**Structural mechanism of two gain-of-function cardiac and skeletal RyR mutations at an equivalent site by cryo-EM**

Kavita A. Iyer¹, Yifan Hu¹, Ashok R. Nayak¹, Nagomi Kurebayashi², Takashi Murayama², Montserrat Samsó¹*

Mutations in ryanodine receptors (RyRs), intracellular Ca\(^{2+}\) channels, are associated with deadly disorders. Despite abundant functional studies, the molecular mechanism of RyR malfunction remains elusive. We studied two single-point mutations at an equivalent site in the skeletal (RyR1 R164C) and cardiac (RyR2 R176Q) isoforms using ryanodine binding, Ca\(^{2+}\) imaging, and cryo–electron microscopy (cryo-EM) of the full-length protein. Loss of the positive charge had greater effect on the skeletal isoform, mediated via distortion of a salt bridge network, a molecular latch inducing rotation of a cytoplasmic domain, and partial progression to open-state traits of the large cytoplasmic assembly accompanied by alteration of the Ca\(^{2+}\) binding site, which concur with the major “hyperactive” feature of the mutated channel. Our cryo-EM studies demonstrated the allosteric effect of a mutation situated ~85 Å away from the pore and identified an isoform-specific structural effect.

**INTRODUCTION**

Ryanodine receptors (RyRs) are large (2.26 MDa) intracellular Ca\(^{2+}\) channels expressed on the endoplasmic reticulum (ER) that play an integral role in excitation–contraction (EC) coupling in skeletal muscle and heart (1–3). More than 300 RyR mutations cause a number of debilitating or fatal disorders, most of them resulting in gain-of-function phenotype. Many mutations in RyR1 cause malignant hyperthermia (MH) triggered by inhalation of volatile anesthetics as well as central core disease (CCD) myopathy due to enhanced ER Ca\(^{2+}\) leakage in skeletal muscle (4–7). In heart, mutations in RyR2 increase the propensity for spontaneous Ca\(^{2+}\) release and cause catecholaminergic polymorphic tachycardia (CPVT) (8) and arrhythmogenic right ventricular dysplasia type 2 (ARVD2) (9). Despite the existence of ample functional evidence of the effect of mutations on RyR channel activity, their structural study lags behind and has been limited to fragments smaller than 3% the size of the entire RyR (10, 11).

Here, we studied the effect of mutations R163C (R164C in rabbit) in RyR1 and R176Q in RyR2, both in the N-terminal domain A (NTDA; residues 1 to 217) located in a highly conserved region of the large cytoplasmic assembly (CytA), using a combination of structural analysis by cryo–electron microscopy (cryo-EM) and functional analysis by Ca\(^{2+}\) imaging and \(^{3}\rm{H}\)ryanodine binding (\(^{3}\rm{H}\)Ry). These single–amino acid mutations in RyR1 and RyR2 represent a loss of a positive charge at the equivalent position in sequence and 3D structure and have been implicated in MH/CCD (12–14), CPVT* (15), and ARVD2 (16). Our cryo-EM studies, conducted under closed-state conditions, revealed that the mutation, despite consisting of an equivalent loss of positive charge, had an isoform-specific effect, on the structure of RyR, with the RyR1 R164C mutant displaying an altered, preactivated conformation.

**RESULTS**

**Effect of the mutations in vivo and in vitro**

We carried out functional studies to assess that the recombinantly expressed RyR channels were functional and had a gain-of-function phenotype aligned with the typical characteristics described previously for these mutants in more native environments (4–7, 15) and to compare quantitatively the two isoforms on the same background. We used RyR-expressing human embryonic kidney (HEK) 293 cells as a model to study RyR activity as previously reported (17, 18). Live-cell Ca\(^{2+}\) imaging on HEK293 cells expressing mutant RyR1 and RyR2 revealed that mutations R164C and R176Q resulted in a significant depletion of Ca\(^{2+}\) in the ER by enhanced Ca\(^{2+}\) release when compared to their wild-type (WT) counterparts (Fig. 1, A to D). In addition, RyR2 R176Q cells showed higher frequency of spontaneous Ca\(^{2+}\) oscillations of lower amplitude compared to WT RyR2 (Fig. 1, B and E).

\(^{3}\rm{H}\)Ry binding is directly related to the channel’s probability of opening (19) and often used as a means to assess the channel’s functionality. We report a different profile of \(^{3}\rm{H}\)Ry binding between WT and mutant RyR1 and RyR2 over a wide range of Ca\(^{2+}\) concentrations, which reveals that the mutation confers significantly higher channel activity than WT. Whereas RyR2 R176Q exhibits a ~30% increased peak activity, RyR1 R164C shows a threefold increase in the peak activity with an enhanced sensitivity to Ca\(^{2+}\) activation (Fig. 1, F and G). The calculated channel activity (17, 18) at resting [Ca\(^{2+}\)] of pCa 7 was ~15- and ~2-fold higher for mutant RyR1 and RyR2, respectively, than WT (Fig. 1, H and I). Thus, both mutations cause gain of function of the channel, with RyR1 R164C exhibiting greater effect than RyR2 R176Q.

**Choice of cryo-EM conditions and 3D reconstruction of RyRs**

Our underlying hypothesis is that in the context of RyR’s large size (2.2 MDa or ~20,000 residues), the mutations situated ~85 Å from the pore and ~50 Å away from the Ca\(^{2+}\) binding site must exert their effect allosterically, and that any structural anomalies leading to a conformation prone to hyperactivity should be more evident in the closed state. In addition, one important determinant of attainable

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¹Department of Physiology and Biophysics, School of Medicine, Virginia Commonwealth University, Richmond, VA, USA. ²Department of Cellular and Molecular Pharmacology, Juntendo University Graduate School of Medicine, Tokyo, Japan.  *Corresponding author. Email: montserrat.sams@vcuhealth.org
resolution in a 3D reconstruction is conformational homogeneity of the dataset. Thus, to attain the best resolution possible, we determined the 3D reconstructions of the RyR under Ca\(^{2+}\)-free conditions ensuring the closed state [because channel activation results in rapid flickering of RyR between closed and open conformations (20)] and in complex with its subunit FK506-binding protein (FKBP) of 12.6 kDa, also known to stabilize the closed state (21, 22). For readability, the structures of the RyR1-FKBP12.6 and RyR2-FKBP12.6 complex (the skeletal and cardiac muscle isoforms, respectively) reported in this paper are referred to as RyR1 or RyR2. As the WT counterpart for our comparative studies, we use the structure for WT RyR2-FKBP12.6 solved here.

