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One SUMO is sufficient to silence the dimeric potassium channel K2P1

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Small ubiquitin modifier 1 (SUMO1) is shown to regulate K2P1 background channels in the plasma membrane (PM) of live mammalian cells. Confocal microscopy reveals native SUMO1, SAE1, and Ubc9 (the enzymes that activate and conjugate SUMO1) at PM where SUMO1 and expressed human K2P1 are demonstrated to colocalize. Silent K2P1 channels in excised PM patches are activated by SUMO isopeptidase (SENP1) and resilenced by SUMO1. K2P1-Lys274 is crucial: when mutated to Gln, Arg, Glu, Asp, Cys, or Ala, the channels are constitutively active and insensitive to SUMO1 and SENP1. Tandem mass spectrometry confirms conjugation of SUMO1 to the ε-amino group of Lys274 in vitro. FRET microscopy shows that assembly of K2P1 and SUMO1 requires Lys274. Single-particle TIRF microscopy shows that wild-type channels in PM have two K2P1 subunits and assemble with two SUMO1 monomers. Although channels engineered with one Lys274 site carry just one SUMO1 they are activated and silenced by SENP1 and SUMO1 like wild-type channels.

KCNK1 | TWIK1 | SAE1 | Ubc9 | SENP1

The activities of many cellular proteins are regulated by covalent conjugation of small ubiquitin modifier (SUMO) proteins on ε-amino groups of specific lysine residues. Such proteins are sumoylated and desumoylated via conserved enzymes that form or hydrolyze isopeptide bonds to these target lysines. This pathway is well-recognized to modulate nuclear import and export, DNA repair, and transcription factor activity (1). Unexpectedly, we found the SUMO pathway to regulate the activity of human K2P1 potassium channels expressed at the PM of *Xenopus laevis* oocytes and COS-7 African Green Monkey fibroblasts (2). At baseline, K2P1 channels in PM were electrically silent due to the indigenous SUMO machinery. Channel activation required exposure to an isopeptidase (SENP1) that removes SUMO or mutation of a specific lysine residue of K2P1, Lys274. Regulatory events were shown to be reversible by "cramming" of channels in PM patches into cells producing SENP1 or SUMO1. Mutation of Lys274 prevented SUMO conjugation producing active channels insensitive to suppression by SUMO1 or activation by SENP1.

Many biochemical and structural studies have examined the interaction of SUMO with transcription factors. In contrast, the mechanisms underlying SUMO regulation at the PM of cells has been controversial in the literature. Even as additional PM substrates for sumoylation were reported [including, Kv2.1 and Kv1.5 voltage-gated potassium channels (3, 4), GluR6 receptors (5), and TRPM4 channels (6)], our description of SUMO action on K2P1 has been questioned (7, 8). Here, we confirm the regulation of human K2P1 channels in PM by sumoylation and characterize the SUMO-channel interaction in detail using CHO cells.

Results

K2P channels are dedicated pathways for background flux of potassium ions that set cellular resting potentials and mediate electrical excitability subject to a broad array of regulatory influences (9). Identified by their unique primary structure of two pore-forming domains in each subunit (10) and their operation as potassium-selective leak pathways (11–13), these channels stabilize excitable cells below firing threshold (14). Encoded by 15 genes in humans, K2P channels have roles in physiology as disparate as sensation of oxygen, mechanical stress, and odor, apoptosis, neuromodulation, and general anesthesia. Two K2P subunits are inferred to assemble as either homo- or heterodimers to form a single, axial potassium-selective pore (15, 16) in vivo (17).

SUMO Machinery, Native SUMO1, and K2P1 at the PM. To form an isopeptide bond, the proform of mammalian SUMO1 ($SUMO1_{101}$) is matured by C-terminal proteolysis to 97 residues ($SUMO1_{97}$), activated by the SUMO activating enzyme complex SAE1/SAE2 and linked via a thioester bond to Cys93 of the conjugating enzyme Ubc9. Ubc9 catalyzes formation of a covalent bond between the terminal Gly of $SUMO1_{97}$ and the ε-amino group of target Lys. To locate native SUMO cascade proteins in CHO cells with confocal microscopy, the PM was identified by expression of GFP targeted to the inner leaflet (18) (Fig. 1*A*). Immunocytochemical staining showed endogenous SAE1, Ubc9, SUMO1 (Fig. 1*A*), and SUMO2/3 (Fig. S1) to colocalize with the PM marker in CHO cells, consistent with prior visualization of native Ubc-9 at the PM of *Xenopus* oocytes (2). As in oocytes (2), expressed human K2P1 channels were observed at CHO PM where they colocalized with native SUMO1 and the PM marker (Fig. 1*B*).

K2P1 Silenced by Native SUMO. K2P1 channels in the CHO cell PM were silent when recorded in whole-cell mode with pipettes containing physiological salt solution (Fig. 2*A*). However, addition of 100 pM recombinant SENP1 isopeptidase into the pipette reliably produced robust potassium currents (Fig. 2*B*). An inactive point mutant of the enzyme, SENP1-C603S, failed to elicit these currents (Fig. 2*C*). Mutating Lys274 to Gln (Fig. 2*D*), Arg, Glu, Asp, Cys, or Ala (Fig. S2) yielded large currents resembling those activated by SENP1 (Fig. 2*E*). As in oocytes (2), SENP1-activated K2P1 and Lys274Gln channels in CHO cells were inhibited by biological levels of external proton unless His122 in the first pore loop was mutated, here to Asn (Figs. 2*F* and Fig. S3). In accord with our study of single K2P1 channels in oocytes (2), both SENP1-activated K2P1 channels and Lys274Gln channels operated in CHO cells as openly-rectifying, potassium-selective pores (Fig. 2 *G* and *H* and Fig. S3).

