 linker, and Nterminus at the cAMP transduction centre."*
>
> *Although this is an interesting theory, there are no data supporting it. Indeed, P545 and T547 at the tip of the C-linker elbow (fig 6A) are crucial for LRMP effect, but these two residues are not involved in the cAMP transduction centre (interface between HCND, S4S5 linker, and Clinker elbow), at least for the data accumulated till now in the literature. Indeed, the hypothesis that LRMP somehow inhibits the cAMP transduction mechanism of HCN4 given the fact that the two necessary residues P545 and T547 are close to the cAMP transduction centre, remains to be proven.*
>
> *Moreover, I suggest analysing the putative role of P545 and T547 in light of the available HCN4 structures. In particular, T547 (elbow) points towards the underlying shoulder of the adjacent subunit and, therefore, is in a key position for the cAMP transduction mechanism. The presence of bulky hydrophobic residues (very different nature compared to T) in the equivalent position of HCN1 and HCN2 also favours this hypothesis. In this light, it will be also interesting to see whether a single T547F mutation is sufficient to prevent the LRMP effect.*

We agree that testing this hypothesis would be very interesting. However, it is challenging. Any mutation we make that is involved in cAMP transduction makes measuring the LRMP effect on cAMP shifts difficult or impossible.

Our simple idea, now clarified in the discussion, is that if you look at the regions involved in cAMP transduction (HCND, C-linker, S4-S5), there are very few residues that differ between HCN4 and HCN2. When we mutate the 5 non-conserved residues in the S5 segment and the C-linker, along with the NT, we are able to render HCN2 sensitive to LRMP. Therefore, something about the small sequence differences in this region confer isoform specificity to LRMP. We speculate that this happens because of small structural differences that result from those 5 mutations. If you compare the solved structures of HCN1 and HCN4 (there is no HCN2 structure available), you can see small differences in the distances between key interacting residues in the transduction centre. Also, there is a kink at the bottom of the S4 helix in HCN4 but not HCN1. This points a putatively important residue for cAMP dependence in a different direction in HCN4. We hypothesize in the discussion that this may be how LRMP is isoform specific.

Moreover, previous work has shown that the HCN4 C-linker is uniquely sensitive to di-cyclic nucleotides and magnesium ions. We are hypothesizing that it is the subtle change in structure that makes this region more prone to regulation in HCN4.

> ***Reviewing Editor (recommendations for the Authors):***
>
> *(1) Exemplar recordings need to be shown and some explanation for the wide variability in the V-half of activation.*

Exemplar currents are now shown for each channel. See the response to Reviewer 3's public comment 2.

> *(2) The rationale for cut sites in LRMP for the investigation of which parts of the protein are important for blocking the effect of cAMP is not logically presented in light of the modular schematics of domains in the protein (N-term, CCD, post-CCD, etc).*

There is limited structural data on LRMP and the HCN4 N-terminus. The cut sites in this paper were determined empirically. We made fragments that were small enough to work for our FRET hybridization approach and that expressed well in our HEK cell system. The residue numbering of the LRMP modules is based on updated structural predictions using Alphafold, which was released after our fragments were designed. This has been clarified in the methods section on pages 5-6 and the Figure 2 legend of the revised manuscript.

> *(3) Role of the HCN4 C-terminus. Truncation of the HCN4 C-terminus unstructured Cterminus distal to the CNBD (Fig. 4 A, B) partially reverses the impact of LRMP (i.e. there is now a significant increase in cAMP effect compared to full-length HCN4). The manuscript is written in a manner that minimizes the potential role of the C-terminus and it is, therefore, eliminated from consideration in subsequent experiments (e.g. FRET) and the discussion. The model is incomplete without considering the impact of the C-terminus.*

We thank the reviewer for this comment as it was a result that we too readily dismissed. We have added discussion around this point and revised our model to suggest that not only can we not eliminate a role for the distal C-terminus, our data is consistent with it having a modest role. Our HCN4-2 chimera and HCN4-S719x data both suggest the possibility that the distal C-terminus might be having some effect on LRMP regulation. We have clarified this in the results (pages 12-13) and discussion (page 19).

> *(4) For FRET experiments, it is not clear why LF should show an interaction with N2 (residues 125-160) but not NF (residues 1-160). N2 is contained within NF, and given that Citrine and Cerulean are present on the C-terminus of LF and N2/NF, respectively,*

> *residues 1-124 in NF should not impact the detection of FRET because of greater separation between the fluorophores as suggested by the authors.*

This is a fair point but FRET is somewhat more complicated. We do not know the structure of these fragments and it's hard to speculate where the fluorophores are oriented in this type of assay. Moreover, this hybridization assay is sensitive to affinity and expression as well. There are a number of reasons why the larger 1-260 fragment might show reduced FRET compared to 125-260. As mentioned in our response to reviewer 2's public comment 2, we have added a limitation section that outlines the various caveats of FRET that could explain this.

