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Anion-Sensitive Fluorophore Identifies the Drosophila Swell-Activated Chloride Channel in a Genome-Wide RNA Interference Screen

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Abstract

When cells swell in hypo-osmotic solutions, chloride-selective ion channels (Clswell) activate to reduce intracellular osmolality and prevent catastrophic cell rupture. Despite intensive efforts to assign a molecular identity to the mammalian Clswell channel, it remains unknown. In an unbiased genome-wide RNA interference (RNAi) screen of Drosophila cells stably expressing an anion-sensitive fluorescent indicator, we identify Bestrophin 1 (dBest1) as the Drosophila Clswell channel. Of the 23 screen hits with mammalian homologs and predicted transmembrane domains, only RNAi specifically targeting dBest1 eliminated the Clswell current (IClswell). We further demonstrate the essential contribution of dBest1 to Drosophila IClswell with the introduction of a human Bestrophin disease-associated mutation (W94C). Overexpression of the W94C construct in Drosophila cells significantly reduced the endogenous IClswell. We confirm that exogenous expression of dBest1 alone in human embryonic kidney (HEK293) cells creates a clearly identifiable Drosophila-like IClswell. In contrast, activation of mouse Bestrophin 2 (mBest2), the closest mammalian ortholog of dBest1, is swell-insensitive. The first 64 residues of dBest1 conferred swell activation to mBest2. The chimera, however, maintains mBest2-like pore properties, strongly indicating that the Bestrophin protein forms the Clswell channel itself rather than functioning as an auxiliary subunit. dBest1 is an anion channel clearly responsive to swell; this activation depends upon its N-terminus.

Introduction

All mammalian cells express chloride channels activated by decreases in extracellular osmolality, albeit with different biophysical properties [1]. The ubiquitous expression of Clswell suggests its essential cellular function. Tightly regulated Clswell channels participate in volume regulation, motility, cell survival, and cell division [1]. In contrast, de-regulated constitutively active Clswell channels exacerbate several cardiac diseases, including myocardial hypertrophy and heart failure [2]. The mammalian Clswell channel-encoding gene has yet to be identified despite the wealth of proteins nominated by candidate approaches [3]. These proteins include CIC-2 [4], CIC-3 [5], P-glycoprotein [6,7], pClh [8,9], p64 [10], phospholemman [11], Best1 and 2 [12], TMEM16A [13], and TMEM16F [14]. The research community has yet to agree on any of these candidates as a bona fide Clswell channel.

In Drosophila, however, accumulating evidence indicates that dBest1 encodes for a Clswell channel. RNAi targeting dBest1 eliminates Drosophila Schneider (S2) cell IClswell, an effect rescued by re-introduction of dBest1 [15]. Further, swell activated dBest1 mutants have altered biophysical properties and reactivity to sulfhydryl reagents [16]. dBest1 likely forms the chloride conducting pore, but it may be an obligate auxiliary subunit of Drosophila IClswell that modifies channel properties akin to CaV β subunits [17].

Assigning chloride channel function to any protein is difficult. The known chloride channel families (e.g., CIC, Anoctamin/TMEM16, CFTR, and ionotrophic GABA A and GlyR) lack structural pore or gating motifs that might form the basis for in silico identification. Expression cloning approaches have also failed due to widespread Clswell channel expression that precludes the separation of endogenous and over-expressed protein activities. Moreover, known chloride channels blockers are non-specific and their affinities are far too low to encourage affinity purification. Finally, previous chloride indicators are poor tools for screening due to loading and retention issues, inconsistent results, and poor reproducibility [18].