SUMO Silencing Is Reversible. PM patches excised from CHO cells permit direct exposure of the cytoplasmic face of channels to various solutions. Fig. 3*A* shows that K2P1 channels remained silent in excised patches until SENP1 was applied. With 250 pM SENP1, K2P1 currents at 50 mV were 392 ± 33 pA, whereas

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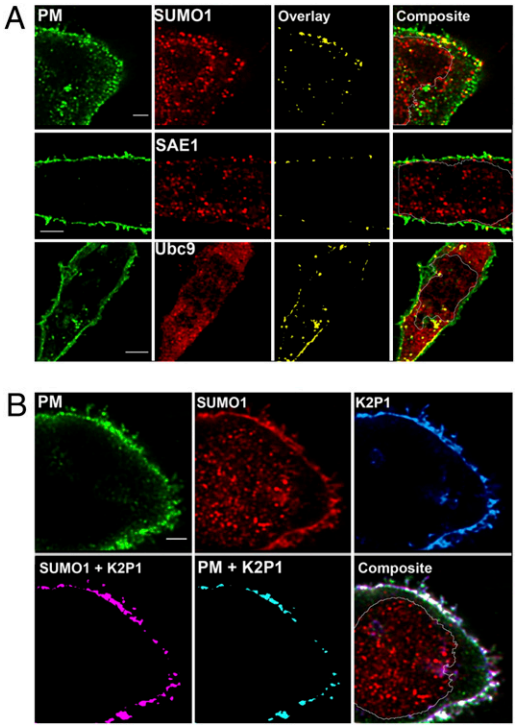

Fig. 1. Native SUMO pathway components at the PM. Colocalization of K2P1, SUMO1, and a PM marker. CHO cells expressing GFP targeted to the PM without (*A*) or with K2P1 carrying a tag (*B*) immunostained to identify GFP (green), SUMO1, SAE1, and Ubc9 (red) and K2P1 (blue) using confocal microscopy. Nuclear stain noted by white outline in composites. (Scale bar: 2 μm.) (*A*) Native SUMO1, SAE1 and Ubc9 (red) are visualized in the nucleus, cytoplasm, and colocalized with PM marker (*Overlay* in yellow). (*B*) Expressed K2P1 (blue) is observed to colocalize with a PM marker (PM + K2P1, light blue) and native SUMO1 (SUMO1 + K2P1, magenta). *Composite* shows colocalization of K2P1, SUMO1, and the PM marker (white).

pretreatment baseline current was just 12 ± 1 pA (Table S1). After removal of SENP1, subsequent exposure to 100 pM $SUMO1_{101}$ decreased current to baseline levels, reapplication of SENP1 released the channels from SUMO suppression restoring current to maximal level, and current magnitude was maintained despite SENP1 wash-out (Fig. 3*A*).

These results demonstrated that excised membrane patches carried, in addition to the K2P channels producing the measured potassium currents, the enzymes required for sumoylation. The ability of $SUMO1_{101}$ to silence K2P1 indicated its maturation to $SUMO1_{97}$, the form able to conjugate, and that SAE1/SAE2 and Ubc9 were available to activate and link $SUMO1_{97}$ to the channel. The need to apply SENP1 to activate the channels argued desumolase was not in the patches.

Consistent with a requirement for sumoylation to silence the channels, 250 pM inactive SENP1-C603S did not restore currents and channels silenced by $SUMO1_{101}$ remained quiescent despite washing away free $SUMO1_{101}$ (Fig. 3*B*). Moreover, neither $SUMO1_{95}$, a truncation mutant lacking the C-terminal motif required to conjugate to targets (Fig. 3*C*) (19), nor heat-inactivated $SUMO1_{101}$ silenced active channels (Table S1). Furthermore, neither the rate of rise nor peak current increased with SENP1 concentrations above 250 pM, silencing was more rapid with $SUMO1_{97}$ than with immature $SUMO1_{101}$, and $SUMO1_{101}$ suppressed currents fully with saturating, concentration-dependent rates. Finally, K2P1 channels with Lys274 mutated to Gln (Fig. 3*D* and Table S1), Arg, or Glu (Fig. S4) were constitutively active and insensitive to SUMO1 and SENP1.