> *(5) For FRET experiments, the choice of using pieces of the channel that do not correlate with the truncations studied in functional electrophysiological experiments limits the holistic interpretation of the data. Also, no explanation or discussion is provided for why LRMP fragments that are capable of binding to the HCN4 N-terminus as determined by FRET (e.g. residues 1-108 and 110-230, respectively) do not have a functional impact on the channel.*

As mentioned in the response to comment 2, the exact fragment design is a function of which fragments expressed well in HEK cells. Importantly, because FRET experiments do not provide atomic resolution for the caveats listed in the revised limitations section on page 20-21, small differences in the cut sites do not change the interpretation of these results. For example, the N-terminal 1-125 construct is analogous to experiments with the Δ1-130 HCN4 channel.

We suspect that residues in both fragments are required and that the interaction involves multiple parts. This is stated in the results "Thus, the first 227 residues of LRMP are sufficient to regulate HCN4, with residues in both halves of the LRMP N-terminus necessary for the regulation" (page 11). We have also added discussion on this on page 21.

> *(6) A striking result was that mutating two residues in the C-linker of HCN4 to amino acids found in HCN channels not affected by LRMP (P545A, T547F), completely eliminated the impact of LRMP on preventing cAMP regulation of channel activation. However, a chimeric channel, (HCN4-2) in which the C-linker, the CNBD, and the C-terminus of HCN4 were replaced by that of HCN2 was found to be partially responsive to LRMP. These two results appear inconsistent and not reconciled in the model proposed by the authors for how LRMP may be working.*

As stated in our answer to your question #3, we have revised our interpretation of these data. If the more distal C-terminus plays some role in the orientation of the C-linker and the transduction centre as a whole, these data can still be viewed consistent with our model. We have added some discussion of this idea in our discussion section.

> *(7) Replacing the HCN2 N-terminus with that from HCN4, along with mutations in the S5 (MCS/VVG) and C-linker (AF/PT) recapitulated LRMP regulation on the HCN2 background. The functional importance of the S5 mutations is not clear as no other experiments are shown to indicate whether they are necessary for the observed effect.*

We have added our experiments on a midpoint HCN2 clone that includes the S5 mutants and the C-linker mutants in the absence of the HCN4 N-terminus (ie HCN2 MCSAF/VVGPT) (Fig. 7). And we have discussed our rationale for the S5 mutations as we believe they may be responsible for the different orientations of the S4-S5 linker in HCN1 and HCN4 structures that are known to impact cAMP regulation.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *A) Comments:*
>
> *(1) Figure 1: Please show some representative current traces.*

Exemplar currents are now shown for each channel in the manuscript.

> *(2) Figure 1: There appears to be a huge number of recordings for HCN4 +/- cAMP as compared to those with LRMP 1-479Cit. How was the number of recordings needed for sufficient statistical power decided? This is particularly important because the observed slowing of deactivation by cAMP in Fig. 1C seems like it may be fairly subtle. Perhaps a swarm plot would make the shift more apparent? Also, LRMP 1-479Cit distributions in Fig. 1B-C look like they are more uniform than normal, so please double-check the appropriateness of the statistical test employed.*

We have revised the methods section (page 7) to discuss this, briefly we performed regular control experiments throughout this project to ensure that a normal cAMP response was occurring. Our minimum target for sufficient power was 8-10 recordings. We have expanded the statistics section (page 9) to discuss tests of normality and the use of a log scale for deactivation time constants which is why the shifts in Fig. 1D (revised) are less apparent.

> *(3) It would be helpful if the authors could better introduce their logic for the M338V/C341V/S345G mutations in the HCN4-2 VVGPT mutant.*

See response to the reviewing editor's comment 7.

> *B) Minor Comments:*
>
> *(1) pg. 9: "We found that LRMP 1-479Cit inhibited HCN4 to an even greater degree than the full-length LRMP, likely because expression of this tagged construct was improved compared to the untagged full-length LRMP, which was detected by co-transfection with GFP." Co-transfection with GFP seems like an extremely poor and a risky measure for LRMP expression.*

We agree that the exact efficiency of co-transfection is contentious although some papers and manufacturer protocols indicate high co-transfection efficiency (Xie et al., 2011). In this paper we used both co-transfection and tagged proteins with similar results.