Here we present an unbiased genome-wide, high-throughput RNAi screen designed to identify the Drosophila Clswell channel and its regulators. Our screen employed H148Q-YFP, a genetically encoded anion-sensitive yellow fluorescent protein [19], to report Clswell activity in Drosophila S2R+ cells. Of our 595 initial hits that altered chloride handling, we concentrated on characterizing proteins with mammalian homology and at least one transmembrane domain as potential Clswell channels. dBest1 emerged from our screen as the lead candidate for Drosophila Clswell. Both RNAi
knockdown of dBest1 and overexpression of a dominant-negative dBest1 eliminated the Cl\textsubscript{swell} current in Drosophila S2R\textsuperscript{+} cells. Conversely, dBest1 overexpression in a mammalian system (HEK cells) produced a Drosophila-like I\textsubscript{Cl\textsubscript{swell}}. To identify domains necessary for swell activation we characterized chimeras between the swell-sensitive dBest1 and the swell-insensitive mBest2. Swell sensitivity is only apparent in mBest2, the closest mammalian homolog of dBest1, when the protein contains the dBest1 amino (N)-terminus. This chimera maintains the pore properties of mBest2, providing additional evidence that the protein itself forms a channel rather than functioning as a necessary auxiliary subunit. We conclude that dBest1 is the channel underlying the Drosophila I\textsubscript{Cl\textsubscript{swell}}.

**Results**

**Drosophila S2R\textsuperscript{+} Cells have Robust I\textsubscript{Cl\textsubscript{swell}}**

*Drosophila* S2 cells are used extensively in genome-wide RNAi screens to dissect signaling pathways, determine protein functions, and assign protein molecular identity [20]. These macrophage-like cells, derived from primary culture of late stage Drosophila melanogaster embryos [21] readily take up RNAi from serum-free media. The subsequent process of targeted mRNA ablation is efficient and highly reproducible [22,23]. For our screen we used S2R\textsuperscript{+} cells [24], an adherent S2 variant well suited for assays that require multiple solution changes. Importantly, S2R\textsuperscript{+} cells have a consistent, large I\textsubscript{Cl\textsubscript{swell}} that activates slowly upon a drop in extracellular osmolality (Figure 1A). I\textsubscript{Cl\textsubscript{swell}} starts to activate within 2 min exposure to hypo-osmotic media, reaching steady state activation by 5 min. The fully activated Cl\textsubscript{swell} conductance is anion selective (Figure 1B). The relative permeability sequence of S2R\textsuperscript{+} Cl\textsubscript{swell} is 1 = SCN\textsuperscript{-} > Cl\textsuperscript{-} > MES\textsuperscript{2-} > aspartate (ASP) while the slope conductance sequence is I = SCN\textsuperscript{-} = Cl\textsuperscript{-} > MES\textsuperscript{2-} > ASP (Table 1 & 2). The Cl\textsubscript{swell} I-V relationship has a slight “S” shape, revealing rectification. An extended step protocol further illustrates features of S2R\textsuperscript{+} I\textsubscript{Cl\textsubscript{swell}} (Figure 1C). I\textsubscript{Cl\textsubscript{swell}} exhibits an initial instantaneous activation followed by a second slow activation phase, suggesting that more than one type of Cl\textsuperscript{-} conductance is turned on in S2R\textsuperscript{+} cells with cell swelling. In contrast, Chien & Hartzell [15] reported a single phase, time-independent I\textsubscript{Cl\textsubscript{swell}} activation in their S2 cells, perhaps indicating Cl\textsuperscript{-} channel expression differences in the two cell lines. Tail currents, normally indicating time dependence of activation of the channel, are evident in our recordings. However, since tail currents were not observed under symmetrical recording conditions [15], we attribute these currents to the exit of intracellular Cl\textsuperscript{-} accumulated during the prolonged steps. S2R\textsuperscript{+} I\textsubscript{Cl\textsubscript{swell}} has an interesting pharmacological profile (Figure 2A). Even at a high concentration (100 \(\mu\)M), the non-specific chloride channel blocker 4,4’-diisothiocyanato-2,2’-stilbenedisulfonic acid (DIDS) [25] blocks less than 25% of the S2R\textsuperscript{+} I\textsubscript{Cl\textsubscript{swell}} (Figure 2A). 4,4’-butyl-6,7-dichloro-2-cyclopropenyl-indan-1-ox-5-yl (DCP1B), a mammalian selective Cl\textsubscript{swell} blocker [26], fails to completely block S2R\textsuperscript{+} I\textsubscript{Cl\textsubscript{swell}} at 30 \(\mu\)M (Figure 2A & E). DCP1B blocks in a voltage-dependent manner (Figure 2E); at 0 mV 52\% of S2R\textsuperscript{+} I\textsubscript{Cl\textsubscript{swell}} is blocked, while 90\% is blocked at 80 mV. Surprisingly, furosemide, a Na-K-2Cl cotransporter (NKCC; SLC12A2) blocker, almost completely inhibits S2R\textsuperscript{+} I\textsubscript{Cl\textsubscript{swell}} at 1 mM (Figure 2A & 9C).