Conjugation of SUMO1 to Lys274. To directly demonstrate sumoylation of K2P1 Lys274, we used an innovative bacterial system developed by others (20) to produce SUMO-target conjugates for structural studies. The method employs a tricistronic plasmid expressing SAE1/SAE2, Ubc9, and SUMO1. To facilitate MS, the construct was modified here to carry $SUMO1_{97}$T95K, a mutant that leaves a Gly-Gly remnant of SUMO on the ε-amino group of target lysine after trypsin treatment (21). $SUMO1_{97}$T95K was confirmed to regulate K2P1 channels like wild-type $SUMO1_{1–97}$ (Table S1 and Fig. S4). The C-terminal portion of K2P1 that is intracellular and carries Lys274 ($K2P1CT_{270–336}$) was coexpressed from a second plasmid. Affinity purification, SDS/PAGE and Coomassie blue staining, showed a band at the expected apparent mass, approximately 30 kDa and MS analysis confirmed the band to be $K2P1CT_{270–336}$–SUMO by excision, trypsin treatment, and spectrometry, yielding sequence coverage of 55% for K2P1 and 74% for SUMO (Fig. S5). The spectrum included a peak at 967.545 Da, the predicted mass of the trypsin fragment bearing

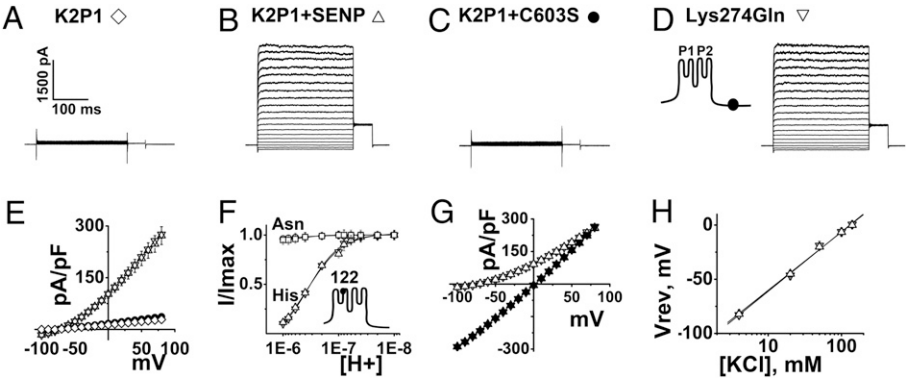

Fig. 2. The native SUMO1 machinery silences K2P1. CHO cells expressing various K2P1 channels were studied in whole-cell mode with 250-ms test pulses from −80 mV to −100 up to 80 mV every 5 s. Mean current-density ± SEM for groups of 8–12 cells; some error bars within symbols. (Scale bars: 1,500 pA, 100 ms.) (*A*) K2P1 is silent without SENP1 in the pipette; current at 50 mV was 25 ± 4 pA/pF (◇). (*B*) K2P1 currents induced by 100 pM SENP1 in the pipette; current at 50 mV was 206 ± 20 pA/pF (△). (*C*) K2P1 is silent with 250 pM SENP1-C603S in the pipette; current at 50 mV was 28 ± 4 pA/pF (●). (*D Left*) Schematic of a K2P1 subunit showing four transmembrane domains, two pore (P) loops and Lys274 on the intracellular C terminus. (*Right*) Lys274Gln channels are constitutively active; current at 50 mV was 210 ± 18 pA/pF (▽). (*E*) Mean current versus voltage plot, symbols as in *A–D*. (*F*) His122 mediates proton block of K2P1. Normalized current at 50 mV with changes in bath pH: K2P1 activated by 100 pM SENP1 (△), Lys274Gln channels (▽), His122Asn K2P1 activated by 100 pM SENP1 (○), and Lys274Gln–His122Asn channels (□). Solid lines are fits to $(1 + [B]/K_i)$-1, where B is proton concentration and K_i indicates half-block (pK_a). K2P1 activated by SENP1 and Lys274Gln channels were blocked with pK_a values of 6.71 ± 0.02 and 6.69 ± 0.03 and Hill coefficients of 0.96 and 0.97. (*Inset*) schematic of His122 in the first pore loop. (*G*) Active K2P1 channels are open-rectifiers. K2P1 with 100 pM SENP1 in 4 mM (△) and 140 mM (▲) bath KCl; Lys274Gln K2P1 channels recorded in 4 mM (▽) and 140 mM (▼) bath KCl. Reversal potentials in 4 mM and 140 mM KCl were −82 ± 4 mV and 0 ± 3 mV for both channels. (*H*) Active K2P1 channels are potassium selective. The reversal potential of K2P1 with 100 pM SENP1 (△) and Lys274Gln (▽) was studied with 4, 20, 50, 100, and 140 mM KCl solutions and linear regressions showed shifts of 53.8 ± 3 mM and 54.8 ± 3 mM per 10-fold change in KCl.