Recombinant mutant rabbit RyR1 and mutant mouse RyR2 were expressed and isolated from HEK293 cells to ensure homogeneity, with the method of protein purification being either affinity chromatography or sucrose gradient (see Materials and Methods; fig. S5, E and F). On one hand, continuous presence of FKBP throughout the purification seemed to improve HD2 integrity as we suggested earlier (26): mutant RyR2 maps obtained here, the entire HD2 is well resolved, with its C-terminal end interacting with the SPRY2 of the neighboring subunit similar to what is customarily observed in RyR1 (fig. S5, D to F). In the present study, we were able to trace de novo the backbone Cα atoms of large portions of the HD2 for the RyR2 R176Q and RyR2 WT structures.

The better resolution of the HD2 domain does not appear to be related to the different species because sequence alignment of porcine and mouse RyR2 reveal ~92% sequence identity (4553 and 141 identical and similar positions overall, and 526 and 18 identical and similar position in the HD2, respectively) with very few stretches of dissimilarity belonging to areas not solved to atomic resolution yet. We attribute the better structural preservation of HD2 to reduced posttranslational modification in our case, because RyRs were expressed in HEK293 cells, and to different purification procedures. The presence of FKBP throughout the purification seemed to improve HD2 integrity as we suggested earlier (26): mutant RyR2 R176Q necessitated collection of two datasets to achieve high resolution, with the method of protein purification being either affinity chromatography or sucrose gradient (see Materials and Methods; fig. S5, E and F). On one hand, continuous presence of FKBP in the
case of affinity chromatography resulted in better preservation of HD2. Nevertheless, the higher yield of sucrose gradients (~4 to 5 versus ~1 mg/ml), with supplementation of FKBP before vitrification, enabled eliminating the carbon support of the cryo-EM grids, which boosted the overall resolution from 4.8 to 3.3 Å (final structure).

**Mutant RyR1 induced unfurling of the CytA**

Channel opening of RyRs exhibit an “unfurling” of the four identical subunits akin to a flower’s petals opening up, first reported by Samsó *et al.* (20) for RyR1 and later confirmed at near-atomic resolution for RyR1 (23, 24) and RyR2 (27). The unfurling involves upward and centrifugal separation of the NTDs in the CytA’s inner ring and a downward movement of the CytA’s corner domains—HD1, HD2, SPRY1, SPRY3, and P1—toward the ER membrane. The three NTD subdomains (A, B, and C) of a single subunit move as an entity during the unfurling, with the distance between adjoining NTDA and NTDB of neighboring subunits increasing during channel opening, to different extents in WT RyR1 and RyR2 (6.5 and 3.3 Å, respectively). While this movement is pronounced when the channel opens, a similar movement of a lesser magnitude has been reported for the “primed” state (23) and for closed RyR1 without FKBP12 (22). In RyR1 R164C, the distance between adjoining NTDB and NTDA+ (“+” refers to the neighboring subunit in the anticlockwise direction as seen from the cytoplasm) increased by 1 Å, indicative of partial unfurling of the CytA of the channel (fig. S6, A to C, and movie S1). This was accompanied by a noticeable rocking of NTDA by 6° (Fig. 2A and movie S1), which is almost comparable to the 8° rocking observed during WT channel opening (Fig. 2B).

![Fig. 2. The flexion angle indicates partial unfurling of the CytA in RyR1 R164C.](image)

(A and B) The NTDA of RyR1 R164C (yellow) undergoes a 6° rocking with respect to WT closed RyR1 (green). This is not far from the 8° rocking observed during channel opening (closed and open WT RyR1 in green and orange, respectively). (C) The plot of flexion angle of multiple RyR structures including ours indicates that the closed and open structures have a positive and negative flexion angle, respectively, for both isoforms. PDB codes are provided in the plot. The presence of FKBP, which stabilizes RyR flexion angle, is indicated. The CytA of RyR1 R164C adopts a conformation that was so far only seen in the presence of activators Ca2+ with ATP and caffeine [PDB: 5T9V (23)] or absence of FKBP [PDB: 4UWA (61)]. (D) The flexion angle (thick blue line) measures the downward movement of the CytA with respect to the ER membrane and correlates with opening and priming. For RyR1 R164C (left), RyR2 R176Q (middle), and RyR2 WT (right), the values were −0.5°, +0.3°, and 0°, respectively, indicating that mutant RyR1 had a conformation between closed and open states, while mutant and WT RyR2 were closed.
On the other hand, there was no measurable change for RyR2 R176Q in the NTDA⁺/NTDB distance (fig. S6, D to F), and the NTDA did not appear to change in orientation.

A measure of the CytA downward movement is the flexion angle (22), which reflects the tilting of each quadrant (formed by the NTDs, SPRY1, SPRY3, P1, and HD1 and HD2 domains) with respect to the ER membrane plane (fig. S1, F and G). The R164C mutation caused RyR1 under closed-state conditions to adopt a CytA conformation that was obtained in the presence of either activators [Ca²⁺ or Ca²⁺ with adenosine triphosphate (ATP) and caffeine (23)] or lack of FKBP, as suggested earlier for other RyR mutants (21). The differential effect of an NTDA mutation in RyR1 and RyR2 suggests that although the mutation neutralizes an Arg at equivalent position, its effect on the channel is further modulated by the nature of the isoforms. The arrow indicates the equivalent Arg residue that is mutated to Cys (C) in RyR1 and to Gln (Q) in RyR2. (Inset images show a zoomed-in view of the NTDA⁺/CD interface, with the NTDA⁺:β8-β9 loop shown in black. A distinct "hook"-like density of R3984 and R3938 is observed in RyR1 R164C (green) and RyR2 R176Q (salmon). The R3984-G160 interaction acts as a "molecular latch" in RyR1 R164C and helps to stabilize a new relative position of NTDA⁺ and CD and appears to hinder the return to the typical closed-state configuration of this domain pair. (C) Overview showing both NTDA⁺/CD and CD/CTD interfaces in RyR1 and to Gln (Q) in RyR2. (B) Inset images show a zoomed-in view of the NTDA⁺/CD interface, with the NTDA⁺:β8-β9 loop shown in black. A distinct "hook"-like density of R3984 and R3938 is observed in RyR1 R164C (green) and RyR2 R176Q (salmon), respectively. The R3984-G160 interaction acts as a "molecular latch" in RyR1 R164C and helps to stabilize a new relative position of NTDA⁺ and CD and appears to hinder the return to the typical closed-state configuration of this domain pair. (C) Overview showing both NTDA⁺/CD and CD/CTD interfaces in RyR1 R164C (green) and RyR2 R176Q (salmon), respectively. (D) Images with emphasis on the Ca²⁺ binding site indicate alteration of the site observed in RyR1 R164C (green) resulting from a decrease in the T5001-E3893 distance to 3.4 Å (from 4.9 Å in closed RyR1), potentially inducing a better fit of the Ca²⁺ ion. In contrast, this is not observed in the RyR2 counterpart. (E) Simple schematic explaining the long-range effect that the NTDA mutation (*) has on HD2 (magenta), CD (green), and CTD (tan), resulting in downward rocking of the distal HD2 (a trait associated with the open-state conformation (22) and shrinking of the Ca²⁺ binding site (#), which facilitates activation (31)). These changes could alter the energy landscape and explain the increased probability of opening.

