**Kelch-like 1 protein upregulates T-type currents by an actin-F dependent increase in α_{IH} channels via the recycling endosome**

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**Abbreviations:** KLHL1, Kelch-like 1 protein; Lat B, latrunculin B; Jas, jasplakinolide; BFA, brefeldin A; ABP, actin-binding protein; ARF, ADP ribosylation factor

The neuronal protein Kelch-like 1 (KLHL1) is a novel actin-binding protein that modulates neuronal structure and function. KLHL1 knockout mice exhibit dendritic atrophy in cerebellar Purkinje neurons and motor dysfunction. Interestingly, KLHL1 upregulates high and low voltage-gated calcium currents (Ca_{2.1} and Ca_{3.2}) and interacts with their respective principal subunits, α_{La} and α_{H}. We reported the mechanism of enhanced Ca_{3.2} (α_{H}) current density (and calcium influx) by KLHL1 is due to an increase in channel number (N) that requires the binding of actin. In this report we further elucidate the role of the actin cytoskeleton in this process using pharmacological tools to disrupt or stabilize actin filaments and to prevent protein trafficking and vesicle recycling. Disruption of the cytoskeleton did not affect the basal activity of α_{H}, but did eliminate its modulation by KLHL1. In contrast, actin-F stabilization on its own increased basal α_{H} activity similar to KLHL1 but without synergy in its presence, suggesting KLHL1 requires actin-polymerization to increase α_{H} currents. Noise analysis revealed that actin polymerization induced an increase in N and P_o, in contrast to increased N in the presence of KLHL1. Interestingly, pharmacological or genetic disruption of endosomal recycling eliminated the increase in channel number by KLHL1 demonstrating this effect occurs via enhanced α_{H} re-insertion through the recycling endosome. Our findings afford insight on a novel mechanism of T-type channel modulation that could have overall functional implications for T-type channel function in the brain.

**Introduction**

The actin cytoskeleton is an important modulator of neuronal structure and function, including cellular excitability by trafficking channels and receptors to the membrane and modulating their function via interactions with actin-binding proteins (ABP).¹-⁵ ABPs bind to actin monomers or to actin filaments to form intracellular structures and networks,² and they also play a crucial role in intracellular trafficking, endocytosis and exocytosis;³ in fact, along with myosin, actin and ABPs may help propel endocytic vesicles through the cytosol.⁴-¹² ABPs can also anchor actin to membrane complexes, act as linkers between actin and other cytoskeletal elements, or indirectly modulate neuronal excitability by influencing channels and transporters.²,¹³ The modulation of voltage-gated ion channels by actin and ABPs is well established for various channel includes sodium, K_Ca, and L-type calcium channels, among others.¹⁴-¹⁷

KLHL1 is a member of the Kelch superfamily of proteins, it contains their two signature motifs: a BTB/POZ domain involved in protein-protein interactions, and a Kelch β-propeller region involved in actin binding.¹⁸,¹⁹ This neuronal ABP is involved in neuronal structure and function; its genetic elimination results in dendritic atrophy in cerebellar Purkinje neurons and motor dysfunction. Interestingly, KLHL1 upregulates high and low voltage-gated calcium currents (Ca_{2.1} and Ca_{3.2}) and interacts with their respective principal subunits, α_{La} and α_{H}. We reported the mechanism of enhanced Ca_{3.2} (α_{H}) current density (and calcium influx) by KLHL1 is due to an increase in channel number (N) that requires the binding of actin. In this report we further elucidate the role of the actin cytoskeleton in this process using pharmacological tools to disrupt or stabilize actin filaments and to prevent protein trafficking and vesicle recycling. Disruption of the cytoskeleton did not affect the basal activity of α_{H}, but did eliminate its modulation by KLHL1. In contrast, actin-F stabilization on its own increased basal α_{H} activity similar to KLHL1 but without synergy in its presence, suggesting KLHL1 requires actin-polymerization to increase α_{H} currents. Noise analysis revealed that actin polymerization induced an increase in N and P_o, in contrast to increased N in the presence of KLHL1. Interestingly, pharmacological or genetic disruption of endosomal recycling eliminated the increase in channel number by KLHL1 demonstrating this effect occurs via enhanced α_{H} re-insertion through the recycling endosome. Our findings afford insight on a novel mechanism of T-type channel modulation that could have overall functional implications for T-type channel function in the brain.

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Here we explored the role of the actin cytoskeleton on this novel form of T-type channel modulation and the possibility that intracellular trafficking may underlie the increase in $\alpha_{1H}$ channel number induced by KLHL1. We used pharmacological tools to stabilize or disrupt the actin cytoskeleton (Jasplakinolide, Jas or Latrunculin B, Lat B). Lat B is a macrolide isolated from sea sponges that sequesters monomeric actin into a non-polymerizable complex, thus promoting the depolymerization of F-actin.23,24 Jas, in contrast, promotes polymerization and stabilizes actin filaments by capping the dissociating end.23,25 We also assayed if disruption of intracellular channel trafficking using brefeldin A (BFA) would interfere with the effect of KLHL1. This macrocyclic lactone inhibits the activation of ARF-GTPases and blocks the recruitment of clathrin-coated vesicles that require class I ADP ribosylation factors (ARFs) 1-3; BFA thus disrupts transport at the trans-Golgi network (TGN) and the function of the recycling endosome, thereby altering de novo protein trafficking, secretion and protein recycling to the plasma membrane.26-30 Endosomal recycling function was also tested using the Rab11 dominant-negative mutant S25N, which specifically prevents the transport of cargo from the recycling endosome to the membrane.31,32

Overall, the biophysical properties and noise analysis of $\alpha_{1H}$ indicate actin polymerization with Jas increases basal $\alpha_{1H}$ current density by increasing the P_{o} of channels already present at the membrane and also by an increase in channel number. In contrast, $\alpha_{1H}$ upregulation by KLHL1 necessitates polymerized actin to exert its effect, which results mainly from an increase of the channel number at the membrane via re-insertion through the recycling endosome.

