Conserved functional consequences of disease-associated mutations in the slide helix of Kir6.1 and Kir6.2 subunits of the ATP-sensitive potassium channel

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Conserved functional consequences of disease-associated mutations in the slide helix of Kir6.1 and Kir6.2 subunits of the ATP-sensitive potassium channel

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Cantu syndrome (CS) is a condition characterized by a range of anatomical defects, including cardiomegaly, hyperflexibility of the joints, hypertrichosis, and craniofacial dysmorphology. CS is associated with multiple missense mutations in the genes encoding the regulatory sulfonylurea receptor 2 (SUR2) subunits of the ATP-sensitive K(+) (KATP) channel as well as two mutations (V65M and C176S) in the Kir6.1 (KCNJ8) subunit. Previous analysis of leucine and alanine substitutions at the Val-65-equivalent site (Val-64) in Kir6.2 indicated no major effects on channel function. In this study, we characterized the effects of both valine-to-methionine and valine-to-leucine substitutions at this position in both Kir6.1 and Kir6.2 using ion flux and patch clamp techniques. We report that methionine substitution, but not leucine substitution, results in increased open state stability and hence significantly reduced ATP sensitivity and a marked increase of channel activity in the intact cell irrespective of the identity of the coassembled SUR subunit. Sulfonylurea inhibitors, such as glibenclamide, are potential therapies for CS. However, as a consequence of the increased open state stability, both Kir6.1(V65M) and Kir6.2(V64M) mutations essentially abolish high-affinity sensitivity to the KATP blocker glibenclamide in both intact cells and excised patches. This raises the possibility that, at least for some CS mutations, sulfonylurea therapy may not prove to be successful and highlights the need for detailed pharmacogenomic analyses of CS mutations.

ATP-sensitive K(+) (KATP)6 channels, found throughout the body, are generated as octameric complexes consisting of four pore-forming Kir6.1 or Kir6.2 subunits with four regulatory sulfonylurea receptor (SUR1 or SUR2) subunits. SUR1 and Kir6.2 are prominently expressed in the pancreas and neurons, and hence mutations underlie hyperinsulinism, diabetes, and neurological disorders (1). Cantu syndrome (CS) is characterized by a range of apparently disparate features, including cardiomegaly, hyperflexibility of the joints, hypertrichosis, and craniofacial dysmorphology as well as multiple cardiovascular features (2–5). Several reports of missense mutations in the genes encoding the SUR2 and Kir6.1 subunits (ABCC9 and KCNJ8, respectively), which are expressed prominently in cardiovascular tissues, provide strong evidence that CS arises from KATP gain of function (GOF) (6–10). Gain of function in SUR2- or Kir6.1-containing KATP channels would be expected to hyperpolarize the membrane potential and decrease excitability, particularly in smooth muscle cells (11). Decreased vascular tone may explain many CS features, including persistent patent ductus arteriosus, dilated and tortuous vessels, lowered blood pressure, increased blood volume, and consequent cardiomegaly.

Although rare, CS is a debilitating syndrome, currently with no specific therapy. Sulfonylureas are potent blockers of KATP channels. These drugs have proven highly beneficial in treatment of neonatal diabetes resulting from GOF in SUR1- and Kir6.2-dependent channels (12, 13). However, as the molecular defect becomes more severe, the drug effect tends to decline and become ineffective in certain cases (14–17). Whether such effects occur in SUR2- or Kir6.1-dependent channels is not known.

To date, two KCNJ8 mutations, encoding Kir6.1(V65M) and Kir6.1(C176S), have been reported in association with CS (5, 10). Analogous to residues Cys-166 and Val-64 in Kir6.2, Cys-176 and Val-65 of Kir6.1 are predicted to be positioned in close proximity at the bottom of transmembrane helix 2 (TM2) and the N-terminal slide helix, respectively (see Fig. 1) (18, 19), raising the possibility that mutation of either one increases channel activity by the same mechanism. Interestingly, previous analysis of leucine and alanine substitutions at the equivalent site (Val-64) in Kir6.2 subunit indicated that these substitutions were tolerated without effects on channel function and that such mutations were therefore not causally associated with neonatal diabetes (20). In this study, we sought to resolve the consequences of methionine versus...
leucine substitutions at this position on channel function and sulfonylurea pharmacology.