**The NTDA⁺/CD interface of mutant RyR1 plays a pivotal role in hyperactivation**

To account for the mechanism underlying the 1 Å shift and 6° rocking of NTDA in mutant RyR1, we examined closely the NTDA⁺/CD interaction, focusing on the highly conserved NTDA⁺:β8-β9 loop (Figs. 3 and 4), which contains the mutation and is closest to the CD, a domain that interacts extensively with NTDA⁺, NTDB, NTDC, HD1, CTD, VSD, Handle, U-motif, and EF-hand, and is a key player in channel activity (fig. S1, F and G, and movie S1) (24). We observed a number of distinct features in this region.

1) A distinct hook-like density originating from R3984 in the CD was very pronounced in the mutant map when compared to the closed and open RyR1 (23) WT maps, which spanned across to the NTDA⁺ (Fig. 3B). In the cardiac isoform, although the equivalent residue R3938 formed a distinct hook density when compared to WT RyR2, there was no bridge of density reaching the NTDA⁺ (Fig. 3B), which agrees with our observation that the overall CytA conformation was basically unchanged. The hook-like density in mutant RyR1 is accounted for by an H-bond interaction between R3984 (CD) and G160 (NTDA⁺:β8-β9 loop), which appears to play a direct role in stabilizing the NTDA⁺/CD orientation (Figs. 3B and 4A and movie S1). This could be a consequence of a more favorable orientation of the β8-β9 loop/CD interface caused by rocking.

2) The β8-β9 loop of mutant RyR2 shows a distinct density for the side chain of R169 that appears to reach the mutated Q176 (Figs. 3B and 4C and movie S2). Contrary to this, in the mutant RyR1 structure, the density for the side chain of the equivalent R157 does not reach the mutated C164 (Figs. 3B and 4D and movie S1). This suggests that the shorter mutated residue might play a greater effect (Fig. 4, C to F) such that going from Arg to Cys (in RyR1)
becomes more determinant in abolishing the H-bond, whereas the change from Arg to Gln (in RyR2) may retain the ability to H-bond with R169 to some degree. Mutations RyR1:R157 (human RyR1:R156K) and RyR2:R169 have been shown to cause MH (28) and bidirectional ventricular tachycardia (29), respectively. This is in agreement with an intricate network of interactions within the β8-β9 loop, with an H-bond between R164-R157 in RyR1 and R176-R169 in RyR2.

3) In addition, we observe a bulge in NTDA+:β8-β9:166-168, along with a 0.4-Å dilation of the loop (Fig. 4, G and H), which could be accounted for by the loss of interaction between the two Arg as indicated above. The displacement of this bulge toward the β9 sheet correlates with the direction of the rocking observed for the entire NTDA+. All three observations converge on the idea that the mutation, by altering the interaction network of the β8-β9 loop and inducing a rocking of the NTDA+ domain, forms a molecular latch that stabilizes a different conformation of the NTDA+/CD domains.

To validate our observations from cryo-EM, we carried out two mutations: RyR1 R164Q and RyR2 R176C. The reasoning for selecting these mutations was to mimic the mutation of the other isoform and observe effects on gain-of-function magnitude. If size of the mutated residue was truly relevant, then mutating Arg to Gln would have a less detrimental effect on the channel as compared to an Arg to Cys mutation. Results from the [3H]Ry binding experiments were in agreement with this hypothesis, with the binding profile of RyR1 R164Q being slightly enhanced compared to that of WT RyR1 across the entire range of Ca2+ concentrations at which RyR1 R164C had a significantly enhanced binding profile (Fig. 5A). On the other hand, for the cardiac isoform, mutation to the smaller Cys residue resulted in a significantly enhanced [3H]Ry binding profile as compared to both WT and RyR2 R176Q (Fig. 5B). At pCa 7, RyR1 R164Q displayed less increased sensitivity to activation by Ca2+ (CICR) than RyR1 R164C (~3-fold versus ~15-fold, respectively, compared to WT; Fig. 5C), while the RyR2 R176C mutant displayed increased sensitivity to Ca2+ activation with respect to RyR2 R176Q (~4-fold and ~2-fold, respectively, compared to WT; Fig. 5D). Ca2+ release activity, as assessed by [Ca2+]ER, was less marked in RyR1 R164Q compared to RyR1 R164C (Figs. 1A and 5, E and G). Likewise, RyR2 expressed in HEK293 cells, showing the typical RyR2-induced spontaneous Ca2+ oscillations, had more enhanced Ca2+ release in RyR2 R176C than in RyR2 R176Q, as assessed by [Ca2+]ER threshold for Ca2+ release (Figs. 1B and 5, F and H) and Ca2+ oscillation frequency (Fig. 5I). Values were statistically significant for all live Ca2+ imaging comparisons (Fig. 5, G and H). All our functional
data—[^3]H[Ry binding and Ca^{2+} imaging—successfully validated our hypothesis that the size of the mutated residue at this position plays a direct role in the degree of hyperactivation.