**H148Q-YFP Reliably Reports the Activity of S2R\textsuperscript{+} Cell Cl\textsubscript{swell} Channels**

Cl\textsubscript{swell} conducts iodide better than chloride, favoring the use of the H148Q-YFP indicator as a reporter of its activity. \(\Gamma^-\) \(K_p = 20\) mM and \(\Gamma^-\) \(K_p = 100\) mM [19,27,28]. Several anion-sensitive YFP variants accurately quantify intracellular Cl\textsuperscript{-} concentration or changes [27,29-31]; anion binding near the YFP chromophore suppresses fluorescence emission by altering chromophore resonance [19]. H148Q-YFP was chosen for Cl\textsubscript{swell} detection because it is bright and potently suppressed by \(\Gamma^-\); these properties are critical for good signal-to-noise ratios during screening. H148Q-YFP (pK\textsubscript{\textit{a}} = 6.7) is also sensitive to intracellular pH changes [19,28]. S2R\textsuperscript{+} cells stably expressing H148Q-YFP maintain their fluorescence in 240 mOSM NaCl however (Figure 3A), indicating that cell swelling does not appreciably alter intracellular pH. Subsequent replacement of bath \(\Gamma^-\) with \(\Gamma^-\) rapidly suppresses indicator fluorescence by 50\% as \(\Gamma^-\) enters the cells through open channels and interacts with the probe. The large fluorescence change and low intrinsic assay variability favor clear separation of potential hits. In the absence of hypo-osmotic solution, \(\Gamma^-\) is unable to enter the S2R\textsuperscript{+} cells and fluorescence is maintained (Figure 3B), indicating that S2R\textsuperscript{+} cells lack alternative constitutively active \(\Gamma^-\) entry pathways that could confound our ability to identify the Cl\textsubscript{swell} channel. Further, furosemide block of open Cl\textsubscript{swell} channels prevents appreciable fluorescence suppression (Figure 3C & D), suggesting that RNAi effectively targeting the Cl\textsubscript{swell} channel will be readily identifiable as hits.

**Genome-wide RNAi Screening of H148Q-YFP S2R\textsuperscript{+} Cells Identifies dBest1 as the Cl\textsubscript{swell} Channel**

The primary screen was conducted at the Harvard/HHMI Drosophila RNAi Screening Center using our stable H148Q-YFP-expressing S2R\textsuperscript{+} cell line. Each well of sixty-six 384-well assay plates contained a dsRNA targeting 1 of 13,900 genes encoding proteins or non-coding RNAs (DRSC 2.0; Figure 4A). Five days after S2R\textsuperscript{+} cells were treated with RNAi, we assessed cellular fluorescence under swell conditions in the presence of \(\Gamma^-\) and \(\Gamma^-\). Wells with fluorescence or ratio (I\textsubscript{Cl}<sub>swell</sub>/CL\textsubscript{-}) fluorescence changes greater than 1.5 times the standard deviation of the plate mean were initially considered as hits (Cl\textsubscript{swell} channel candidates or regulators of its activation pathway). We pared the list of 595 hits to genes with mammalian homologs and those with predicted transmembrane domains (Figure 4B, Table S1). In a secondary screen, we confirmed that each RNAi significantly reduced swelling-induced fluorescence and targeted only the mRNA from the identified gene (qPCR). We then directly measured I\textsubscript{Cl\textsubscript{swell}} via whole-cell voltage clamp. Candidates genes, whose RNAi significantly reduced the S2R\textsuperscript{+} cell I\textsubscript{Cl\textsubscript{swell}} were cloned and expressed in HEK293 or CHO-K1 cells. I\textsubscript{Cl\textsubscript{swell}} was then measured via whole-cell recording and compared with currents from untransfected cells. The only candidate of our screen to satisfy all the criteria for a Cl\textsubscript{swell} channel was dBest1 (Table S1).