Results

Kir6.1(V65M), but not Kir6.1(V65L), results in $K_{ATP}$ gain of function

To study recombinant channels containing mutant subunits, COSm6 cells were transfected with WT or mutant Kir6.1 and either SUR1 or SUR2A. Channel activity was first assayed using a radioactive Rb$^+$ ($^{86}$Rb$^+$) efflux assay. When expressed with SUR1, Kir6.1(V65M) exhibited markedly increased $K_{ATP}$-dependent efflux rates compared with WT in both basal conditions (Ringer’s solution) and in the presence of the SUR1-selective $K_{ATP}$ activator diazoxide as shown in Fig. 2, A and B. In contrast, $K_{ATP}$-dependent efflux rates for Kir6.1(V65L) were not significantly different from WT in either condition (Fig. 2, A and B).

When $K_{ATP}$ channels were activated by incubation with oligomycin and 2-deoxyglucose (metabolic inhibition (MI)), no significant effect on efflux rate was observed for either V65M or V65L (Fig. 2C). MI typically leads to maximal activation of all available channels; hence, these results suggest that neither mutation affects the maximal available conductance and therefore that channel density was unaffected. When expressed with SUR2A, the $K_{ATP}$-dependent basal flux rate was very low for both WT and mutant Kir6.1 subunits (Fig. 3A), likely due to lower expression level and decreased Mg-nucleotide activation of the SUR2A subunit (21). However, when channels were activated with the SUR2-selective activator pinacidil, a marked increase in $K_{ATP}$-dependent flux was seen for V65M-containing, but not V65L-containing, channels, when compared with WT (Fig. 3B). Maximum $K_{ATP}$-dependent efflux rates in MI were significantly higher for V65M than for WT when expressed with SUR2A (Fig. 3C). This suggests that V65M increases maximal conductance, although this analysis does not distinguish between increased channel density or more complete activation of available channels.

The conserved effects of valine-to-methionine or -leucine substitution at the equivalent residue in Kir6.2

Val-65 in Kir6.1 and the homologous residue in Kir6.2 (Val-64) lie within the amphipathic N-terminal slide helix, which is highly conserved in Kir channels. As shown in Fig. 4, when coexpressed with SUR1, Kir6.2(V64M) also significantly
increased basal and pinacidil-activated $K_{ATP}$-dependent efflux rates. Conversely, the V64L mutation had no significant effect, consistent with the previous report that this mutation does not alter channel function (20). When coexpressed with SUR2A, marked increases in basal and pinacidil-activated efflux rates were again observed for Kir6.2(V64M) but not for Kir6.1(V65L) (Fig. 5, A and B). The same maximum efflux rates in MI for WT, V64M, and V65L, irrespective of the SUR subunit (Figs. 4C and 5C), imply no effects of either mutation on channel density.

The molecular mechanism of $K_{ATP}$ GOF conferred by valine-to-methionine mutations

Taken together, the above data demonstrate that substitution of valine by methionine at residue 65 in Kir6.1 or residue 64 in Kir6.2 results in gain of function of expressed $K_{ATP}$ channels.

Figure 2. Kir6.1(V65M), but not Kir6.1(V65L), increases SUR1-dependent $K_{ATP}$ channel activity in intact cells. Cumulative $86\text{Rb}^+$ efflux as a function of time was measured in GFP-transfected control COSm6 cells and in cells transiently expressing WT or mutant Kir6.1 with SUR1. Experiments were performed in basal conditions in Ringer’s solution (A) or in the presence of the $K^+$ channel opener diazoxide (B) or the metabolic inhibitors (MI) oligomycin and 2-deoxy-D-glucose (C). Data points and error bars represent mean and S.E. of eight to 18 experiments. Summary representations of the mean cumulative flux at 25 min are shown on the right (∗ denotes statistical significance as determined by unpaired Student’s t tests; $p < 0.05$).
To investigate the molecular mechanism, we examined nucleotide sensitivity of channels in excised, inside-out patch clamp experiments. As shown in Fig. 6, channels comprising WT Kir6.2 and SUR2A were inhibited by Mg$^{2+}$/H$^{11001}$/ATP-free ATP with an IC$_{50}$ of 30 M, which was not significantly altered by the V64L mutation. In contrast, the V64M mutation resulted in 6-fold reduction in ATP sensitivity (Fig. 6). Decreased ATP sensitivity could arise from a change in the affinity of the channel for ATP or, because ATP binds to and stabilizes closed states of the channel, a change in intrinsic open state stability. Recent cryo-EM structures (18, 19) confirm that the slide helix of Kir6.2 is structurally distinct from the ATP-binding site, and thus a direct effect on ATP binding is not expected. In contrast, multiple studies have demonstrated that mutations in the slide helix act to stabilize open conformations of Kir channels (22–24). PIP$_2$ in the cytoplasmic leaflet is essential for Kir channel activity (25). At ambient levels in the cytoplasmic leaflet, WT Kir6.2/SUR1 channel open probability is ~0.5 but approaches 1