**The Ca^{2+} binding site of mutant RyR1 is altered**

The disruption of the NTDA/CD interaction is accompanied by an upward movement of the CTD in mutant RyR1, which, in turn, alters the Ca^{2+} binding site. In both isoforms, the Ca^{2+} binding site (23, 27, 30, 31) consists of key negative residues in the CTD and CD that reorient to coordinate the Ca^{2+} ion causing channel opening. In RyR1 R164C, the upward movement of the CTD brings the backbone carbonyl of T5001 closer to the side-chain carbonyls of E3893 and E3967 of the CD [Fig. 3D and movie S1; T5001-E3893 distance of 3.4 Å compared to 4.9 and 1.7 Å in closed and open WT RyR1 (23), respectively; fig. S7, A to C]. It is notable that a single-point mutation in the NTDA of RyR1 affects the Ca^{2+} binding site under closed-state conditions, which might explain the increased sensitivity of the channel to Ca^{2+} observed previously (17) and in our functional studies (Fig. 1). In contrast, RyR2 R176Q did not show any shrinking of the Ca^{2+} binding site as compared to WT closed RyR2 (Fig. 3D and fig. S7, D to F). Of note, our functional studies show that while RyR1 R164C has a ~15-fold increased CICR, RyR2 R176Q displays only a ~2-fold increase (Fig. 1, H and I) at pCa 7, the condition used for our cryo-EM reconstructions. This, along with the significantly greater[^3]H[Ry binding of RyR1 R164C with respect to RyR1 WT at submicromolar Ca^{2+} as compared to similar binding of[^3]H[Ry to RyR2 R176Q and RyR2 WT (Fig. 1, F and G), is compatible with the structure-based observation that the RyR1 R164C mutation has a greater effect on channel conformation than the equivalent RyR2 R176Q mutation.

**Mutant RyR1 exhibits a closed-dilated pore under closed-state conditions**

Detailed analysis of the pore revealed an increased radius from 0.8 Å (PDB: 5TB0) to ~1.2 Å at the gating residue, I4937, in going from WT to R164C (fig. S8, A to C). Related to this slight separation, ~10 Å away from the gating residue, R4944, also in the S6 helix, has a distinct loop-like density in the RyR1 R164C map, with its distal N atom interacting with the O atom of D4938 in the neighboring helix (distance = 3.3 Å) as in the WT closed state, instead of the backbone of G4941 observed in the WT open state (fig. S8D) (23). These lateral interactions appear to stabilize the S6 helices in the open and closed states, respectively. All this suggests that the S6 helices of R164C, although closed, share some characteristics with the open conformation. While the equivalent change in lateral interactions was also observed for RyR2 R176Q (fig. S8H), the pore remained unchanged with a radius of ~1.0 Å (fig. S8, E to G), in agreement with the emerging pattern of isoform-specific effects.

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**Fig. 5. Validation of cryo-EM–derived models using[^3]H[Ry binding and Ca^{2+} imaging of reciprocal mutations.** Mutation to larger Gln (Q) as compared to smaller Cys (C) from Arg (R) resulted in a[^3]H[Ry binding profile and live Ca^{2+} handling more similar to WT RyR, likely due to better preservation of the putative H-bond network.[^3]H[Ry binding values from Fig. 1 included for easier comparison. (A and B) Ca^{2+}-dependent[^3]H[Ry binding of RyR1 R164C (green) and RyR1 R164Q (blue) and of RyR2 R176Q (red) and RyR2 R176C (blue) with respect to WT counterparts (black). (C and D) Calculated CICR activity of RyR1 R164C (green bar) and RyR1 R164Q (blue bar) and of RyR2 R176Q (red bar) and RyR2 R176C (blue bar) at resting Ca^{2+} (pCa 7) with respect to WT counterparts (empty bar). Data are means ± SD (n = 3). (E and F) Representative traces of [Ca^{2+}]_{cyto} (upper) and [Ca^{2+}]_{ER} (lower) signals determined with G-GECO1.1 and R-CEPIA1er, respectively, for RyR1 WT (black) and RyR1 R164Q (blue) and for RyR2 WT (black) and RyR1 R164C (blue) and for RyR2 WT (black) and RyR1 R164C (blue) and for RyR2 WT (black) and RyR2 R176Q (red bar), and RyR2 R176C (blue bar) expressed in HEK293 cells. Data are means ± SD (n = 67 to 121). ***P < 0.001 versus WT, *P < 0.05 versus WT, ###P < 0.001 versus original disease mutant, and #P < 0.05 versus original disease mutant.
Quantification of local and long-range allosteric effects

Because we observed conformational changes spanning from the NTDA to the TmD (schematics in Fig. 3E), we further assessed whether the long-range allosteric effect in mutant RyR1 was via a deformation of individual domains, via a change in their relative orientation, or both. To this effect, we compared (i) the root mean square deviation (RMSD) between each individual domain (or α helix in the TmD) of mutant RyR1 with the corresponding domains in the closed and open states of WT RyR1 (pairwise 1-domain comparison) and (ii) the RMSD between paired neighboring domains in the mutant and equivalent paired domains in the closed and open states of WT RyR1 (pairwise 2-domain comparison). To assess the mutation-induced conformational changes under closed-state conditions, the RMSDs of the mutant channel with respect to closed and open WT channels were subtracted such that negative values corresponded to greater similarity to open conformation.

The “pairwise 1-domain comparison” revealed that among the CytA domains that differ between open and closed conformations (NTDA, NTDC, CD, EF hands, U motif, CTD, VSD, and TmD), RyR1 R164C’s individual domains show greater resemblance to those in the closed structure (Fig. 6A). Subsequently, the “pairwise 2-domain comparison” revealed greater similarity of most interdomain interfaces to closed WT RyR1 (RMSDs below 1 Å), with some interfaces differing largely from open WT RyR1 (RMSDs between 1 and 4 Å; Fig. 6A). The only exception to this trend was the NTDA+/CD interface, with a difference in RMSD of −0.105 Å, suggesting a conformation more akin to the open conformation. The fact that the pairwise 1-domain comparison of NTDA and CD has positive values compared to negative values for the NTDA+/CD pair suggests that rocking at the interface between these two domains caused by the R164C mutation propagated to the rest of the channel via minor but important rearrangements. Thus, the long-range allosteric change could arise by combination of direct domino effect and compensatory movements due to the linkage of all the domains (Fig. 6B).