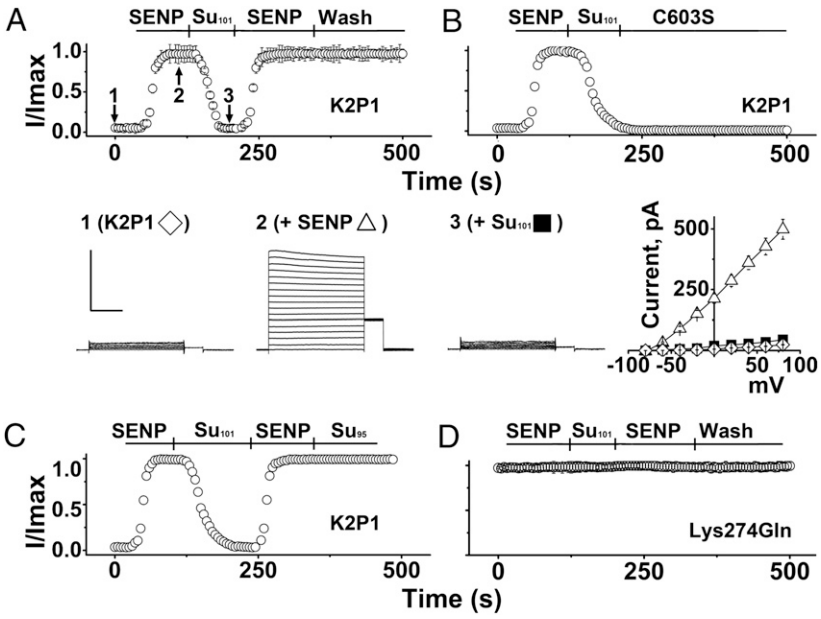

Fig. 3. SENP1 and SUMO1 reversibly modulate K2P1 via Lys274. K2P1 or K2P1 Lys274Gln channels in PM patches were excised from CHO cells and plots of normalized current ± SEM at 50 mV produced from 250-ms steps from a holding voltage of −80 mV every 5 s (or current families recorded by steps from −80 to 80 mV), n = 5–6. Some error bars are within symbols. (*A*) K2P1 current activated by 250 pM SENP1, suppressed by 100 pM $SUMO1_{101}$ (Su_{101}), and reactivated by 250 pM SENP1 is stable when peptides are washed away. (*Lower Insets*) Exemplar current families at times noted by arrows (1–3) and mean current-voltage plots for multiple patches (*Far Right* and Table S1). (*B*) K2P1 activated by 250 pM SENP1 and suppressed by 100 pM $SUMO1_{101}$ (Su_{101}) remains silent on application of 250 pM SENP1-C603S. (*C*) K2P1 activated by 250 pM SENP1, suppressed by 100 pM $SUMO1_{101}$ (Su_{101}), and reactivated by 250 pM SENP1 is insensitive to 250 pM $SUMO1_{95}$ (Su_{95}). (*D*) K2P1 Lys274Gln is active without added SENP1 and insensitive to application of 250 pM SENP1 or 100 pM $SUMO1_{101}$ (Su_{101}) (Table S1).

Lys274 linked via its side-chain to the Gly-Gly remnant of $SUMO1_{97}$T95K. Sequencing by MS/MS identified this three-ended product uniquely.

FRET Shows K2P1 and SUMO1 Interact via Lys274 at the PM in Live Cells. FRET between proteins tagged with GFP variants reports on separations of less than 100 Å. In live cells, this proximity is achieved only when proteins interact directly. Here, we measured macroscopic, donor decay time-course, a FRET strategy that limits inaccuracies due to differences in initial acceptor and donor intensities and acceptor bleaching. First, FRET between channel subunits was studied by tagging K2P1 at the N terminus, a site that tolerates GFP insertion (2), with yellow or cyan fluorescent protein (YFP-K2P1 and CFP-K2P1). The tagged subunits reached the PM when coexpressed (Fig. 4*A*). Demonstrating subunit assembly, donor decay was far longer for CFP-K2P1 expressed with YFP-K2P1 rather than untethered, cytosolic YFP (Fig. 4 *B* and *C*).

Demonstrating assembly of K2P1 channels and SUMO1, CFP-K2P1 showed FRET with YFP-SUMO1 but only when both proteins were capable of forming isopeptide bonds (Fig. 4 *C* and *D*). Thus, the CFP-K2P1 channel decay time-course was prolonged by YFP-$SUMO1_{101}$ but not by linkage-incompetent YFP-$SUMO1_{95}$. Further, CFP-K2P1 Lys274Gln channels failed to show FRET with YFP-$SUMO1_{101}$ even though they assembled at the PM like wild-type channels based on microscopy, FRET, and measurements of potassium currents. So, too, CFP-K2P1 subunits with Lys274 altered to Arg or Glu formed channels at the PM but failed to FRET with YFP-$SUMO1_{101}$ (Fig. S6).

The classical SUMO target RanGAP1 is sumoylated on Lys526 and must bear this residue to bind Ubc9 in vitro (22). A similar observation was made here for K2P1 in live CHO cells (Fig. 4*E*). FRET was measured between YFP-Ubc9 and CFP-K2P1 channels but not channels formed by CFP-K2P1 Lys274Gln subunits. Demonstrating that SUMO1 was not required for Ubc9 binding to K2P1, a point mutant of Ubc9 unable to forming thioester linkages also showed FRET with wild-type CFP-K2P1.

Counting Two SUMO Monomers on Single K2P1 Channels. If dimeric, K2P1 channels carry two Lys274 sites, one on each subunit. To confirm the stoichiometry of channels inferred by indirect methods (15, 16) and determine occupancy of K2P1 Lys274 sites by SUMO, we proceeded as follows. Total internal reflection fluorescence microscopy was used to excite single fluorescent channel particles in CHO cell PM and single-particle photobleaching was employed to quantify GFP-tagged proteins (Fig. 5 *A* and *B*). This method has been used to count subunits in ion channels in the PM of oocytes (23). First, the channels were confirmed to be dimeric: 90% of single channel particles formed with either GFP-K2P1 or GFP-K2P1 Lys274Gln subunits showed two stepwise decreases in fluorescence and 10% showed one bleaching step (Fig. 5*C*); with dimeric complexes one step is expected in 10–15% of particles based on prebeaching, simultaneous bleaching of two fluorophores and missed events, given the time resolution of the system (23) but could include channels with a single GFP-$SUMO1_{101}$. To count SUMO1 monomers on single K2P1 channels, untagged K2P1 and GFP-$SUMO1_{101}$ were coexpressed (Fig. 5*D*). In this case, two bleaching steps were observed for 85% of particles and 15% showed one step, indicating two GFP-$SUMO1_{101}$ proteins per channel. As expected, K2P1 Lys274Gln channels expressed with GFP-$SUMO1_{101}$ yielded no fluorescent PM particles, providing additional evidence that linkage at Lys274 was required for stable assembly of SUMO1 with K2P1 channels at the PM.