**DRSC26457 RNAi Targeting dBest1 Eliminates I\textsubscript{Cl\textsubscript{swell}}**

dBest1 is a protein of 769 amino acids containing 4 transmembrane domains [32,33] (Figure 4C). It is one of four Bestrophin family members in Drosophila, with highest homology to mBest2/hBest2 (51\% identity and 67\% similarity; BLAST). Hartzell and colleagues first proposed that dBest1 was a chloride channel activated by high intracellular Ca\textsuperscript{2+} and cell swelling [15,16]. In our H148Q-YFP fluorescence assay dBest1 RNAi DRSC26457 abrogated the fluorescence change normally observed when \(\Gamma^-\) enters the S2R\textsuperscript{+} cells through activated Cl\textsubscript{swell} conductances (Figure 5A, B). Interestingly, DRSC26457 also decreased the baseline fluorescence variability of S2R\textsuperscript{+} cells (Figure 5B), suggesting that I\textsubscript{Cl\textsubscript{swell}} contributes to resting intracellular Cl\textsuperscript{-} concentrations. S2R\textsuperscript{+} I\textsubscript{Cl\textsubscript{swell}} was essentially eliminated by dBest1 RNAi DRSC26457 treatment (Figure 5C). This RNAi specifically and effectively reduced dBest1 mRNA by 91.5\% ± 0.5. 

Identifying the Swell Activated Chloride Channel
mRNA levels for the 3 remaining Bestrophin members and other CICwell candidates were unaffected. A second RNAi targeting dBest1 (DRSC16909; corresponds with dB1S [15]) was part of our initial screen. It was less effective at knocking down dBest1 mRNA (85% reduction, n = 3; significantly less than DRSC26457; p<0.001, Student’s t-test) and had two predicted off-target hits: CG4623 (20/20) and CG16711 (18/18). DRSC16909 did not significantly alter H148Q-YFP induced

Figure 1. Characterization of the S2R+ cell I_{CICwell}. (A) Hypo-osmotic solutions slowly activate I_{CICwell}. I_{CICwell} begins to activate 1.7±0.3 min after exposure to 200 mOSM solution and reaches steady state activation within 5±0.3 min (n = 13). I_{CICwell} was assessed in ramp protocols and reported at +84.5 mV (upper trace) and -115.5 mV (lower trace). 240 mOSM stimulates I_{CICwell} activation slightly more slowly (1.8±0.3 min to initiation and 5.2±0.6 min to steady state, n = 12; data not shown). (B) The S2R+ cell I_{CICwell} is anion-selective. I_{CICwell} was activated by 200 mOSM solution; relative permeability and slope conductance sequences were determined for the steady state I_{CICwell} by replacing Cl\(^-\) with equimolar anion concentrations. (C) An extended step protocol (red inset) reveals more than one set of activation kinetics with offset activation initiation times.