DISCUSSION

In the present study, we report the cryo-EM structures of two gain-of-function RyR mutants, RyR1 R164C and RyR2 R176Q, as well as the WT counterpart of RyR2, all in complex with FKBP12.6. For protein purification and functional and structural characterization, we generated stably expressing HEK293 cell lines for the two mutants—rabbit RyR1 R164C and mouse RyR2 R176Q—as well as WT mouse RyR2. Ca²⁺ imaging and [³H]Ry binding revealed significant Ca²⁺ depletion of the ER, greater degree of channel open probabilities for mutant RyR1 and RyR2 versus their WT counterparts, as well as an increased spontaneous Ca²⁺ release for mutant RyR2 (Fig. 1). All these characteristics constitute well-known hallmarks of most RyR disease mutations including these studied here (4–7, 13, 15, 16).

Fig. 6. RMSD analysis reveals the mechanism of the long-range allosteric effect. RMSD between mutant and closed WT RyR1 was subtracted from RMSD between mutant and open WT RyR1 such that greater similarity to the closed conformation results in a positive value. (A) Pairwise 1- and 2-domain comparisons indicate that, in most cases, domains are more akin to the closed conformation except for the NTDA+/CD interface of RyR1 R164C (inset), which bore greater similarity to open RyR1 (PDB: 5T9W). The two domains change in relative orientation with respect to each other near the site of the mutation, while individually they were more similar to the closed state. (B) Schematic representation of domain movements observed in RyR1 during channel opening (top), visualized for one monomer. The size and direction of the arrows indicate magnitude of movement observed. In RyR1 R164C closed (bottom), domain translocations, mainly confined to the large CytA, are similar to the opening motion near the site of the mutation (red asterisk). Other domains are differentially affected, changing the configuration of the closed channel. These motions may also affect the DHPR, which is physically connected to RyR1’s CytA.

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Both mutations are located in the NTDA, more than 85 Å away from the pore. How may the mutation act through such long molecular distance? Structurally, RyR1 R164C adopts a conformation of the CytA between fully opened and closed, as evidenced by a flexion angle of −0.5° [versus +1.7° for closed RyR1 (22); Fig. 2]. A further distinct change with respect to WT RyR1 is an overall translation and rotation (1 Å shift, 6° rotation) of NTDA⁺ with respect to NTDB (of the neighboring subunit; Fig. 2A and fig. S6A) that causes an overall separation of domains from the fourfold axis, a trait associated with separation of the S6 helices in the TmD and channel opening (20). In addition, domain separation from the fourfold axis with a closed pore was associated with channel priming (23). Furthermore, we observed a slight dilation of RyR1’s ion gate despite remaining a closed pore (fig. S8, A to D). All of these changes observed in the RyR1 R164C structure appear to be a direct consequence of the mutation because no other ligand (activator or inhibitor) besides the constitutive ligand FKBP was used in our cryo-EM studies. These changes are remarkable because they were observed under conditions that lead to the closed state (submicromolar Ca²⁺, which is typical of skeletal muscle resting state and cardiac diastolic phase [Ca²⁺]cyt), which suggest that despite being closed, the R164C mutation changed the energy landscape of the RyR1 channel so that it would take less energy to open it, thus increasing the probability of channel opening. In contrast, under the same closed-state conditions, RyR2 R176Q maintained the closed conformation of WT RyR2 including the CytA (Fig. 2D and fig. S6D).

In both disease mutations, the positively charged Arg has mutated to a residue that lacks charge (Cys in RyR1 and Gln in RyR2). This is indicative of the importance of ionic interactions mediated by the Arg residue in the conserved NTDA:β-β loop. As summarized in Fig. 3E, disruption of the intricate network of ionic/H-bond interactions in the NTDA⁺:β-β loop further appears to cause a long-range dendro effect transduced through the CD: The upward movement of the CTD toward the CD observed in mutant RyR1 R164C alters the Ca²⁺ binding site to a configuration that promotes coordination of a Ca²⁺ ion (Figs. 3D and 6B and fig. S7), which may explain its increased sensitivity to Ca²⁺, in agreement with our earlier findings probing residues in the vicinity of the Ca²⁺ binding site (31). The effects of the mutations on the structures of mutant RyR1 and RyR2 were reproducible, because earlier datasets collected for both mutants on a Titan Krios equipped with a post-Gatan Imaging Filter (GIF) K2 summit detector gave similar results. In addition, for mutant RyR2, the effect of the nanodisc on the open/closed state of the channel can be excluded as the channel remained “closed” in the presence of detergent.

We discovered that although the two mutations being studied here—RyR1 R164C and RyR2 R176Q—are a loss of a positive charge at an equivalent site, the single-point mutation in RyR1 has a greater structural effect than the equivalent single-point mutation in RyR2 at pCa 7 (Figs. 2 and 3). On one hand, the size of the mutated residue matters, as the reciprocal mutation affected the channel’s function with the opposite tendency (Fig. 5), in agreement with our hypothesis that Gln can preserve to some extent the hydrogen bond network as a residue that lacks charge (Cys in RyR1 and Gln in RyR2). On the other hand, the effects of these mutations are further transduced in an isoform-specific manner: Despite similar trend in the decrease in [Ca²⁺]ER concentrations (Fig. 1, C and D), at submicromolar Ca²⁺, the RyR1 mutants increased the probability of channel opening with respect to WT RyR1 more than the RyR2 counterparts, both for the Gln mutation and for the Cys mutation (Fig. 5, C and D), suggesting that the effects of these mutations are further transduced in an isoform-specific manner.

RyR1 and RyR2 are primarily activated by physical coupling with DHPR (32, 33) and CICR (3, 34), respectively. By inducing an altered conformation of the CytA, which includes the interface that interacts with DHPR, the NTDA:β-β loop mutation in RyR1 could influence orthograde and retrograde altered signaling between RyR and DHPR. Modified signaling involving DHPR was measured previously for this mutation in different functional assays (35, 36). In addition, this dysfunction could be further influenced by the remodeled Ca²⁺ binding site and increased Ca²⁺ sensitivity shown here, structurally and functionally, in RyR1 R164C. The mutant cardiac isoform, RyR2 R176Q, appears to require additional triggers such as isoproterenol administration (15) or other stimuli (37, 38) to cause ventricular tachycardia in the in vivo studies, suggesting that post-translational modifications such as phosphorylation or other factors might play a role in channel leakiness. Besides MH, CCD, and heart failure, RyR mutations and remodeling of RyR-mediated Ca²⁺ signaling pathways have also been implicated in certain forms of cancer (39, 40). An important factor in drug design is to achieve selectivity, targeting one isoform over the other, and thereby minimize off-target side effects. The structural study here shows that within each isoform, the structural effects caused by the mutation have distinct characteristics, which are potentially attractive avenues that could be further exploited to guide the design of selective inhibitors.