One SUMO Is Sufficient to Silence a K2P1 Channel. To determine if SUMO-induced silencing required sumoylation of both Lys274 sites, tandem constructs were produced encoding two K2P1 subunits in series (Fig. 6*A*). Channels with two Lys274 sites (Lys–Lys) showed two bleaching steps when coexpressed with GFP-$SUMO1_{101}$ indicating two SUMO1 molecules on each channel (Fig. 6*B*). In contrast, tandems with one Lys274 and one Lys274Gln site (in either order) showed a single bleaching step, indicating a single associated SUMO1 monomer.

As expected, Lys–Lys subunits formed channels that operated like those formed of wild-type K2P1 subunits and Gln–Gln channels were constitutively active and insensitive to SENP1 and $SUMO1_{101}$ (Fig. S7). Notably, Lys–Gln and Gln–Lys channels that carried just one SUMO1 also functioned like wild type (Fig. 6*C*). Thus, both channels with a single Lys274 site were silent in excised PM patches, activated by 250 pM SENP1, insensitive to linkage-incompetent $SUMO1_{95}$, rapidly resilenced by $SUMO1_{101}$, and unresponsive to catalytically-inactive SENP-C603S (C603S).

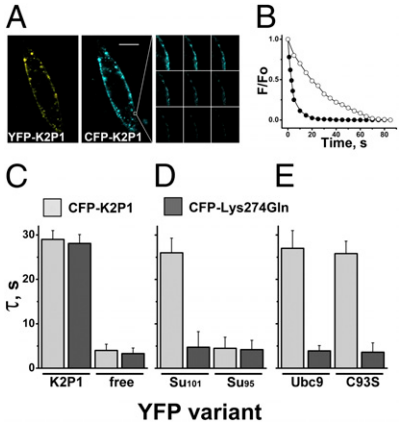

Fig. 4. SUMO1 interacts with K2P1 via Lys274 in PM of live CHO cells. CFP-fused to K2P1 or Lys274Gln was studied with YFP-fused to K2P1, SUMO1, or Ubc9 variants. Data are mean time-constant for CFP decay ± SEM for 2–5 areas of 5–7 cells with continuous excitation. (*A*) CHO cells coexpressing YFP-K2P1 (*Left*) and CFP-K2P1 (*Center*). (*Right*) An expanded view of the indicated area and shows decay of CFP-K2P1 fluorescence. Images are captured every 5 s. (Scale bar: 10 μm.) (*B*) Fit for study in *A*. Normalized fluorescence intensity versus time for CFP-K2P1 coexpressed with YFP-K2P1 (○) or free YFP (●) fit by a single exponential to give a decay time-constant (τ). (*C*) Mean τ ± SEM shows assembly of channels with CFP-K2P1 and YFP-K2P1 (gray, τ = 28.4 ± 3.3 s) or CFP-Lys274Gln and YFP-Lys274Gln (black, τ = 27.6 ± 2.8 s) when compared to decay for the CFP-subunits expressed instead with free YFP (τ = 4.5 ± 3.1 s). (*D*) Mean τ ± SEM shows interaction of K2P1 and SUMO to require Lys274 and a SUMO competent to form isopeptide bonds. CFP-K2P1 interacts with YFP-SUMO1$_{101}$ (Su$_{101}$) (gray, τ = 26.8 ± 2.0 s) but not YFP-SUMO1$_{95}$ (Su$_{95}$) (gray, τ = 5.1 ± 3.9 s), whereas CFP-Lys274Gln does not interact with YFP-SUMO1$_{101}$ (black, τ = 4.8 ± 2.4 s) or YFP-SUMO1$_{95}$ (black, τ = 4.3 ± 2.2 s). (*E*) Mean τ ± SEM shows assembly of YFP-Ubc9 with CFP-K2P1 (gray, τ = 28.4 ± 4.0 s) but not CFP-Lys274Gln (black, τ = 4.1 ± 1.8 s). YFP-Ubc9-Cys93Ser (C93S) with a point mutant that precludes thioester linkage to SUMO interacts with CFP-K2P1 (gray, τ = 25.4 ± 3.2 s) but not CFP-Lys274Gln (black, 4.3 ± 2.8 s).