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Figure 2. Pharmacological profiles of S2R+ I_{Clswell} and I_{dbest1} match and differ from those of HEK I_{Clswell} and I_{db4m}. (A–D) % Block of S2R+ I_{Clswell}, HEK I_{Clswell} and I_{db4m}, by 1 mM furosemide, 100 µM DIDS, and 30 µM DCPIB. Block at 0 mV is presented to emphasize the incomplete voltage-dependent DCPIB block of S2R+ I_{Clswell} and I_{dbest1}. (A) Steady state S2R+ I_{Clswell} activated by 200 mOsm stimulation was blocked 96% ± 1.6 by furosemide, 19% ± 4 by DIDS, and 52% ± 10.6 by DCPIB. * no difference compared to I_{dbest1} block and significantly different compared to HEK I_{Clswell} and I_{db4m} (ANOVA, p < 0.05). (B) I_{dbest1}, stimulated for 2 min by 200 mOsm, was blocked 96% ± 1.7 by furosemide, 44% ± 10 by DIDS, and 47% ± 10.9 by DCPIB. * no difference compared to S2R+ I_{Clswell} block and significantly different compared to HEK I_{Clswell} and I_{db4m} (ANOVA, p < 0.05). (C) Steady-state HEK I_{Clswell} activated by 200 mOsm stimulation was blocked 7% ± 3.5 by furosemide, 77% ± 3 by DIDS, and 99% ± 0.7 by DCPIB. (D) Constitutive I_{db4m} (320 mOsm) was blocked 77% ± 3 by furosemide, 98% ± 1.5 by DIDS, and 98% ± 1.2 by DCPIB. * significantly different compared to S2R+ I_{Clswell} HEK I_{Clswell} and I_{db4m} (ANOVA, p < 0.05). (E–F) I–V relations for S2R+ I_{Clswell} and I_{dbest1} demonstrate DCPIB voltage-dependent block. At 80 mV, DCPIB block of S2R+ I_{Clswell} is 90% ± 3.6 (n = 6), and 82% ± 6.5 for I_{dbest1} (n = 7).

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fluorescence suppression (Figure 5A), and was not a hit in our initial screen. It is possible that the 15% remaining mRNA translated sufficient amounts of functional dBest1/Clswell channels to exclude it as a hit in our screen. This prospect emphasizes the importance of validated, effective RNAi for accurate screening.

Mutant dBest1 W94C Significantly Reduces S2R+ I_{Clswell}

To substantiate the conclusion that dBest1 is an essential component of the Clswell channel, we tested whether a mutant dBest1 would act as a dominant negative regulator of I_{Clswell}. In humans, Bestrophin 1 is mutant in vitelliform macular dystrophy

![Fluorescence Suppression](image1.png)

**Figure 3.** H148Q-YFP stably expressed in S2R+ cells reports the entry of Cl⁻ through activated Clswell channels. (A) Cellular swelling in 240 mOsm Cl⁻ did not alter fluorescence intensity as Clswell Channels activate (Student's t-test, p = 0.65; n = 76). Replacement of Cl⁻ with I⁻ evoked a 51% ± 1.3 decrease in fluorescence (Student's t-test, p < 0.001; n = 76). Imaging assay; fluorescence is in arbitrary units (a.u.). (B) Clswell channels must be open for I⁻ induced fluorescence suppression to occur. 320 mOsm NaI suppresses fluorescence by 16% ± 0.7 (Student's t-test, p = 0.1; n = 54). (C) Furosemide, an NKCC2 blocker, completely inhibits the S2R+ cell Clswell channels at 1 mM (n = 3). (D) 1 mM Furosemide block of Clswell prevents significant I⁻ induced suppression of H148Q-YFP fluorescence (Student's t-test, p = 0.54; n = 16).

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**Table 1.** Relative Permeabilities.