Figure 3. Kir6.1(V65M), but not Kir6.1(V65L), increases SUR2A-dependent K$_{ATP}$ channel activity in intact cells. Cumulative $^{86}$Rb$^+$ efflux as a function of time was measured in GFP-transfected control COSm6 cells and in cells transiently expressing WT or mutant Kir6.1 with SUR2A. Experiments were performed in basal conditions in Ringer’s solution (A) or in the presence of the K$^+$ channel opener pinacidil (B) or the metabolic inhibitors (MI) oligomycin and 2-deoxy-D-glucose (C). Data points and error bars represent mean and S.E. of three experiments. Summary representations of the mean cumulative flux at 25 min are shown on the right (* denotes statistical significance as determined by Mann-Whitney U test; p < 0.05).
as PIP$_2$ is increased in the membrane (26). Mutations that intrinsically stabilize or destabilize the channel open state increase or decrease, respectively, the basal open probability, which can be assessed by maximizing the open probability by adding exogenous PIP$_2$ (26). To estimate the effect of the V64M mutation on channel open state stability, the activating response to exogenous PIP$_2$ was therefore assessed for WT Kir6.2- and Kir6.2(V64M)-containing channels (see “Experimental procedures” and Ref. 14). The analysis indicates that the V64M mutation significantly increases the intrinsic apparent $P_o$ under ambient conditions following patch excision (from ∼0.6 in WT to ∼1.0 in Kir6.2(V64M); Fig. 7, A–C).

**The open state-stabilizing valine-to-methionine substitutions decrease sulfonylurea sensitivity in both Kir6.2 and Kir6.1**

Second generation sulfonylurea drugs, such as glibenclamide, inhibit SUR2-containing $K_{ATP}$ channels with moderate affinity (27, 28) and therefore may serve as a potential pharma-
cotherapy for CS. However, it has previously been demonstrated that open state-stabilizing mutations in Kir6.x subunits can impair sulfonylurea inhibition (14–16). The effect of 10 μM glibenclamide on K\(_{ATP}\)-dependent rubidium effluxes was assessed in cells expressing either WT or V64M mutant Kir6.2 with SUR2A. As shown in Fig. 8, MI-activated WT channel fluxes were inhibited 75% by glibenclamide, but there was no significant inhibition of Kir6.2(V64M) channels. All known CS patients are heterozygous, and we modeled heterozygosity by cotransfecting WT and V64M mutant Kir6.2 with SUR2A subunits. Glibenclamide inhibition was again markedly reduced by the V64M mutation with only ~20% inhibition of MI-activated Rb\(^+\) fluxes (Fig. 8, A and B). We also examined the sensitivity of Kir6.2 + SUR2A and Kir6.2(V64M) + SUR2A channels to glibenclamide inhibition in inside-out patch clamp recordings (Fig. 8, C and D). In agreement with the above results, sensitivity was again markedly reduced by the V64M mutation (Fig. 8, C and D) with almost no inhibition even at 10 μM glibenclamide.