**Results**

Actin polymerization mediates the effect of KLHL1 on $\alpha_{1H}$. We previously reported that a KLHL1 mutant lacking the actin-binding kelch domains fails to elicit the increase in $\alpha_{1H}$ current density, N and $\tau_{\text{deactivation}}$ seen with wild type KLHL1; although changes in calcium influx upon high frequency stimulation remain (reviewed in ref. 21), suggesting actin has an important role in this regulation. To evaluate the role of the actin cytoskeleton on the channel modulation by KLHL1, we first assessed whether actin-F is necessary using the destabilizing cytoskeleton on the channel modulation by KLHL1, we first assessed whether actin-F is necessary using the destabilizing agent Latrunculin B.23,24,38,39 Incubation with 2 $\mu$M Lat B for 20 min prior to electrophysiological tests induced the expected de-polymerization,39,40 seen as a decrease in actin staining at the membrane with punctate distribution of actin in the cytosol,39,41 as shown in Figure 1A. This treatment did not affect basal $\alpha_{1H}$ currents, as observed in the example current traces shown in panel B. However, disruption of actin-F by Lat B clearly prevented the increase in current density observed in the presence of KLHL1 (panel C). Figure 1D shows the I-V relationships in the presence of Lat B. Currents peaked at -25 mV with a mean value of -30.3 ± 2.6 pA/pF in control cells ($\alpha_{1H}$ only, n = 21, solid black line), compared to -30.2 ± 3.1 pA/pF in the presence of $\alpha_{1H}$ + Lat B (empty circles; n = 15, p > 0.05). KLHL1-expressing cells treated with Lat B had a peak current density of -32.6 ± 3.9 pA/pF (grey circles) (n = 13; p > 0.05); the effect of KLHL1 is shown for comparison in dashed lines (-42.2 ± 2.7 pA/pF, n = 19; p = 0.007). Likewise, the effect of KLHL1 on $\alpha_{1H}$ activity-dependent calcium influx was prevented by Lat B (Suppl. Fig. S1A) as was the change in $\tau_{\text{deactivation}}$ seen in the presence of KLHL1 (Suppl. Fig. S1B); no other biophysical properties were altered ($\tau_{\text{on}}$, $\tau_{\text{off}}$, steady-state activation and inactivation; Suppl. Fig. S1C–F), including single channel properties studied with noise analysis (Suppl. Table 1). These data suggest the actin cytoskeleton does not normally modulate $\alpha_{1H}$ currents and that the presence of actin polymers is necessary for the effect of KLHL1 on T-type currents.

We next tested the effect of the actin filament-stabilizing agent Jasplakinolide. Again, a dose-response curve was performed to find the minimum incubation time necessary to elicit polymerization; care was taken to use the lowest dose and incubation times necessary to elicit the stabilizing effect.23 Treatment with 1 $\mu$M Jas 1 hour prior to recordings elicited the expected thickening of actin filaments located near the cell membrane, as revealed by the phalloidin staining shown in Figure 2A.23,35,42,43 Interestingly, Jas treatment significantly increased $\alpha_{1H}$ current density on its own (Fig. 2B and D), with peak values of -42.3 ± 3.8 pA/pF for $\alpha_{1H}$ + Jas vs. -30.3 ± 2.6 pA/pF in control conditions (n = 21 and 16; p < 0.05). The current density increase was indistinguishable from that observed in the presence of KLHL1 (-42.2 ± 2.7, n = 19) (Fig. 2B); in fact, when KLHL1-expressing cells were subjected to Jas treatment, the peak $\alpha_{1H}$ current density could not be further enhanced in the presence of the ABP (Fig. 2C), and
Figure 2. Stabilization of filamentous actin mimics the effect of KLHL1 on α1H current density. (A) Visualization of the actin cytoskeleton using TRITC-conjugated phalloidin with and without treatment with 1 μM of Jasplakinolide (Jas) (size bar = 10 μm). (B) Example α1H current traces (Vt = -25 mV) of cells treated with Jas (grey trace); control cells (α1H, black line) and α1H + KLHL1 (dashed line) are shown for comparison (B and C). (C) KLHL1 and Jas do not synergize; +KLHL1 + Jas, grey circles; α1H + KLHL1 and Jas (grey circles); α1H + KLHL1 (dashed line) overlaps with the Jas and KLHL1 + Jas I-V curves.

Figure 3. Biophysical property changes induced by Jas. (A) Effect of Jas on calcium entry at 10 and 100 Hz stimulus frequency. Data is normalized to control values; 1, 10 and 100 = spike number. (B) Steady-state inactivation and activation properties. Treatment with Jas induces a left-shift in steady-state activation. (C) τ on is decreased in the presence of Jas; α1H (black line), α1H + Jas and α1H + +Jas + KLHL1 (grey trace). Right, Summary of τ on at various voltages: α1H + Jas (empty circles); α1H + KLHL1 + Jas (grey circles).

Figure 4A shows representative current traces used for noise analysis and their was similar to that of each individual treatment (-42.7 ± 3.9 pA/pF, n = 13; p < 0.05 vs. control) (Fig. 2D). Thus, actin polymer stabilization induces current density increases to a plateau level that cannot be further augmented by the presence of KLHL1, suggesting that stabilization of the actin cytoskeleton underlies the KLHL1-mediated enhancement of α1H current density.