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Identifying the Swell Activated Chloride Channel
Figure 4. Genome-wide RNAi screening of H148Q-YFP S2R+ cells identifies \( \text{Cl}_{\text{swell}} \) channel candidates and regulators. (A) RNAi treatment alters S2R+ cell H148Q-YFP fluorescence levels. Heat map plate reader data following 5d RNAi treatment (240 mOsm \( \text{Cl}^- \)). Fluorescence was subsequently measured in 240 \( \text{Cl}^- \) Wells where the \( \text{I}^- \) to \( \text{Cl}^- \) fluorescence ratio was high are hits. Control RNAis are found in columns 13 and 14. Thread RNAi is in 13B, 13G, 14K, and 14N. Rho RNAi is in 13D, 13E, 14J, and 14O. GFP RNAi is in 13C, 13F, 14L, and 14M. Wells with elevated fluorescence in 240 mOsm \( \text{Cl}^- \) are shown in red and orange. (B) Functional classification of the 595 hits identified in our screen. 21 hits were transmembrane proteins of unknown function, putative ion channels, or transporters. 9 candidates with human homology were further evaluated (Table S1). (C) Protein sequence alignment of dBest1 and mBest2 (Multalign; multalin.toulouse.inra.fr/multalin/). Green bars indicate transmembrane domains. Yellow bars indicate other putative \( \alpha \)-helices (SOSUI; bp.nuap.nagoya-u.ac.jp/sosui/). A red star indicates the W94C mutation. A blue star indicates the \( \text{Ca}^{2+} \)-binding bowl. A pink arrow indicates Stop 383. A pink box outlines the region switched in the d64m chimera.

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Figure 5. DRSC26457 identifies dBest1 as \( \text{Cl}_{\text{swell}} \). (A & B) RNAi efficiently targeting dBest1 prevents significant \( \text{I}^- \) induced H148Q-YFP suppression following hypo-osmotic stimulation. Fluorescence is in arbitrary units (a.u.). (A) Plate reader assay. DRSC26457 RNAi treatment resulted in a fluorescence decrease of 6.4% ± 1.9. Control and DRSC16909 RNAi treatment resulted in fluorescence decreases of 40.5% ± 9.1 and 43.7% ± 6.2 respectively. * 240 mOsm NaCl and NaI fluorescence levels are significantly different (Student’s t-test, p < 0.05). (B) Imaging assay. The fluorescence levels of individual S2R+ cells treated with control or dBest1 DRSC26457 RNAi were measured during hypo-osmotic stimulation in the sequential presence of \( \text{Cl}^- \) and \( \text{I}^- \). The fluorescence of control cells decreased by 56% (n = 44); in contrast, the fluorescence of dBest1 DRSC26457 RNAi treated cells was suppressed by 15% (n = 174). (C) dBest1 DRSC26457 RNAi eliminated \( \text{I}_{\text{swell}} \) in S2R+ cells. Following dBest1 RNAi treatment \( \text{I}_{\text{swell}} \) is not significantly different from \( \text{I}_{\text{osm}} \) (Student’s t-test, p = 0.1). * control and RNAi treated \( \text{I}_{\text{osm}} \) are significantly different (Student’s t-test, p = 0.02). (D) dBest1 W94C-gfp overexpression suppresses S2R+ \( \text{I}_{\text{swell}} \). * control and W94C-gfp \( \text{I}_{\text{osm}} \) are significantly different (Student’s t-test, p = 0.02). (E) Confocal images of dBest1 W94C-gfp overexpression in S2R+ cells. Images were obtained before (320 mOsm) and after swell (200 mOsm). Scale bar indicates 10 \( \mu \)m.

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(Best’s disease [34,35]). One mutation, W93C, occurs in a conserved sequence of the channel’s putative pore [36,37] (Figure 4C). When we expressed the homolog dBest1 W94C-gfp in S2R+ cells, I_{Cl\text{swell}} was significantly reduced (Figure 5D). Interestingly, the late activating component of I_{Cl\text{swell}} remained clearly evident at depolarized potentials (Figure S1). We could not study this current in more detail as a loss of cell membrane integrity rapidly ensued. We conclude that dBest1 is responsible for the early activating S2R+ cell I_{Cl\text{swell}}. W94C might interact with WT dBest1 to disrupt the Cl_{swell} channel pore or it may prevent proper protein trafficking [38]. In S2R+ cells, dBest1 W94C-gfp has a distinct intracellular expression pattern unaltered by osmotic changes (Figure 5E), suggesting the latter explanation over the former. Regardless, dBest1 W94C has a dominant negative impact on I_{Cl\text{swell}}, further evidence that dBest1 is integral to the Cl_{swell} channel.