**MATERIALS AND METHODS**

**Reagents**

All chemicals were purchased from Thermo Fisher Scientific or Sigma-Aldrich with the exception of MOPS (1-(piperazin-1-yl)ethanesulfonic acid (MOPSO; pH 6.8), 2 mM dithiothreitol (DTT), and 1 mM adenosine monophosphate (AMP) for 5 hours for RyR1 and 0.17 M NaCl, 20 mM MOPS (pH 7.0), 2 mM DTT, 1 mM AMP, and 1 mM MgCl₂ for 1 hour for RyR2. Free Ca²⁺ was adjusted with 10 mM EGTA using WEBMAXC STANDARD (42). The [³H]Ry binding

**Construction of cDNAs and generation of stable and inducible HEK293 cell lines expressing RyR**

Complementary DNA (cDNA) cassettes encoding rabbit RyR1 were gifted by H. Oyamada, Showa University. Mutant (R164C) rabbit RyR1 (equivalent to mutant R164C in humans) was generated as described previously (41). cDNAs for mouse RyR2 were generated as described (18), and R176Q mutation was introduced by inverse polymerase chain reaction (PCR). The cDNAs were cloned into pcDNA5/FRT/TO vector. HEK293 cells stably and inducibly expressing RyRs were generated using the Flp-In T-REX System (Thermo Fisher Scientific, USA).

**³H]Ry binding**

The assay was carried out as described previously (18, 41). Briefly, microsomes prepared from HEK293 cells expressing RyR1 and RyR2 were incubated with 5 nM [³H]Ry at 25°C in reaction media. Because RyR1 exhibits much lower channel activity than RyR2, reaction media and incubation periods were different between the two isoforms: 0.17 M NaCl, 20 mM 3-(N-morpholino)-2-hydroxypropane sulfonic acid (MOPS; pH 6.8), 2 mM dithiothreitol (DTT), and 1 mM adenosine monophosphate (AMP) for 5 hours for RyR1 and 0.17 M NaCl, 20 mM MOPS (pH 7.0), 2 mM DTT, 1 mM AMP, and 1 mM MgCl₂ for 1 hour for RyR2. Free Ca²⁺ was adjusted with 10 mM EGTA using WEBMAXC STANDARD (42). The [³H]Ry binding
data ($B$) were normalized to the maximum number of functional channels ($B_{\text{max}}$). The $B_{\text{max}}$ values were separately determined by Scatchard plot analysis using various concentrations (3 to 20 nM) of $[^{3}H]$Ry in a high-salt medium and –2 and –4 pmol/mg of protein for RyR1 and RyR2, respectively. The resultant $B/B_{\text{max}}$ represents the averaged activity of each mutant.

The $Ca^{2+}$-dependent $[^{3}H]$Ry binding data were fitted to the following equation

$$A = A_{\text{max}} \times \frac{[Ca^{2+}]^{n_A}}{(\left[Ca^{2+}\right]^{n_A} + K_{i}^{n_A})} \times \left(1 - \frac{[Ca^{2+}]^{n_I}}{([Ca^{2+}]^{n_I} + K_{i}^{n_I})}\right)$$

where $A$ is the activity at the specified $Ca^{2+}$, $A_{\text{max}}$ is the gain that determines the maximal attainable activity, $K_{A}$ and $K_{I}$ are dissociation constants, and $n_{A}$ and $n_{I}$ are Hill coefficients for $Ca^{2+}$ of activation and inactivation, respectively. $n_{A}$ and $n_{I}$ were fixed at 1.2 and 1.5, respectively, for RyR1 (41) and 2.0 and 1.0, respectively, for RyR2 (18). Curve fitting was performed with Prism (GraphPad Software, CA, USA). To estimate ryanodine binding at resting $Ca^{2+}$ concentrations, $A$ at $pC7$ for each mutant was calculated by Eq. 1 using the obtained parameters ($K_{A}$, $K_{I}$, and $A_{\text{max}}$).

$Ca^{2+}$ imaging studies of mutant and WT RyR in HEK293 cells

Single-cell $Ca^{2+}$ imaging was carried out in HEK293 cells expressing WT or mutant RyR as described (17, 18). Cells were cotransfected with R-CEPIA1er (43) and G-GECO1.1 (44) for luminal $Ca^{2+}$ ($[Ca^{2+}]_{\text{ER}}$) and cytosolic $Ca^{2+}$ ($[Ca^{2+}]_{\text{cyto}}$), respectively. Doxycycline was added to the culture medium at the time of transfection. $Ca^{2+}$ measurements were carried out 26 hours after induction in a Hepes-buffered Krebs solution [140 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 11 mM glucose, and 5 mM Hepes (pH 7.4)], G-GECO1.1 and R-CEPIA1er were excited by 488 and 568 nm, respectively, and fluorescence images were simultaneously recorded by using the W-VIEW System (Hamamatsu Photonics, Hamamatsu, Japan) at a rate of one frame every 0.7 s. At the end of each experiment, $F_{\text{min}}$ and $F_{\text{max}}$ were obtained with 0 mM $Ca^{2+}$ Krebs solution containing 20 mM iminomycin and 5 mM BAPTA [1,2-bis(2-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid], and 20 mM $Ca^{2+}$ Krebs solution containing 20 mM iminomycin and 20 mM CaCl$_2$, respectively (18). Regions of interest (ROIs) corresponding to individual cells were selected, and the average fluorescence intensity ($F$) of each ROI minus the background intensity ($F - F_{\text{min}}$) was normalized to the maximal fluorescence intensity ($F_{\text{max}} - F_{\text{min}}$). Measurements were carried out at 26°C.