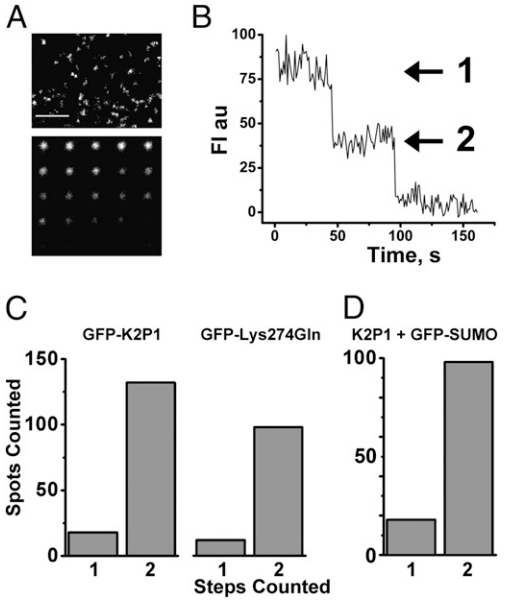

Fig. 5. Two SUMO1 monomers associate with K2P1 channels in CHO cell PM. GFP-tagged subunits were studied in CHO cell PM by TIRF and single-particle photobleaching to count subunits. Discrete particles in cells expressing GFP-K2P1 and GFP-SUMO1$_{101}$ were similar (n = 5–7 cells). (*A Upper*) Fluorescent particles in a cell expressing GFP-K2P1. (*Lower*) Decrease in fluorescence of one particle at 10 s intervals with continuous excitation. (Scale bar: 60 pixels.) (*B*) The time-course of photobleaching for a representative GFP-K2P1 fluorescent particle showing two bleaching steps as expected for a dimeric channel. (*C*) A histogram showing that 88% of 150 GFP-K2P1 (*Left*) and 89% of 110 GFP-Lys274Gln particles (*Right*) had two bleaching steps. (*D*) Bleaching steps for GFP-SUMO1$_{101}$ expressed with untagged K2P1 channels show that 85% of 120 fluorescent particles had two bleaching steps. No discrete, immobile particles were observed when GFP-SUMO1$_{101}$ was coexpressed with K2P1 Lys274Gln channels.

Discussion

In this report, we directly demonstrate that the SUMO pathway operates at the PM of living mammalian cells to regulate K2P1 channels, a conclusion we presented previously (2) that has been subject to challenge by Feliciangeli et al. (7, 8). Here, aspects of this controversy are considered.

Is the SUMO Cascade at the PM? The answer is yes. Immunocytochemical reporters unambiguously reveal native components of the SUMO conjugation machinery (SAE1, Ubc9, SUMO1, and SUMO2/3) to be resident at the PM of CHO cells (Fig. 1 and Fig. S1). This accords with our observation of Ubc9 at the PM of oocytes (2), work by others showing Ubc9 in dendrites of cultured hippocampal neurons (5), and experiments demonstrating SUMO attached to septins that form a filamentous ring encircling yeast bud neck during mitosis (24). Microscopy further demonstrates expressed human K2P1 localizes at the CHO cell PM with a membrane marker where it colocalizes with native SUMO1 (Fig. 1). Electrical silencing of SENP1-activated K2P1 channels in excised PM patches by applied SUMO1$_{97}$ (Table S1) demonstrates SAE1/SAE2 that activates SUMO1 and Ubc9 that conjugates it to the channel remain in membrane patches after excision. Channel silencing by direct application of SUMO1$_{101}$ (Fig. 3 and Table S1) shows that a protease that matures the protein to SUMO1$_{97}$ is also stably associated with the PM. Classically, SENPs are held responsible for both SUMO maturation and cleavage of isopeptide bonds to desumoylate target (25). That K2P1 channels in off-cell patches remain inactive until soluble SENP1 is applied suggests maturation and desumoylation are mediated by separate enzymes in CHO cell PM.

Why Do Feliciangeli and Coworkers Fail to Observe SUMO Regulation? Controversy has attended K2P1 from the outset. A year after its cloning, Lesage et al. (26) described K2P1 in oocytes as small currents that were insensitive to lowered bath pH and showed weak inward rectification (TWIK); we and others attributed this to endogenous flux arguing the channel to be silent (27). A decade later, we showed in oocytes and COS-7 cells (2), that SENP1 treatment activated K2P1 to yield robust, openly-rectifying, potassium-selective currents like those of the canonical K2P background channel in *Drosophila melanogaster*, K2PØ (11–13, 16). Thus, K2P1 rectified as expected for an open, potassium-selective portal exposed to unequal levels of permeant ion on opposite sides of the membrane. In further distinction, K2P1 current was blocked by external proton (pK_a approximately 6.7) by a mechanism held in common with the "acid-sensitive" K2P channels K2P3 and K2P9 (also called TASK1 and TASK3) (15), that is, via titration of a His122 in the first pore loop (2). We also demonstrated that mutation of K2P1 Lys274 activated the channels and made them resistant to SUMO-mediated silencing (2).

Subsequently, in several reports (7, 8), Feliciangeli and colleagues failed to observe an effect of SENP1 on K2P1 in oocytes and COS-7 cells. Although they did observe currents with the K2P1 mutant Lys274Glu, substitution to Arg did not yield currents in their hands. This led them to propose that K2P1 currents were small due to intracellular sequestration of the channels, an apparent contradiction to their own prior reports. We suggest the discrepancies can be rationalized by unrecognized technical