Use-dependency. Co-expression of KLHL1 with α1H induced increases in calcium influx during trains of action potentials, and not surprisingly Jas also increased calcium influx at all spikes during 10 and 100 Hz trains of action potentials. Figure 3A shows the summary of use-dependent calcium influx data in the presence of Jas. It induced calcium influx increases ranging from 64 to 88% at all spikes compared to control, statistically similar to the effect of KLHL1 (66–89% increase) and to the co-treatment with KLHL1 and Jas (60–80%), as seen in the figure and in Table 1.

Biophysical properties. We further explored the effect of actin polymer stabilization on the biophysical properties of α1H. Despite the fact that Jas exerts a similar increase in current density as KLHL1, there were significant differences with respect to their effects on the current properties. KLHL1 altered the τ deactivation of α1H without further effects on its activation and inactivation kinetics (τ on and τ off) or its steady-state properties. In contrast, treatment with Jas did not alter the τ on or τ deactivation of α1H on its own but eliminated the τ deactivation changes induced by KLHL1 (Suppl. Fig. S2A and B). In addition, Jas induced a decrease in α1H, τ off value at -30 mV to 18.5 ± 1.2 ms (n = 14) from 24.1 ± 1.9 ms in control (n = 13; p < 0.05); co-expression of KLHL1 and Jas treatment also resulted in τ off values of 17.8 ± 1.4 ms (n = 14; p < 0.04), as depicted in Figure 3C (for reference KLHL1 τ off = 24.1 ± 1.5 ms). Overall, the results suggest that chemically induced polymerization is a stronger stimulus than KLHL1 and overrides its effect when Jas is also present.

Steady-state activation. As seen in Figure 3B, treatment with Jas (empty circles) induced a left shift in V50 from -32.7 ± 0.9 mV (n = 21) to -38.2 ± 1.7 mV (n = 11; p < 0.01), in contrast to KLHL1 treatment, which did not alter the SSA properties (-33.5 ± 0.8 mV, n = 26). Co-expression of KLHL1 and Jas treatment similarly resulted in V50 of -40.5 ± 1.4 mV (grey circles) (n = 12; p < 0.001). The slope factor (k) was not affected in the presence of KLHL1 alone (6.6 ± 0.2 vs. 6.3 ± 0.4; n = 21 and 26; p = 0.32) or Jas (5.7 ± 0.5 n = 11; p > 0.05); however, KLHL1 co-expression and Jas treatment changed this value to 4.6 ± 0.4 (n = 12; p < 0.001).

Steady-state inactivation V50 values, on the other hand were not altered with any of the treatments. V50 values were -59.3 ± 1.3, -58.5 ± 1.2 and -58.9 ± 1.4 mV for control α1H, α1H + Jas and α1H + KLHL1 + Jas (n = 13, 10 and 12, respectively; p > 0.05). Slope values did not change either (k = -6.9 ± 0.4, -5.8 ± 0.3 and -6.0 ± 0.3 for control, Jas and KLHL1 + Jas; n = 13, 10, 12; p > 0.05), for reference, the slope value of α1H + KLHL1 was -6.2 ± 0.3, n = 16, p = 0.11.

Non-stationary noise analysis. Figure 4A shows representative current traces used for noise analysis and their
Figure 4. Non-stationary noise analysis. (A) Example traces elicited with steps from $V_h = -90$ mV to $V_t = -30$ mV (top) with their corresponding variance vs. current plots ($\alpha_1h$, $\alpha_1h + Jas$ and $\alpha_1h + KLHL1 + Jas$) fitted with the function $\sigma^2 = i - i^2/N$. (B) Summary of noise analysis properties.

| Panel B summarizes the data obtained when cells co-expressing $\alpha_{1H}$ or $\alpha_{1H} + KLHL1$ were treated with 1 $\mu$M Jas (rows 3 and 4); the data obtained for $\alpha_{1H}$ and $\alpha_{1H} + KLHL1$ are included in the first two rows for comparison. As discussed earlier, KLHL1 co-expression with $\alpha_{1H}$ induced an increase in the channel number N (column 4) without modifying the channels’ unitary current ($i$), conductance ($\gamma$) or open probability ($P_o$) (this latter value showed a non-significant tendency to increase). In contrast, the macroscopic current increases seen by actin polymerization with Jas resulted from increases in both N and $P_o$ values.

As expected from the current-density data, co-treatment of $\alpha_{1H} + KLHL1$ with Jas did not further increase the number of channels, suggesting this value has indeed reached a plateau. The $P_o$ was still higher than control, in line with a direct effect of Jas on the channels present at the membrane.

In summary, actin polymerization by itself induced a similar macroscopic effect on $\alpha_{1H}$ current density and calcium influx as KLHL1. However, the biophysical changes evoked by KLHL1 or Jas were different from each other: actin polymerization directly affects basal $\alpha_{1H}$ currents, and its mechanism of action involves an increase in $P_o$ and an increase in channel number, in addition to a decrease in $\tau_{off}$. KLHL1, on the other hand, requires actin-F to exert its action which is mediated by an increase N; KLHL1 also directly interacts with $\alpha_{1H}$ and induces an increase in $\tau_{deactivation}$. When both actin polymerization with Jas and KLHL1 were present there was no synergy.