> *(2) pg 9: "LRMP 1-227 construct contains the N-terminus of LRMP with a cut-site near the Nterminus of the predicted coiled-coil sequence". In Figure 2 the graphic shows the coiledcoil domain starting at 191. What was the logic for splitting at 227 which appears to be the middle of the coiled-coil?*

See response to the reviewing editor's comment 2.

> *(3) Figure 5C: Please align the various schematics for HCN4 as was done for LRMP. It makes it much easier to decipher what is what.*

Fig. 5 has been revised as suggested.

> *(4) pg 12: I assume that the HCN2 fragment chosen aligns with the HCN4 N2 fragment which shows binding, but this logic should be stated if that is the case. If not, then how was the HCN2 fragment chosen?*

This is correct. This has been explicitly stated in the revised manuscript (page 14).

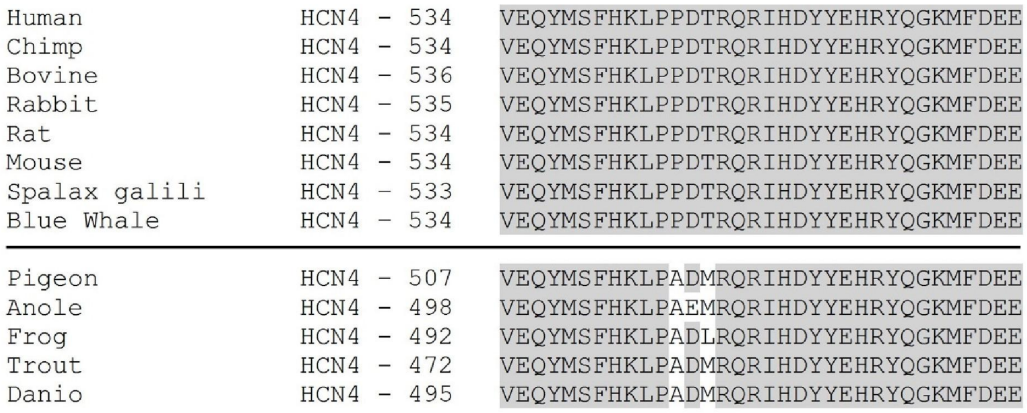
> *(5) Figure 7: Add legend indicating black/gray = HCN4 and blue = HCN2.*

This has been stated in the revised figure legend.

> *(6) pg 17: Conservation of P545 and T547 across mammalian species is not shown or cited.*

This sentence is not included in the revised manuscript, however, for the interest of the reviewer we have provided an alignment of this region across species here.

**Author response image 1.**




> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) It is not clear whether in the absence of cAMP, LRMP also modestly shifts the voltagedependent activity of the channels. Please clarify.*

We have clarified that LRMP does not shift the voltage-dependence in the absence of cAMP (page 10). In the absence of cAMP, LRMP does not significantly shift the voltagedependence of activation in any of the channels we have tested in this paper (or in our prior 2020 paper).

> *(2) Resolution of Fig. 8b is low.*

We ultimately decided that the cartoon did not provide any important information for understanding our model and it was removed.

> *(3) Please add a supplementary figure showing the amino acid sequence of LRMP to show where the demarcations are made for each fragment as well as where the truncations were made as noted in Fig 3 and Fig 4.*

A new supplementary figure showing the LRMP sequence has been added and cited in the methods section (page 5). Truncation sites have been added to the schematic in Fig. 2A.

> *(4) In the cartoon schematic illustration for Fig. 3 and Fig.4, the legend should include that the thick bold lines in the C-Terminal domain represent the CNBD, while the thick bold lines in the N-Terminal domain represent the HCN domain. This was mentioned in Liao 2012, as you referenced when you defined the construct S719X, but it would be nice*

> *for the reader to know that the thick bold lines you have drawn in your cartoon indicate that it also highlights the CNBD or the HCN domain.*

This has been added to figure legends for the relevant figures in the revised manuscript.

> *(5) On page 12, missing a space between "residues" and "1" in the parenthesis "...LRMP L1 (residues1-108)...".*

Fixed. Thank you.

> *(6) Which isoform of LRMP was used? What is the NCBI accession number? Is it the same one from Peters 2020 ("MC228229")?*

This information has been added to the methods (page 5). It is the same as Peters 2020.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) "Truncation of residues 1-62 led to a partial LRMP effect where cAMP caused a significant depolarizing shift in the presence of LRMP, but the activation in the presence of LRMP and cAMP was hyperpolarized compared to cAMP alone (Fig. 3B, C and 3E; Table 1). In the HCN4Δ1-130 construct, cAMP caused a significant depolarizing shift in the presence of LRMP; however, the midpoint of activation in the presence of LRMP and cAMP showed a non-significant trend towards hyperpolarization compared to cAMP alone (Fig. 3C and 3E; Table 1)".*
>
> *This means that sequence 62-185 is necessary and sufficient for the LRMP effect. I suggest a competition assay with this peptide (synthetic, or co-expressed with HCN4 full-length and LRMP to see whether the peptide inhibits the LRMP effect).*

We respectfully disagree with the reviewer's interpretation. Our results, strongly suggest that other regions such as residues 25-65 (Fig. 3C) and C-terminal residues (Fig. 6) are also necessary. The use of a peptide could be an interesting future experiment, however, it would be very difficult to control relative expression of a co-expressed peptide. We think that our results in Fig. 7E-F where this fragment is added to HCN2 are a better controlled way of validating the importance of this region.