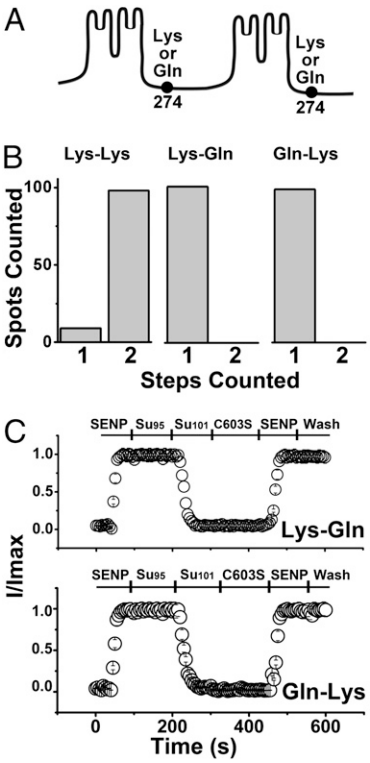

Fig. 6. One SUMO1 is sufficient to silence K2P1 channels. Tandem channels with K2P1 (Lys) and/or Lys274Gln (Gln) subunits were studied in CHO cells with GFP-$SUMO1_{101}$ as in Figs. 3 and 5. (*A*) Drawing of K2P1 subunits linked in tandem noting the location of the first and second Lys274 sites. (*B Left*) As for wild-type K2P1 (Fig. 5B), two bleaching steps were recorded in 90% of 90 spots with GFP-$SUMO1_{101}$ and Lys–Lys channels. (*Center*) One step was observed in all 105 particles studied with GFP-$SUMO1_{101}$ and Lys–Gln channels. (*Right*) One step was also seen for all 102 particles studied with GFP-$SUMO1_{101}$ and Gln–Lys channels. (*C*) Lys–Gln channels (*Upper*) and Gln–Lys channels (*Lower*) are silent at 50 mV in excised patches (18 ± 6 pA and 22 ± 4 pA, respectively) until activated by 250 pM SENP1 (332 ± 15 and 345 ± 21 pA, respectively). Activated channels were insensitive to 250 pM $SUMO1_{95}$ (Su_{95}) but suppressed by 100 pM $SUMO1_{101}$ (Su_{101}). Silent channels were insensitive to application of 250 pM SENP1-C603S (C603S), whereas reapplication of 250 pM SENP1 restored channel activity in a manner that was stable to extended washing (Wash). Electrophysiological data are normalized, mean ± SEM currents. Error bars are within some symbols; $n = 5$ for each channel type.

difficulties in their experiments. The K2P1 currents they report in mammalian cells are very small (2-8 pA/pF) even after mutation of Lys274 to Glu (10-16 pA/pF) compared to our values for wild-type channels exposed to SENP1 (approximately 280 pA/pF) (Fig. 2) or Lys274 mutation (approximately 275–300 pA/pF) (Fig. 2 and Fig. S2). Further, the currents they describe are neither shown to be carried by potassium ions or sensitive to external acidification. Notable differences between our approaches include their use of K2P1 subunits bearing a unique protein tag (HcRed) and whole-cell recording (7, 8). Whenever feasible, we use untagged wild-type K2P1 and record in excised patch mode (as previously with oocytes with patch-cramming (2) or here using CHO cells and direct application of purified reagents) to mitigate confounding effects of mutations and/or over-expression of SUMO pathway components. We also prefer to study K2P channels in mammalian cells to avoid batch and seasonal variation that attends use of oocytes and favor CHO cells that have low natural levels of PM potassium current. Feliciangeli and colleagues (8) posit K2P1 silencing is brought about by dynamin-mediated trafficking into endosomes and see little protein in the PM compared to our studies (Fig. 1). We agree that trafficking contributes to modulation of K2P channel surface expression (28), as it does for many membrane proteins, but consider this peripheral to functional regulation of K2P1 proteins present in PM by the SUMO pathway.

The inability of Feliciangeli et al. (7) to obtain biochemical evidence of K2P1 sumoylation is unsurprising. Isolation of conjugates from live cells often yields a small fraction of target bearing SUMO when functional evidence indicates near complete sumoylation. This discrepancy is dubbed the SUMO enigma (1). Possible explanations include lysis of isopeptide bonds on cell fractionation and rapid turnover because some SUMO-target linkages are transient and in dynamic equilibrium with stable, noncovalent assemblies (19). Indeed, even the established substrates p53 and Stat1 are difficult to isolate as SUMO conjugates unless synthesized as fusion products with Ubc9 (29). We purified K2P1 subunits conjugated to SUMO1 monomers via Lys274 from oocytes (2) but have not isolated them using CHO, COS-7, or HeLa cells. This mirrors studies of other PM proteins purified from mammalian cells by detergent solubilization (3–6).

This work offers five lines of evidence for SUMO regulation of K2P1 channels in PM, supporting our prior conclusions (2), using mammalian cells and tools that overcome the enigmatic limitations of studying SUMO biology via cell solubilization: microscopic colocalization, off-cell patch recording with recombinant SUMO pathway components, mass spectrometry, FRET, and single particle TIRF. Three of the five methods allow direct, real-time evaluation of channel populations or single protein complexes in living cells and are applicable to other PM proteins regulated by sumoylation.

Additional Insights into SUMO Regulation of K2P1. This article supports four mechanistic conclusions beyond those reported previously (2). Loss of FRET between CFP-K2P1 and YFP-SUMO1 with conjugation-defective channels or SUMO variants indicates that Lys274 is the only site that mediates SUMO regulation (Fig. 4). The absence of polymeric chain formation and of non-Lys274 sites of SUMO attachment in CHO cells is established by observation of no more than two single-particle photobleaching steps for channels with two Lys274 sites and no more than one for those with one Lys274 (Figs. 5 and 6). One SUMO1 can silence a K2P1 channel (Fig. 6). That antibody-recognizing native SUMO2 and SUMO3 stain CHO cell PM (Fig. S1) and that both SUMO2 and SUMO3 can silence K2P1 (Fig. S8) indicates SUMO1 is not unique among SUMOs in its operation at the PM or ability to regulate K2P1.