Figure 4. Non-stationary noise analysis. (A) Example traces elicited with steps from $V_h = -90$ mV to $V_t = -30$ mV (top) with their corresponding variance vs. current plots ($\alpha_1h$, $\alpha_1h + Jas$ and $\alpha_1h + KLHL1 + Jas$) fitted with the function $\sigma^2 = i - i^2/N$. (B) Summary of noise analysis properties.

### Table I. Calcium influx (-pC/pF)

|       | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Hz    | 10    | 100   | 10    | 100   |       |       |       |       |       |
| %     |       |       |       |       |       |       |       |       |       |
| $\alpha_{1H}$ | 349.6 ± 38.6 | -     | 118.9 ± 9.9 | -     | 235.1 ± 21.3 | -     | 14.6 ± 1.3 | -     | 21   |
| + KLHL1 | 624.1 ± 71.9* | 78    | 225.7 ± 17.2* | 89    | 410.7 ± 40.3* | 74    | 24.3 ± 2.5* | 66    | 21   |
| + Jas  | 638.3 ± 66.5* | 82    | 195.3 ± 17.8* | 64    | 398.4 ± 51.8* | 69    | 27.5 ± 2.6* | 88    | 10   |
| + KLHL1 + Jas | 613.3 ± 96.5* | 75    | 190.2 ± 31.4* | 60    | 408.1 ± 71.2* | 74    | 26.3 ± 3.8* | 80    | 13   |
| + KLHL1 + BFA | 420.7 ± 89.1 | 20    | 163.6 ± 39.1 | 37    | 319.9 ± 84.9 | 36    | 17.5 ± 3.3 | 20    | 10   |
| + Jas + BFA | 365 ± 55.5  | 5     | 155.5 ± 17.9  | 31    | 381.1 ± 53.2* | 62    | 25.5 ± 2.9* | 75    | 17   |
| + KLHL1 + BFA + Jas | 397.1 ± 62.1 | 14    | 160.1 ± 24.7  | 35    | 380.9 ± 59.1* | 62    | 24.2 ± 4.1* | 66    | 14   |
| + BFA  | 306.6 ± 30.2 | 0     | 139.1 ± 14.1  | 17    | 264.0 ± 30.2  | 12    | 16.7 ± 1.8  | 14    | 10   |

% = % increase compared to $\alpha_{1H}$; *p < 0.05 compared to $\alpha_{1H}$

### Noise Analysis Properties

| 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| I ($\mu$A) | 0.14 ± 0.01 | 1.53 ± 0.09 | 0.50 ± 0.04 | 569 ± 74 | 299.9 ± 28.7 | 23    |
| $\gamma$ ($\mu$S) | 1.30 ± 0.03 | 1.44 ± 0.08 | 0.64 ± 0.03 | 1237 ± 125 | 369.1 ± 59.4 | 27    |
| $P_o$ (tails) | 0.13 ± 0.14 | 1.47 ± 0.10 | 0.75 ± 0.03 | 1258 ± 150 | 787 ± 72.2 | 16    |
| N      | 0.13 ± 0.14 | 1.47 ± 0.10 | 0.84 ± 0.03* | 1175 ± 125 | 664.3 ± 79.2 | 20    |
| $\tau_{deactivation}$ | 0.11 ± 0.02 | 1.19 ± 0.21 | 0.54 ± 0.07 | 565 ± 127 | 338.0 ± 68.5 | 9     |
| $\tau_{off}$ | 0.13 ± 0.01 | 1.46 ± 0.08 | 0.64 ± 0.05 | 906 ± 178 | 484.6 ± 36.7 | 11    |
| $\tau_{on}$ | 0.13 ± 0.01 | 1.49 ± 0.16 | 0.71 ± 0.04* | 589 ± 210 | 387.0 ± 65.2 | 5     |
| $\tau_{activation}$ | 0.11 ± 0.01 | 1.28 ± 0.15 | 0.53 ± 0.03 | 395 ± 72 | 307.2 ± 32.3 | 8     |

*p<0.05 compared to $\alpha_{1H}$

Corresponding current-variance plots for control ($\alpha_{1H}$) and the experimental conditions ($\alpha_{1H} + Jas$ and $\alpha_{1H} + KLHL1 + Jas$). Panel B summarizes the data obtained when cells co-expressing $\alpha_{1H}$ or $\alpha_{1H} + KLHL1$ were treated with 1 $\mu$M Jas (rows 3 and 4); the data obtained for $\alpha_{1H}$ and $\alpha_{1H} + KLHL1$ are included in the first two rows for comparison. As discussed earlier, KLHL1 co-expression with $\alpha_{1H}$ induced an increase in the channel number N (column 4) without modifying the channels’ unitary current ($i$), conductance ($\gamma$) or open probability ($P_o$) (this latter value showed a non-significant tendency to increase). In contrast, the macroscopic current increases seen by actin polymerization with Jas resulted from increases in both N and $P_o$ values.

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In summary, actin polymerization by itself induced a similar macroscopic effect on $\alpha_{1H}$ current density and calcium influx as KLHL1. However, the biophysical changes evoked by KLHL1 or Jas were different from each other: actin polymerization directly affects basal $\alpha_{1H}$ currents, and its mechanism of action involves an increase in $P_o$ and an increase in channel number, in addition to a decrease in $\tau_{off}$. KLHL1, on the other hand, requires actin-F to exert its action which is mediated by an increase N; KLHL1 also directly interacts with $\alpha_{1H}$ and induces an increase in $\tau_{deactivation}$. When both actin polymerization with Jas and KLHL1 were present there was no synergy.
in $\alpha_{1H}$ function upregulation, yet the biophysical effects of Jas dominated over KLHL1’s (Jas prevented the effect of KLHL1 on $\tau_{deactivation}$), suggesting the former non-reversible treatment also involves a region of $\alpha_{1H}$ that interacts with KLHL1, and that the interaction of $\alpha_{1H}$ with Jas is stronger than that of KLHL1 (see summary Fig. 8).