As the open state-stabilizing effects appear to be similar for the Kir6.2(V64M) and Kir6.1(V65M) mutations, the effects of

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**Figure 5.** Kir6.2(V64M), but not Kir6.2(V64L), increases SUR2A-dependent K\(_{ATP}\) channel activity in intact cells. Cumulative 86Rb\(^+\) efflux as a function of time was measured in GFP-transfected control COSm6 cells and in cells transiently expressing WT or mutant Kir6.2 with SUR2A. Experiments were performed in basal conditions in Ringer’s solution (A) or in the presence of the K\(^+\) channel opener pinacidil (B) or the metabolic inhibitors (MI) oligomycin and 2-deoxy-D-glucose (C). Data points and error bars represent mean and S.E. of three to six experiments. Summary representations of the mean cumulative flux at 25 min are shown on the right (* denotes statistical significance as determined by Mann-Whitney U test; \(p < 0.05\)).
Conserved gain-of-function Kir6.1 and Kir6.2 mutations

Figure 6. Gain of function in Kir6.2(V64M) results from decreased ATP sensitivity. Representative excised patch clamp recordings from COSm6 cells coexpressing WT or mutant Kir6.2 subunits with SUR2A are shown. A, membrane potential was held at ~50 mV, and currents were recorded continuously in inside-out excised patches exposed to KINT in the absence or presence of 0.01, 0.1, or 5 mM Mg\(^{2+}\)-free ATP. B, summary dose-response data (data points and error bars represent mean and S.E.; 10 patches each) was fit using a four-parameter Hill equation to estimate the ATP concentration for half-maximal inhibition. "IC\(_{50}\)" values were 32.2 ± 6.6 \(\mu\)M (Hill coefficient, 2.2 ± 0.2) for WT, 198 ± 31.1 \(\mu\)M (Hill coefficient, 2.9 ± 0.3) for Kir6.2(V64M), and 31.0 ± 2.2 \(\mu\)M (Hill coefficient, 2.2 ± 0.1) for Kir6.2(V64L) (* denotes statistical significance as determined by unpaired Student's t tests; \(p < 0.05\)). In this and in representative current recordings in subsequent figures, the dashed lines represent zero channel current.

Figure 6: Conserved gain-of-function Kir6.1 and Kir6.2 mutations on inhibitor sensitivity are also likely to be conserved. However, a previous report suggested that sulfonylurea sensitivity may be differentially affected by mutations in the slide helix of Kir6.2 versus Kir6.1 (15). Thus, we also sought to confirm the effect of the Kir6.1(V65M) mutation itself on glibenclamide inhibition. Due to very low Rb\(^{+}\) fluxes and low density in patch-clamp recordings for Kir6.1-only-containing channels, we coexpressed Kir6.1 WT or Kir6.1(V65M) with WT Kir6.2 to yield heterotetramers as described previously (10). As expected, heteromeric Kir6.2/Kir6.1(V65M) channels exhibited ~5-fold lower ATP sensitivity in excised patch clamp recordings than Kir6.2/Kir6.1 WT channels (Fig. 9A). In Rb\(^{+}\) flux experiments, the effect of 10 \(\mu\)M glibenclamide on \(K_{\text{ATP}}\) dependent efflux was also reduced from ~50% for WT Kir6.1-containing channels to ~30% for V65M-containing channels (Fig. 9, C and D), and glibenclamide sensitivity was markedly reduced in Kir6.2/Kir6.1(V65M) channels in inside-out patch clamp recordings (Fig. 9, E and F).