Implications for the K2P1 Biology. As K2P1 is prominent in the heart and central nervous system, SUMO is anticipated to influence these tissues. Indeed, ongoing studies show sumoylation to regulate K2P1 in the PM of cerebellar granule neurons and, via heteromeric assembly, K2P3 and K2P9 subunits that are implicated in responses to general anesthetics, oxygen tension, and noxious stimuli (9, 30). Factors that modulate the SUMO pathway in the nucleus and cytosol are expected to impact PM targets. We postulate, therefore, that changes in temperature and oxidative stress will alter sumoylation of K2P1 channels. SUMO is also expected to vary K2P1 trafficking and half-life as with classical targets, GluR6 receptors (5) and TRPM4 channels in a progressive form of heart block (6). Our demonstrations that SUMO controls rapid PM responses of ion channels and its known role in regulating transcription suggests the pathway may serve to couple acute and long-term cellular responses to environmental change.

Materials and Methods

Please see *SI Materials and Methods* for further information.

Molecular Biology and Production of Peptides. Human K2P1 [NP_002236, $SUMO1_{101}$ (Acc. BCOO5899) and SENP1 (Acc. BC045639)] was subcloned into

pMAX, a vector with a CMV promoter as described (2). YFP and CFP genes (Clontech) were inserted into $SUMO1_{101}$ or K2P1 at the N termini as described for EGFP (2). SENP1 and SENP1-C603S were produced as GST fusions as before (2). $SUMO1_{101}$, $SUMO1_{95}$, $SUMO1_{97}$, $SUMO_{97}$-Thr95Lys were produced using pET28a with 6 His-tags and a tobacco itch virus (TEV) cleavage domain.

Cell Culture, Heterologous Expression, and Immunocytochemistry. CHO cells (ATCC) were transfected with Lipofectamine 2000 (Invitrogen). To label PM, lck-GFP (18) was expressed. For triple labeling, K2P1 carrying a nine-residue rho1d4 tag at the C terminus was also expressed. Cells were fixed with 4% paraformaldehyde and permeabilized with Triton X-100. Primary antibodies were chicken anti-GFP (1:10,000, ab13970; Abcam), mouse anti-rho1d4 (1:5,000, University of British Columbia), and rabbit antibodies to SUMO1 (1:250; ab32058; Abcam), SUMO2/3 (1:100, ab3742; Abcam), SAE1 (1:100, ab58383; Abcam) and Ubc9 (1:100, ab75854; Abcam). Antibody binding was detected with cross-adsorbed, species-specific secondary antibodies labeled with Alexa 488, 594, or 647. A Leica SP5 laser scanning confocal microscope was used and images analyzed with ImageJ.

Electrophysiology. An Axopatch 200B amplifier and pCLAMP software (Molecular Devices) at filter and sampling frequencies of 5 and 25 kHz were used. Excised patch studies used a bath solution (in mM): KCl 136, $MgCl_2$ 1, K_2ATP 2, EGTA 5, hepes 10, pH 7.2 (KOH). Electrodes were borosilicate glass (Clark) with resistances of 1.5–2 MΩ when filled with (in mM) $CaCl_2$ 1.3, $MgCl_2$ 0.5, $MgSO_4$ 0.4, KCl 3.56, KH_2PO_4 0.44, NaCl 139.7, Na_2HPO_4 0.34, glucose 5.5, Hepes 10, pH 7.4 with NaOH. For whole-cell recording, 4- to 5-MΩ electrodes were filled with bath solution and CHO cells superfused with electrode solution. Electrodes were coated with Sigmacote (Sigma).

FRET. Using an automated Olympus IX81 fluorescence microscope, CFP was excited at 458 nm and the emission collected through a 470- to 500-nm bandpass filter. YFP was excited at 514 nm and the emission collected through a 525- to 575-nm filter. Experiments used Metamorph (Molecular Devices) and images captured using a CCD camera and analyzed with ImageJ.

Single Particle Photobleaching. GFP was excited by a 488-nm, 10-mW Argon laser (Melles Griot) and the critical angle for TIRF obtained with a micrometer and a 150×, 1.45-NA apochromat objective (Olympus) on an Olympus IX70 microscope. To observe single particles, GFP-fused subunits were expressed at low levels. Constant excitation induced photobleaching and movies of 300–500 frames acquired at 10 Hz using a back-illuminated EM-CCD camera. Data were analyzed as per ref. 23. Average background fluorescence was determined from the first five images, subtracted from the movie, fluorescence summed for a 4×4 pixel region around the peak of each particle and analyzed over time with ImageJ and Origin v6 software.

Mass Spectrometry. K2P1 residues 270–336 were coexpressed in *Escherichia coli* with pT-E1E2S1(Thr95Lys), a vector carrying mouse SAE1 and SAE2 (as a linear fusion of Aos1 and Uba2), Ubc-9 (20) that was altered to bear $SUMO1_{97}$(Thr95Lys) (21). Proteins were purified, digested and analyzed on a Dionex Ultimate 3000 Nano-HPLC and LTQ-FT tandem MS (Thermo Instruments) running Xcalibur ver2.2.

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