The role of the TGN and recycling endosome on $\alpha_{1H}$ upregulation by KLHL1. KLHL1 induced an increase in the number of $\alpha_{1H}$ channels at the membrane which requires polymerization of the actin cytoskeleton. Given the pivotal role that actin filaments and ABPs play in receptor trafficking and membrane protein re-insertion, we investigated the role of these processes in the modulation of $\alpha_{1H}$ by KLHL1 using pharmacological (BFA) and dominant-negative tools.

BFA can disrupt TGN trafficking and/or recycling endosome activity depending on the concentration and treatment time used. Overall, low concentrations (2–50 ng/ml) of BFA are used for long time periods (16–50 hrs) to disrupt trafficking from the Golgi to the membrane, whereas higher concentrations (10 μg/ml) are often used for short time periods (30–60 min) to study recycling of proteins back to the membrane. We first assessed BFA’s effects on the basal $\alpha_{1H}$ current expression in our $\alpha_{1H}$ stably expressing cell-line by performing a dose-response curve. As seen in Figure 5A and B, overnight treatment with increasing BFA concentrations (0–300 ng/mL) progressively affected basal $\alpha_{1H}$ currents: 0.03 μg/mL BFA did not alter $\alpha_{1H}$ current density compared to untreated samples (-33.1 ± 3.7 vs. -30.3 ± 2.6 pA/pF in control; n = 6 and 21; p > 0.05); treatment with 0.05 μg/mL BFA resulted in $\alpha_{1H}$ peak current density of -16.3 ± 2.8 pA/pF (n = 4; p < 0.05); 0.1 μg/mL BFA elicited a peak current density of -7.5 ± 0.5 pA/pF (n = 3; p < 0.01); and 0.3 μg/mL BFA completely eliminated $\alpha_{1H}$ currents (n = 9). Thus overnight incubation with the drug eliminated basal $\alpha_{1H}$ current expression with an IC$_{50}$ = 0.21 μM (0.06 μg/ml) (Fig. 5C), likely by preventing channel trafficking from the Golgi network. Noticeably, overnight treatment with 30 ng/mL BFA, which does not affect basal $\alpha_{1H}$ current expression, completely prevented the effect of KLHL1: $\alpha_{1H}$ current density in the presence of KLHL1 + BFA was -30.4 ± 5.9 vs. -30.3 ± 2.6 pA/pF in $\alpha_{1H}$ control (n = 21 and 8; p > 0.05), as observed in Figure 5E (18 hours).

This result suggests the effect of KLHL1 results from channel re-insertion from recycling endosomes and not from de novo channel trafficking from the Golgi network. The former is a faster process with kinetics of tens of minutes and indeed, short-term treatment (1 hour incubation) with 1 μg/mL BFA also resulted in the elimination of the effect of KLHL1 on $\alpha_{1H}$ current density (-33.8 ± 2.0 pA/pF in control; n = 10; p > 0.05), as seen in the examples shown in Figure 5D and the summary panel in E (for reference, KLHL1 treatment is also shown). Likewise, under these conditions N was no longer increased (Fig. 4B, row 5). These data corroborates the idea that increased endosomal recycling is likely involved in the effect of KLHL1.
Treated with Jas and BFA, the current density increase was rescued in macroscopic current density.

was also intermediate and was also not significantly different additional effect on and to KLHL1 (p > 0.05 for both sets). Calcium influx increases was similarly affected to both control (p > 0.05) and to Jas-treated cells (p > 0.05); N reversed of the Jas effect on current density yielding an intermediate value (the effect of KLHL1 is included for comparison). At 10 Hz, calcium entry increased by 20 and 38% similar to (Fig. 4B, row 2), resulting in a now significant change in current 

Figure 6A. BFA disrupts the effect of KLHL1 on current density and calcium influx and partially alters the effect Jas on αi. (A) BFA precludes the current density increases seen with KLHL1 and Jas on their own; but not when +KLHL1 + Jas are together; the effects of KLHL1 and Jas on αi are shown for comparison. (B) Normalized charge entry at 10 and 100-Hz stimuli. Treatment of KLHL1-transfected cells with BFA (K + BFA) precluded the effect induced by KLHL1 (K), but BFA only partially precludes the effects of Jas (J) and KLHL1 + Jas (K + J). (C) τ_deactivation Changes induced by KLHL remain after treatment with BFA. (D) BFA does not change the effects of Jas on inactivation kinetics (KLHL1 was not affected).

Figure 6B. Treatment with Jas and BFA (Fig. 6A) resulted in a partial reversal of the Jas effect on current density yielding an intermediate value of -36.9 ± 3.3 pA/pF (n = 18), statistically similar to both control (p > 0.05) and to Jas-treated cells (p > 0.05); N was similarly affected (Fig. 4B, row 6). Interestingly, the P0 value was also intermediate and was also not significantly different from control or +Jas (Fig. 4B, row 6) implying that BFA has an additional effect on αi-actin-F at the membrane. Thus, partial decreases in the effect of Jas on N (via the recycling endosome) and in αi (possibly by a direct effect) by BFA abolished the increase in macroscopic current density.