Purification of recombinant mutant and WT RyR1 and RyR2 from HEK293 cells

$Vesicle$ $purification$

HEK293 cells expressing rabbit RyR1 R164C and mouse RyR2 (R176Q or WT) were cultured in Dulbecco’s modified Eagle medium (DMEM) (high glucose, glutamine, and no sodium pyruvate) supplemented with fetal bovine serum and bovine growth serum (Gibco, Thermo Fisher Scientific) and penicillin-streptomycin. Cells were harvested using trypsin (0.05%) in phosphate-buffered saline (PBS) and separated by centrifugation (1800 rpm, 10 min) using a Beckman Coulter Avanti J-E centrifuge (Rotor F10B1C-6 × 500 fiber-lite). Cells were lysed using a Qsonica sonicator (98% amplitude, ~3 to 5 pulses of 20 s each), and nuclei fraction was separated by centrifugation (4400 rpm, 10 min) using a Beckman Coulter Avanti J-E centrifuge (Rotor F10B1C-6 × 500 fiber-lite). The supernatant was subject to ultracentrifugation (43,000 rpm, 1 hour) using a Beckman Coulter Optima MAX-TL ultracentrifuge (rotor: TLA 100.3) to pull down the vesicles. The pellet was resuspended in homogenization buffer using a Dounce homogenator, frozen using liquid nitrogen, and stored at –80°C for subsequent protein purification.

$Protein$ $purification$ $for$ $rabbit$ $RyR1$ $R164C$ $and$ $mouse$ $RyR2$ $R176Q$

ER vesicles were first solubilized in 20 mM Na-Mops (pH 7.4), 5% CHAPS, 1.25% PC, 1 mM NaCl, 2 mM DTT, and protease inhibitor cocktail [aprotinin (5 µg/ml), leupeptin (5 µg/ml), and pefabloc (2.5 µg/ml)] for 25 min. This was followed by ultracentrifugation (43,000 rpm, 1 hour) to remove unsolubilized material. The supernatant was applied to a 10 to 20% (v/v) discontinuous sucrose gradient and centrifuged (27,000 rpm, 23 hours). Fractions containing RyR were confirmed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), pooled, and diluted fivefold in 20 mM Na-Mops (pH 7.4), 0.5% CHAPS, 2 mM DTT, and protease inhibitor cocktail. RyRs were then concentrated on a HiTrap Heparin HP column (Amersham Biosciences) and eluted with 20 mM Na-Mops (pH 7.4), 1.5% NaCl, 0.5% (v/v) CHAPS, 2 mM DTT, and protease inhibitor cocktail. The elution buffer was changed to 20 mM Mops-Na (pH 7.4), 0.5% NaCl, 2 mM DTT, 2 mM EGTA, and 0.015% Tween-20 (v/v; EMD Millipore) without protease inhibitor cocktail. The purity and quality of RyR1 R164C and RyR2 R176Q were assessed by 10% SDS-PAGE and by negative staining EM. Aliquots were flash-frozen in liquid nitrogen and stored at –80°C for subsequent cryo-EM grid preparation. Yields of protein purification for the two mutants RyR1 R164C and RyR2 R176Q were 0.21 mg/Triple Nunc flask and 0.1 mg/150 mm plate, respectively.

$Protein$ $purification$ $for$ $mouse$ $RyR2$ $WT$

ER vesicles (1 ml) were solubilized in 1 mM NaCl, 20 mM Mops (pH 7.4), 2 mM DTT, and protease inhibitors using 2.5% CHAPS and 1.25% PC for 20 min at 4°C with mild agitation. This was followed by ultracentrifugation (10,000 rpm for 1 hour) to remove unsolubilized material. Supernatant was diluted fivefold before loading onto a 1-ml StreptTrap column (GE Healthcare Life Sciences) preloaded with SBP-FKBP12.6 (45). The RyR2-FKBP12.6 complex bound to the StreptTrap column was washed with 20 mM Mops (pH 7.4), 0.2 mM NaCl, 2 mM DTT, 2% CHAPS, 1.5% PC, and protease inhibitors and eluted (aliquots of ~120 µl) using 20 mM Mops (pH 7.4), 0.4 mM NaCl, 2 mM DTT, 0.02% Tween 20, and 5 mM desthiobiotin. The purity and quality of RyR2 WT were assessed by 10% SDS-PAGE and by negative staining EM. Aliquots were flash-frozen in liquid nitrogen and stored at –80°C for subsequent cryo-EM grid preparation.

$Reconstitution$ $into$ $nanodiscs$

For reconstitution of mouse RyR2 R176Q into lipid nanodiscs, membrane scaffold protein (MSP) 1E3D1 (MSP1E3D1) and POPC were added to RyR2 R176Q (1:2.50 RyR:MSP:POPC molar ratio) and incubated for 3 hours at 4°C, followed by detergent removal by dialysis at 4°C in 20 mM Mops (pH 7.4), 600 mM KCl, 2 mM DTT, and 2 mM EGTA. The reconstituted channels, mouse RyR2 R176Q nanodiscs, were examined by negative staining for quality and used directly for preparation of cryo-EM grids.

$Cryo-EM$ $grid$ $preparation$

Grids were prepared using a FEI Vitrobot Mark IV plunge as previously described (26). Quantifoil Cu or Au grid (1.2/1.3; 300 mesh; Quantifoil) was either coated with a thin layer of carbon using a...
Denton vacuum evaporator (DV-500) and glow-discharged for 20 s at 25 mA or used as such. For RyR1 R164C, FKBP12.6 was added just before adsorption to the grid, and 3.0 μl of RyR1 R164C-FKBP12.6 complex (~5.0 mg/ml) was adsorbed onto a Quantifoil Au grid that had been glow-discharged for 40 s at 25 mA. RyR2 R176Q (~4.0 mg/ml) was incubated with FKBP12.6 before grid preparation, and 2.0 μl of the complex was adsorbed onto each side of a Quantifoil Au grid (no glow discharge). The RyR2 WT protein solution (4 μl) was incubated with ~0.1 to 0.2% fluorinated octyl maltoside (f-OM) just before grid preparation and applied to a thin carbon-coated Quantifoil Au grid glow-discharged for 20 s at 25 mA and adsorbed for 60 s. For all three samples, adsorption was followed by removal of the excess protein solution by blotting (blot force, 1 to 3; blot time, 0.5 to 2 s) using Whatman 540 filter paper and by plunge freezing into liquid ethane cooled by liquid nitrogen. Grids were screened on an in-house Tecnai F20 electron microscope.