Discussion

The recent identification of multiple missense mutations in SUR2 (ABCC9) and Kir6.1 (KCNJ8), which all result in \(K_{\text{ATP}}\) GOF, demonstrates that CS arises primarily from \(K_{\text{ATP}}\) channel GOF (2, 10). Brownstein et al. (5) initially reported the Kir6.1(V65M) mutation in a case report with the prediction that the mutation was causal. However, a previous report showed that other substitutions at the equivalent (Val-64) residue in Kir6.2 are tolerated without significant effects on \(K_{\text{ATP}}\) function (20). This raises the possibility that the Kir6.1(V65M) mutation may actually be benign. To address this, we systematically characterized the effects of valine-to-methionine and valine-to-leucine substitutions in both Kir6.1 and Kir6.2 and show that methionine substitution, but not leucine substitution, results in marked gain of function for either channel, whether coexpressed with SUR1 or SUR2A regulatory subunits. We show that substitution by methionine, but not by leucine, results in reduced ATP sensitivity for both Kir6.2(V64M) and Kir6.1(V65M) channels, and for Kir6.2(V64M), we show that this results from an increase in the open state stability such that the intrinsic open probability is higher. As a consequence, \(K_{\text{ATP}}\) channels that include this mutation will exhibit increased activity under physiological regulation by intracellular nucleotides. Similar increases in open state stability have previously been reported for other slide helix mutations (e.g. Q52R (14)), reflecting an important role of this domain in controlling channel gating. Intriguingly, in light of recently reported structures of the Kir6.2-SUR1 \(K_{\text{ATP}}\) complex (18, 19), the single other known CS Kir6.1 mutation, Kir6.1(C176S), is located very close to Val-65 in a cluster of hydrophobic residues (Fig. 1). Previous analyses have demonstrated that the equivalent Kir6.2(C166S) also increases intrinsic open state stability (29, 30). This raises the possibility that both V65M and C176S mutations act to destabilize the closed channel by disrupting this closed-state hydrophobic cluster. In contrast, Männikkö et al. (20) reported that the Kir6.2(V64L) mutation ameliorated the deleterious effects of the nearby pathogenic F60Y mutation when expressed on the same subunit. Thus, it is also possible that V64M may result in pathogenic reduction of ATP sensitivity by disrupting the interaction that is normally present between these two slide helix residues.

The potential utility of sulfonylurea drugs in the treatment of CS remains to be tested clinically, but “second-generation” sulfonylureas, such as glibenclamide (glyburide), which demonstrate moderate potency for inhibiting SUR2-containing \(K_{\text{ATP}}\) channels, may offer promise for a specific therapy. However,
Sulfonylurea sensitivity can be markedly decreased by K_{ATP} mutations that increase the intrinsic open probability of channels (14–16). Consistent with this, we show here that both the Kir6.1(V65M) and Kir6.2(V64M) mutations essentially abolish high-affinity glibenclamide sensitivity in both intact cells and excised patches. This finding raises the possibility that, at least for some CS mutations, sulfonylurea therapy may not be successful and highlights the need for detailed pharmacogenomic analyses of the effects of individual CS mutations on K_{ATP} inhibitor sensitivity.

Figure 7. Kir6.2(V64M) increases channel open state stability. Representative K_{ATP} currents recorded from patches expressing WT (A) or V64M mutant Kir6.2 (B) with SUR2A following excision at the arrow and in the presence of 10 mM ATP, 1 mM ATP, or 5 μg/ml PIP_{2} as indicated are shown. C, relative P_{o} determined as a ratio of steady-state current in the patch upon excision in the absence of nucleotides to the maximum current measured following PIP_{2}. Individual patch data are represented by symbols (n = 8–14); bars and error bars are the means and S.E. Relative P_{o} = 0.59 ± 0.07 (WT) and 1.05 ± 0.07 (Kir6.2(V64M)) (* denotes statistical significance as determined by unpaired Student’s t tests; p < 0.05).
Experimental procedures

Modeling of Kir6.1 tetrameric channels

Kir6.1 was modeled by homology to the recently published Kir6.2 structures Protein Data Bank code 5TWV (19) and Protein Data Bank code 5WUA (18). Molecular dynamics (MD) simulations were carried out using Gromacs software version 5.1.1 and the Amber 99 force field as described previously (31). Mutations were introduced using Swiss-PdbViewer (32). Three 100-ns MD simulations were performed for WT Kir6.1 and V65L and V65M mutants. All structures were embedded in a lipid bilayer consisting of 588 1-palmitoyl-2-oleoylphosphatidylcholine lipids using the g_membed tool (33) and solvated using the extended simple point charge water model (34). K⁺ and Cl⁻ ions were randomly placed within the solvent to neutralize the system and to obtain an ion concentration of 150 mM. The root mean square deviation of simulated protein structures all converged to \( \approx 3.5 \) Å for each structure at around 20 ns, indicating that the simulated systems were stable and at equilibrium (Fig. 1D).