Noticeably, when cells were transfected with KLHL1 and co-treated with Jas and BFA, the current density increase was rescued (-43.3 ± 4.8 pA/pF; n = 14, p < 0.05) (Fig. 6A), suggesting that the partial effects of BFA on αi + Jas were perhaps additive with the slight rise in P0 originally detected in the presence of KLHL1 (Fig. 4B, row 2), resulting in a now significant change in current density due to increased P0 (Fig. 4B, row 7). As expected, noise analysis corroborated that treatment with BFA alone did not alter any of the basal values for αi (Fig. 4B, row 8).

Use dependency. Treatment of αi with BFA did not alter the basal calcium influx values; however, the calcium influx increases in αi + KLHL1 during high frequency stimulation were prevented with the addition of BFA, resulting in intermediate values between control and +KLHL1, as seen in Figure 6B and in Table 1 (the effect of KLHL1 is included for comparison). At 10 Hz, calcium entry increased by 20 and 38% similar to αi basal values and to KLHL1 (p > 0.05 for both sets). Calcium influx increases at 100 Hz frequency were 36 and 20% for the first and last spikes, comparable to control and to αi + KLHL1 (p > 0.05). Interestingly, the increases in calcium influx seen in the presence of αi + Jas were prevented by BFA at 10 Hz, resulting in levels similar to control (9 and 31% increase at the first and last spike) (Fig. 6B and Table 1). However, at 100 Hz the increase in influx was again detectable (62 and 75% higher than control). Co-treatment of αi + KLHL1 with Jas and BFA induced the same effect as αi + Jas + BFA (Fig. 6B and Table 1).

Overall these results suggest that in addition to preventing endosomal recycling, BFA may have a direct effect on αi, which interferes with the interaction between αi + KLHL1 at the plasma membrane. This BFA-αi interaction appears to be use-dependent, since high-frequency stimulation tends to rescue part of the αi-KLHL1 interaction resulting in intermediate influx values. Moreover, stimulation at 100 Hz can completely rescue the effect of Jas, in line with the idea that αi-Jas is a stronger interaction than that of KLHL1.

Biophysical properties. We also tested the effect of BFA on the kinetics of deactivation and inactivation of αi. As observed in Figure 6C, τ_deactivation in the presence of BFA alone did not affect the basal αi current kinetics: τ_deactivation = 2.9 ± 0.2 ms compared to 2.9 ± 0.1 ms in control (n=8, 22; p > 0.05). Interestingly, treatment with BFA did not eliminate the effect of KLHL1 on the τ_deactivation (3.3 ± 0.3 ms; n = 9, p < 0.05 vs. αi); consistent with a direct effect of KLHL1 on αi at the membrane independent of changes to N; for reference KLHL1 induced τ_deactivation = 3.4 ± 0.2 ms (n = 24, p = 0.004). BFA and Jas co-treatment did not affect τ_inactivation or τ_deactivation values (Suppl. Fig. S2A and B).

For completeness we also investigated the effect of BFA on τ_deactivation since this value changed with Jas treatment (Fig. 3), but not with KLHL1.21 As seen in Figure 6D, BFA did not eliminate the Jas effect, τ_deactivation = 19.8 ± 0.8 ms vs. 24.1 ± 1.9 ms in αi (n = 14, 13; p < 0.05); and co-treatment of KLHL1-expressing cells with Jas + BFA did not alter this result either (18.8 ± 0.8 ms, n = 9; p < 0.05; not shown in graph). The first three bars in Figure 6D have been included for comparison (τ_deactivation: 24.1 ± 1.9 ms, n = 14; + KLHL1: 24.1 ± 1.5 ms, n = 18 p = 0.36; and + Jas: 18.1 ± 1.3 ms, n = 14; p < 0.01).

Steady-state properties were not affected by BFA alone, yet the negative shift in SSA V50 seen with Jas could no longer be observed in the presence of BFA (Suppl. Fig. S2C). Again, this observation suggests BFA may have an additional effect on the interaction of Jas-polymerized actin with the channel that is independent of the drug’s effect on channel trafficking.

Targeting of endosomal recycling using the dominant-negative Rab11 S25N. To corroborate that our findings with BFA are indeed mainly due to interruption of endosomal recycling, we specifically targeted this pathway using the dominant negative
Rab11 mutant S25N. The small GTPase Rab11 plays an critical role in the recycling of proteins from the endosome to the plasma membrane, among other functions. Its activation can be prevented with the Rab11 mutant S25N, which blocks the GDP to GTP nucleotide exchange step thus blocking recycling endosome activity. As seen in Figure 7, overexpression of WT Rab11 did not affect the increase in current density seen with KLHL1 expression (-45.5 ± 3.9 pA/pF vs. -42.2 ± 2.7 pA/pF for α1H + KLHL1 n = 12 and 19, p > 0.05), if anything, a slight increase in current density was observed (not significant). However, overexpression of Rab11 S25N precluded the KLHL1-induced current density increase: current density was similar to the values seen with BFA incubation or with α1H alone (-32.6 ± 3.9 pA/pF for Rab11 S25N vs. -30.3 ± 2.6 pA/pF for α1H, n = 13 and 21, p > 0.05), confirming that KLHL1 increases current density via increased recycling of channels from the endosome to the membrane.

Discussion

Modulation of ion channel function by the actin cytoskeleton has been well documented for various channel types including HVA calcium channels: actin filament disruption can alter current activity and/or induce channel internalization of calcium-activated potassium channels, L-type calcium channels, CFTR, CIC-2 and ENaC channels [4,15]. Here we show a novel form of modulation of T-type currents by the ABP KLHL1; disruption of actin filaments by sequestering globular actin with the sponge agent Swinholide A. Overall, these data suggest polymerization with Jas induces a stronger conformational change, different than that seen with KLHL1, despite the fact that both experimental conditions can cause increase in N by similar mechanisms involving endosomal recycling.