Data collection
R164C rabbit RyR1-FKBP12.6 and R176Q mouse RyR2-FKBP12.6 data were collected on a Titan Krios at 300 kV at the National Cancer Institute Cryo-EM facility equipped with a post-GIF K3 direct electron detector at ×81,000 magnification and 1.08 Å per pixel using the software Leginon (46). Exposure time was 3.5 and 3.4 s, respectively, with an electron dose of 50 e⁻/Å² spread over 40 frames. Movies (4923 and 8655) were collected with defocus values in the range of −1.0 to −2.0 μm.

WT mouse RyR2-FKBP1.6 data were collected on a Titan Krios at 300 kV at Purdue University Cryo-EM facility equipped with a post-GIF K2 Summit direct electron detector at a ×105,000 magnification and 0.69 Å per pixel using the software Leginon (46). Exposure time for WT mouse RyR2 was 14 s with an electron dose of 60 e⁻/Å² spread over 70 frames. Movies (4470) were collected with defocus values in the range of −2.0 to −4.5 μm (see table S2).

Image processing

**R164C RyR1-FKBP12.6 dataset**
All image processing was performed using cryoSPARC v2.9 (fig. S9A, table S2) (47). Of the 4923 motion-corrected micrographs, ~587 micrographs were eliminated due to either ice contamination or drift. The final 4336 movies were subject to full-frame motion correction and contrast transfer function (CTF) estimation using CTFFIND4.1 inside cryoSPARC v2.9 with a low-pass–filtered (30 Å) 3D structure, resulting in a “good” class containing 385,324 particles from the 452,952 particles. The 385,324 particles were submitted to 3D reconstruction in a real_space_refine, and quality of models was assessed using MolProbity scores (58) (see table S2).

**R176Q RyR2-FKBP12.6 dataset**
All image processing was performed using cryoSPARC v2.11 (fig. S9B, table S2) (47). Motion correction resulted in a total of 8655 micrographs, of which ~1141 micrographs were eliminated due to either ice contamination or drift. Movies (7514) were separated into two independent batches—PART A (4755) and PART B (2759)—and were subjected to patch motion correction and patch CTF estimation inside cryoSPARC v2.11, resulting in further elimination of 179 micrographs with CTF fit resolution above 6.5 Å. Approximately 464 particles were manually picked from ~13 micrographs and subjected to 2D classification. Selected 2D classes were then used to perform automated particle picking on the final 7335 micrographs, resulting in a total of ~860,841 particles (PART A, 550,544; PART B, 310,287). The results from autopicking were subjected to four rounds of 2D classification to remove false positives, resulting in 492,083 particles (PART A, 315,827; PART B, 176,256). The particles were subjected to 3D classification, resulting in two good classes from each of the two parts A and B, which reproduced the same 3D features, and then combined to give a final particle stack consisting of 282,778 particles. The combined stack was used for 3D reconstruction with a low-pass–filtered (30 Å) 3D ab initio reconstructed structure as a reference in cryoSPARC v2.11.

**WT RyR2-FKBP12.6 dataset**
Dose-weighted micrographs were inter- and in-frame motion-corrected with Motioncor2 (fig. S9C, table S2) (48). Out of 4470 motion-corrected micrographs, only ~63% of the dataset could be retained due to ice contamination or drift. Approximately 500 particles were manually picked using Boxer (49) and used for 2D classification in Relion 1.4 (50). Selected 2D classes were then used to perform automated particle picking on the final 2831 micrographs, resulting in ~260,000 particles, and the results from automated picking were manually cleaned to remove false positives, resulting in 240,724 particles. Prevalence of top views in the dataset required elimination of ~56% of particles to limit the top views to at most 10× side views by performing multireference alignment using Spider17.05 (51), which resulted in 104,792 particles with better distribution of particle orientations. Further elimination of particles with shifts greater than 5 Å resulted in a final particle stack of 103,845 particles, which were submitted to 3D reconstruction using FREALIGN v9.11 (52) with a low-pass–filtered (100 Å) 3D structure of the RyR2-FKBP12.6 complex [EMD:8303 (26)] as a reference. Local resolution estimation for all cryo-EM maps was performed using ResMap1.1 (53). Image processing was performed using the software from the SBGrid suite of programs (54).

Model building
Initial model building of an RyR monomer was performed using phenix.real_space_refine function of Phenix (55). For the RyR1 R164C structure, a monomer of WT RyR1 [PDB: 5TB0 (23)] was aligned to the map and used as the starting model. Refinement was performed iteratively with application of secondary structure and Ramachandran restraints and with morphing and simulated annealing. In addition, the monomer of WT RyR1 (PDB: 5TB0) was used as reference model in subsequent rounds of iteration, with reference model restraints applied. For the RyR2 R176Q structure, the input model was a monomer of WT RyR2 [PDB: 5L1D (26)] to which HD2 of WT RyR1 (PDB: 5TB0) was attached using Chimera (56). The same method was followed to model RyR2 WT, using the final RyR2 R176Q structure as the input model. The sequence was updated for mouse RyR2 (UniProt: E9Q401) for both the mutant and WT models. Residues R164 and R176 in the RyR1 R164C and RyR2 R176Q structures were mutated, and all residues were manually fitted using Coot (57). Validation was performed as part of phenix.real_space_refine, and quality of models was assessed using MolProbity scores (58) (see table S2).

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Pore analysis
Detailed pore analysis was performed using HOLLE2.11 (59) and MOLEonlne update 2018 (60). Only residues of the CTD (4957 to 5033 in RyR1 and 4889 to 4969 in RyR2) and S6 helices (4908 to 4956 in RyR1 and 4840 to 4888 in RyR2) of the four subunits were retained from the atomic models for pore analysis.

High-resolution images
All high-resolution images were generated using Chimera (56).

Statistical analysis
Data are expressed as means ± SD. Statistical comparisons have been made using GraphPad Prism 8 software. Student’s t test was used for comparisons between two groups. One-way analysis of variance (ANOVA) was used to compare three or more groups. P < 0.05 was considered significant.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/3/eabb2964/DC1

View/request a protocol for this paper from Bio-protocol.

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