Mutagenesis and heterologous expression of K<sub>ATP</sub> channels

Mutations were introduced in rat Kir6.1-pcDNA3.1 and mouse Kir6.2-pcDNA3.1 using the QuikChange II site-directed mutagenesis kit (Agilent Technologies) and confirmed by direct sequencing of the coding region. For channel expression, COSm6 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum,
105 units/liter penicillin, and 100 mg/liter streptomycin. At 60–70% confluence, cells were transfected with the relevant plasmids using FuGENE 6 transfection reagent (Promega). For experiments with homomeric channels, cells were cotransfected with pcDNA3.1-mKir6.2 or pcDNA3-Kir6.1 (0.6 μg) and pECE-hamsterSUR1 cDNA or pCMV6-ratSUR2A (SUR2) (1 μg). For experiments using heteromeric channels, cells were cotransfected with WT and mutant Kir6.x with WT SURx at ratios of 0.3:0.3:1.0 (w/w/w). Cells transfected with GFP-pcDNA3.1 served as controls. A small amount of GFP DNA was coexpressed for identification of transfected cells in electrophysiology experiments.

**Macroscopic 86Rb⁺ efflux assays**

Transfected cells were incubated overnight at 37 °C in DMEM containing 1 μCi/ml 86RbCl (PerkinElmer Life Sciences). Bathing medium was then replaced by room temperature Ringers solution (118 mM NaCl, 10 mM HEPES, 25 mM NaHCO3, 4.7 mM KCl, 1.2 mM KH2PO4, 2.5 mM CaCl2, 1.2 mM MgSO4, adjusted to pH 7.4 with NaOH) immediately before
two 1-min washes in assay medium. $^{86}$Rb efflux was then assessed in 1) the absence (basal) or 2) presence of 2.5 mg/ml oligomycin and 1 mmol/liter 2-deoxy-D-glucose (metabolic inhibition; applied during two 1-min washes prior to assay) or 3) the presence of SUR1- or SUR2-specific $K^+$ channel opener, diazoxide or pinacidil, respectively, at 100 μM (applied during two 1-min washes prior to assay). At selected time points, the solution was collected and replaced with fresh solution. Upon completion of the assay, cells were lysed with 2% SDS, and radioactivity in these samples was measured by liquid scintillation. A nonspecific efflux pathway was assumed to be present in all experiments. In metabolic inhibition in particular, both the nonspecific efflux rate and $K_{ATP}$-specific efflux rates decreased with time. Data are shown as mean cumulative Rb$^+$ efflux (±S.E.) relative to total initial Rb$^+$ content. Data were tested for statistical significance using Student’s t test where normal distribution of data could be confirmed or by the non-parametric Mann-Whitney U test where normal distribution could not be confirmed; a p value <0.05 was considered significant for both tests.

**Excised patch clamp**

After 24–48 h, transfected fluorescent cells were selected for analysis by excised patch clamp experiments using a perfusion chamber that allows for the rapid switching of solutions (35). The bath and pipette solutions (KINT) contained 140 mM KCl, 10 mM HEPES, 1 mM EGTA, pH 7.4 with KOH. $K_{ATP}$ currents were recorded from inside-out patches at −50 mV. Current levels in solutions of varying nucleotide or glibenclamide concentrations were normalized to the basal current in the absence of inhibitors, and dose-response data were fit with a four-parameter Hill equation.

Normalized current $= I_{min} + (I_{max} - I_{min})/(1 + (X/I_{50})^n)$

(Eq. 1)

where the current in KINT $= I_{max}$, $I_{min}$ is the minimum current observed in high ATP, $X$ refers to the concentration of ATP or glibenclamide, $I_{50}$ is the concentration of half-maximal inhibition, and $H$ denotes the Hill coefficient.

For experiments assessing PIP$_2$ activation, an ammonium salt of 1-α-phosphatidylinositol 4,5-bisphosphate from porcine brain (Avanti Polar Lipids) was dissolved in KINT to prepare a 5 μg/ml working solution. For each membrane patch, $P_o$ was estimated by dividing the steady-state current in zero ATP by the maximum steady-state current after exposure to PIP$_2$ (14). Data were tested for statistical significance using Student’s t test where normal distribution of data could be confirmed or by the non-parametric Mann-Whitney U test where normal distribution could not be confirmed. A p value <0.05 was considered significant for both tests. Experiments were performed at 20–22°C.

Author contributions—P. C. and C. G. N. conceived the project. P. C., C. M., and C. G. N. designed the experiments. P. C., C. M., and X. C. performed the experiments and analyzed the data. P. C., C. M., A. S.-W., and C. G. N. wrote the paper.

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