Enhancement of surface channel expression via increased recycling endosome activity is well documented in the case of AMPA receptors, ENaC, cardiac pacemaker and outward-rectifying K channels. We targeted this process for further analysis due to the critical role actin plays in endocytosis, exocytosis and in trafficking and reinsertion of channels and receptors from the recycling endosome. Brefeldin A disrupts retrograde recycling and protein trafficking in the Golgi network by disrupting the function of class I ARFs, thereby preventing the association of coatamers with the donor membrane. We found that BFA prevented the basal expression of α1H with IC50 = 210 nM, likely through BFA-inhibition of ER-Golgi and post-Golgi channel transport. Low doses of BFA that do not affect these processes; and similarly, short-term incubation with BFA targeting the kinetics of recycling from the sorting endosome to the recycling endosome and plasma membrane completely eliminated the increase in channel number, suggesting KLHL1 increases the number of channels via increased channel recycling.

The effect of BFA on trafficking and recycling are clearly documented. BFA did not eliminate the changes in τd with KLHL1, consistent with the idea that BFA did not affect the interaction of KLHL1 with α1H already present at the plasma membrane. However, further analysis shows the drug does appear to have a direct effect on α1H. As evidenced by the observations that BFA precluded the effect of KLHL1 at both 10 and 100
vesicles, thus favoring the re-insertion process over degradation and modulate channel internalization and recycling, as in the case of TWIK1-K channels and ARF-6.73

In summary, here we determined the process by which KLHL1 increases T-type $\alpha_{1H}$ activity that requires—but is distinct from—actin polymerization (Fig. 8). Our data is consistent with actin polymerization with Jas inducing increased current and calcium influx in $\alpha_{1H}$ by increased N and P of channels already present at the membrane; whereas KLHL1 mode of action is mainly by increasing the actual number of channels at the membrane, although data obtained with the mutant $\Delta$Kelch81 and from this study indicate a direct effect of the ABP on channels already present at the plasma membrane also occurs.

The lack of modulation of $\alpha_{1G}$ by KLHL1 follows the same trend as other types of modulation of LVA channels, where $\alpha_{1G}$ but not $\alpha_{1H}$ is affected, such as the modulation by ascorbate, reducing agents, and G-protein $\beta\gamma$ subunits; likely reflecting the structural differences between these two isoforms.74-77 The fact that KLHL1 modulates Ca$_{V3.2}$ and Ca$_{V2.1}$ channels is more puzzling, for these channels belong to different channel families with a higher degree of structural divergence. Motif analysis has not shown any similarity in critical motifs between these two channel types, thus we hypothesize the involvement of actin filaments in the modulation of $\alpha_{1H}$ and $\alpha_{1A}$ by KLHL1 somehow facilitates the interaction among the two other components perhaps necessitating only a minimal actin-filament interacting motif with a low homology consensus region. So far, analysis of candidate motifs has not yielded any useful information.78-84

Interestingly, GABA receptor immunolocalization is decreased and diffuse rather than punctate in post-synaptic sites in cerebellar neurons from SCA8 CTG-expanded mice;85 thus the possibility that KLHL1 may regulate the function of a wider number of neuronal channels and receptors by modulating their membrane insertion via recycling should be considered. This novel modulation mechanism of voltage-gated calcium channels may be important in neuronal function.

Materials and Methods

Cell culture. Ca$_{V3.2}$ stably transfected human embryonic kidney 293 cells ($\alpha_{1H}$ HEK-293)33 were maintained in a 5% CO$_2$-humidified atmosphere at 37°C in high glucose minimum essential medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin and streptomycin and 600 mg/ml of G-418 (all reagents from Cellgro, Herndon, VA).

cDNA transfection. $\alpha_{1H}$ HEK-293 cells at 60% confluency were transfected using Polyethyleneimine (PEI).34 Cells were transfected with 1 $\mu$g of human EGFP-KLHL1 cDNA,35 EGFP cDNA was used to maintain equal transfection concentrations in control experiments. PEI was mixed with the DNA and allowed to incubate with the cells for three hours before media was replaced. Cells were used for experiments 1–3 days post-transfection.

Drugs and chemicals. Stock solutions were freshly made in DMSO (di-methylsulfoxide) or methanol (Sigma, St. Louis, MO) and diluted to their final concentrations in Tyrode: 1 $\mu$M Jas (Molecular Probes, Eugene OR), 2 $\mu$M Lat B (Biomol,

Hz frequency stimulation, as it did the effect of Jas at 10 Hz; BFA also eliminated the shift in SSA $V_{50}$ value and/or P of channels in the presence of Jas. This effect appears to depend on the presence of actin polymerization (either by Jas or KLHL1) since it did not affect the properties of basal $\alpha_{1H}$ currents; the interaction of BFA with $\alpha_{1H}$ appeared to be more labile than that of Jas since stimuli at 100 Hz rescued its increased calcium influx effect.

In view of this secondary effect of BFA on actin-F-$\alpha_{1H}$ and/or actin-F-$\alpha_{1H}$ KLHL1, the involvement of the recycling endosome was confirmed using the dominant negative Rab11 S25N mutant, which specifically blocks the endosomal recycling pathway; this mutant completely prevented the KLHL1 induced increase in current density, corroborating the BFA data. The activation of ARF-1 can stimulate the actin cytoskeleton assembly in Golgi membranes,65 so it is conceivable that KLHL1 could enhance actin-polymerization and assembly in $\alpha_{1H}$-containing
Plymouth Meeting, PA), and 1 μg/ml BFA (Sigma). Control experiments using the maximal DMSO (0.2%) and methanol (0.02%) concentrations showed these solvents do not affect Ca²⁺ currents or cell viability.