> *(2) "Truncation of the distal C-terminus (of HCN4) did not prevent LRMP regulation. In the presence of both LRMP and cAMP the activation of HCN4-S719X was still significantly hyperpolarized compared to the presence of cAMP alone (Figs. 4A and 4B; Table 1). And the cAMP-induced shift in HCN4-S719X in the presence of LRMP (~7mV) was less than half the shift in the absence of LRMP (~18 mV)."*
>
> *On the basis of the partial effects reported for the truncations of the N-terminus of HCN4 162 and 1-130 (Fig 3B and C), I do not think it is possible to conclude that "truncation of the distal C-terminus (of HCN4) did not prevent LRMP regulation". Indeed, cAMP-induced shift in HCN4 Δ1-62 and Δ1-130 in the presence of LRMP were 10.9 and 10.5 mV, respectively, way more than the ~7mV measured for the HCN4-S719X mutant.*
>
> *As you rightly stated at the end of the paragraph:" Together, these results show significant LRMP regulation of HCN4 even when the distal C-terminus is truncated, consistent with a minimal role for the C-terminus in the regulatory pathway". I would better discuss this minimal role of the C-terminus. It is true that deletion of the first 185 aa of HCN4 Nterminus abolishes the LRMP effect, but it is also true that removal of the very Cterm of HCN4 does affect LRMP. This unstructured C-terminal region of HCN4 contains isotype-specific sequences. Maybe they also play a role in recognizing LRMP.*

> *Thus, I would suggest further investigation via truncations, even internal deletions of HCN4-specific sequences.*

Please see the response to the reviewing editor's comment 3.

> *(3) Figure 5: The N-terminus of LRMP FRETs with the N-terminus of HCN4.*
>
> *Why didn't you test the same truncations used in Fig. 3? Indeed, based on Fig 3, sequences 1-25 can be removed. I would have considered peptides 26-62 and 63-130 and 131-185 and a fourth (26-185). This set of peptides will help you connect binding with the functional effects of the truncations tested in Fig 3.*

Please see the response to the reviewing editor's comment 2 and 5.

> *Why didn't you test the C-terminus (from 719 till the end) of HCN4? This can help with understanding why truncation of HCN4 Cterminus does affect LRMP, tough partially (Fig. 4A).*

Please see the response to the reviewing editor's comment 3.

> *(4) "We found that a previously described HCN4-2 chimera containing the HCN4 N-terminus and transmembrane domains (residues 1-518) with the HCN2 C-terminus (442-863) (Liao et al., 2012) was partially regulated by LRMP (Fig. 7A and 7B)".*
>
> *I do not understand this partial LRMP effect on the HCN4-2 chimera. In Fig. 6 you have shown that the "HCN4-P545A/T547F was insensitive to LRMP (Figs. 6B and 6C; Table 1), indicating that the unique HCN4 C-linker is necessary for regulation by LRMP". How can be this reconciled with the HCN4-2 chimera? HCN4-2, "containing" P545A/T547F mutations, should not perceive LRMP.*

Please see the response to the reviewing editor's comment 6.

> *(5) "we next made a targeted chimera of HCN2 that contains the distal HCN4 N-terminus (residues 1-212) and the HCN2 transmembrane and C-terminal domains with 5 point mutants in non-conserved residues of the S5 segment and C-linker elbow (M338V/C341V/S345G/A467P/F469T).....Importantly, the HCN4-2 VVGPT channel is insensitive to cAMP in the presence of LRMP (Fig. 7C and 7D), indicating that the HCN4 Nterminus and cAMP-transduction centre residues are sufficient to confer LRMP regulation to HCN2".*
>
> *Why did you insert also the 3 mutations of S5? Are these mutations somehow involved in the cAMP transduction mechanism?*
>
> *You have already shown that in HCN4 only P545 and T547 (Clinker) are necessary for LRMP effect. I suggest to try, at least, the chimera of HCN2 with only A467P/F469T. They should work without the 3 mutations in S5.*

Please see the response to the reviewing editor's comment 7.