**Immunocytochemistry.** Cells were incubated in Jas, Lat B, or vehicle control before staining with TRITC-phalloidin (Sigma St. Louis, MO) for 20 minutes. Coverslips were fixed and mounted on slides with anti-quenching agent (Citiflour, Ted Pella Inc., Redding, CA). Images were acquired with a Biorad Confocal Microscope using a planapochromatic lens (40X oil objective), numerical amplitude 1.3. Images were analyzed with ImageJ freeware (NIH).

**Electrophysiology.** Currents were recorded 1–3 days post-transfection using the whole cell patch clamp technique with an Axopatch 200B amplifier (Axon instruments, Union City, CA) at room temperature as previously described. Data were acquired at 1 kHz and digitized at 20 kHz. Noise analysis currents were filtered at 10 kHz. αIH Ca²⁺ currents were recorded in external solution containing (in mM): 5 CaCl₂, 140 TEACl, 10 HEPES and 10 glucose (pH 7.4; 300 mM); the intracellular solution composition was (in mM): 108 CsMeSO₃, 4 MgCl₂, 1 Cs-EGTA, 9 HEPES, 5 ATP-Mg, 1 GTP-Li, 15 phosphocreatine-TRIS (pH 7.4, 280 mM). Pipettes pulled from borosilicate glass (Warner Instruments Inc., Hamden, CT) had resistances of 4–6 MΩ when filled with intracellular solution. Cell capacitance was measured from a transient current evoked by a 5 mV depolarizing pulse from a holding potential of -90 mV (12.4 ± 0.4 pF, n = 51). Cells with series resistance (Rs) <10 MΩ were used; Rs was compensated online (>80%).

**Current density.** Currents were held at (Vₜ) = -100 mV and depolarized for 150 ms to test potentials (Vₜ) = -70 to +70 mV, in 5 mV increments.

**Current kinetics.** Current inactivation (τᵢ) was measured by applying a 1 second-long pulse at Vₜ from -90 to +10 mV and current deactivation (τᵢₐₐ₉) was measured from tail currents elicited from Vₜ = -90 mV to Vₜ = -30 mV and back to Vₜ = -60 to -140 mV. All time constants were obtained by fitting their respective phase of the current with a single exponential.

Steady-state activation (SSA) was analyzed with protocols from Vₜ = -90 mV depolarized to Vₜ = -90 to 0 mV (ΔV = 10 mV) for 12 ms and re-polarized to -100 mV to evoke inward tail currents. Steady-state inactivation (SSI) was determined by stepping the membrane potential to various pre-pulse voltage levels (Vₚₑᵢ₉: -110 to 0 mV, ΔV = 10 mV) for 1 s prior to depolarization to a fixed test level (-30 mV) to evoke channel opening. Data was fitted by a single Boltzmann function of the form

\[ I_{\text{MAX}}/(1 + \exp^{(V_{50} - V)/k}) + m \]

where \( I_{\text{MAX}} \) = maximal current, \( V_{50} \) = half voltage of activation, \( k \) = slope factor and \( m \) = baseline.

**Use-dependency.** Use-dependent effects were investigated at 10 and 100 Hz by digitizing a single neuronal action potential (AP) waveform at those frequencies; to achieve 100 Hz, this stimulus protocol was constructed by truncating the APs causing a shortened re-polarization phase compared to 10 Hz. These single waveforms resulted in smaller calcium influx compared to APs at 10 Hz or a single AP. This technical difference was controlled for by comparing experimental groups to their corresponding controls at each frequency, or when calculating inactivation by normalizing against the first AP for each frequency. Total calcium influx was measured as the total charge elicited (in picocoulombs, pC) obtained by the integral of individual traces and normalized to cell size (pC/pF).

**Non-stationary noise analysis.** Experimental points were generated using a set of 50 or 100 individual current traces elicited from \( V_{\text{h}} = -100 \) to \( V_{\text{t}} = -30 \) mV that were used to calculate the trace average; the variance or “noise” of each experimental trace was then measured with respect to this average. Thermal noise was measured in the absence of channels at the resting potential of the trace and was subtracted from the traces. Single channel current values \( i \) were calculated from 2 ms-long sections along the whole decay phase of the current traces. The number of channels was then calculated by fitting the variance vs. mean current amplitude data to a parabola with the equation

\[ \sigma^2 = i - (1/N) \]

where \( i \) = single channel current amplitude, \( N \) = number of channels and \( i \) = mean current amplitude. Single channel conductance \( (\gamma) \) was calculated with the equation

\[ i = \gamma(E - E_{\text{rev}}) \]

where \( i \) = single channel current, \( \gamma \) = single channel conductance, \( E \) = the test voltage and \( E_{\text{rev}} \) = the reversal potential. Channel open probability \( (P_o) \) was calculated from steady-state activation experiments and from noise analysis; the \( P_o \) values given in Figure 4B are from the tail current data set since they are considered more accurate than the set from noise analysis, as the latter reflect the presence of channel inactivation; both sets of data showed consistent responses with experimental treatments.

Data were analyzed with Clampfit 9 software (Axon Instruments). Results are presented as mean ± SEM. Statistical significance was determined by \( p < 0.05 \) using Student’s t-test or Analysis of Variance (ANOVA) was used with post-hoc Tukey’s t-test where significance was \( p < 0.05 \).

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**Note**

Supplementary materials can be found at: www.landesbioscience.com/supplement/AmorolaranCHAN3-6-Sup.pdf
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