Another disease-associated Bestrophin mutation, D308A, occurs in a putative Ca_{2+} -binding bowl located in the channel’s C-terminus (Figure 4C; blue star). D308A is proposed to eliminate Bestrophin activation by disruption of calcium binding [39]. We introduced this mutation into dBest1 to determine if activation by calcium and cell swelling could be separated. Unfortunately dBest1 D308A-gfp was not functional in HEK cells (data not shown). Three possible explanations may underlie this result: 1) activation by multi-modal stimuli is simultaneously disrupted by the mutation; 2) the mutation causes protein misfolding and the channel function has been eliminated for reasons unrelated to activation; 3) the mutant channel is mislocalized. Our GFP-tagged protein was expressed (data not shown), but we cannot exclude the possibility that it mislocalizes or fails to interact appropriately with other proteins necessary for I_{Cl\text{swell}} activation or channel function [34].

Exogenous dBest1 Expression Creates a Drosophila-like I_{Cl\text{swell}}

Exogenous expression of a candidate protein substantiates whether the protein is necessary and/or sufficient in a given process. Our secondary screen assessed whether candidate protein expression resulted in a novel I_{Cl\text{swell}} or augmented the endogenous HEK I_{Cl\text{swell}} (Table S1). The HEK cell line chosen for candidate over-expression lacked constitutive I_{Cl} and I_{SCN} (potentially contaminating conductances attributable to SLC1A family member expression [40]; data not shown). The endogenous HEK I_{Cl\text{swell}} develops very slowly (Figure 6A & B); a two fold increase was noted within the first 2 min of swell. Once the HEK I_{Cl\text{swell}} reaches steady state, however, it has increased more than forty fold (44.4±10.7 fold, n = 29; Student’s t-test, p<0.000005). Tail currents are absent (Figure 6B & C). Characteristic voltage-dependent inactivation develops during steps to positive potentials (Figure 6C). HEK I_{Cl\text{swell}} is anion selective; its permeability and conductance sequences match closely to those of S2R+ I_{Cl\text{swell}} (Figure 6D; Table 1 & 2). The HEK I_{Cl\text{swell}} pharmacological profile (Figure 2C) correlates well with the literature. 100 μM DIDS, slightly above the reported IC_{50} [41], blocks 78% of the HEK I_{Cl\text{swell}} at +80 mV (Figure 2C). DCPIB has an IC_{50} of 4 μM [26]; at 30 μM 100% of HEK I_{Cl\text{swell}} is blocked (Figure 2C). 1 mM furosemide barely inhibits HEK I_{Cl\text{swell}} (Figure 2C). The endogenous HEK I_{Cl\text{swell}} recapitulates the key features noted for the mammalian I_{Cl\text{swell}} [1].

Bestrophin proteins are not universally accepted as bona fide chloride channels; alternatively they are intracellular ion channel regulators [33,42,43]. dBest1-gfp is clearly observed on or near the surface of HEK-293 cells (Figure 7A). Its expression results in a Drosophila-like I_{Cl\text{swell}} (Figure 7B–E). Constitutively active I_{Best1} is apparent in iso-osmotic 320 mOsm solution and is significantly increased 16±4.5 fold (Student’s t-test, p<0.005) during the first 2 min of hypo-osmotic stimulation (Figure 7B). I_{Best1} has the same “S”-shaped rectification as Drosophila I_{Cl\text{swell}} during ramps (Figure 7C); tail currents and time-dependent activation are both apparent in the step protocol (Figure 7D). I_{Best1} is anion selective; it has the same permeability and conductance sequences as S2R+ I_{Cl\text{swell}} and HEK I_{Cl\text{swell}} (Figure 7E; Table 1 & 2). Strikingly, IdBest1 and the endogenous S2R+ I_{Cl\text{swell}} share a similar pharmacological profile that differs significantly from HEK I_{Cl\text{swell}} (Figure 2A-C). 100 μM DIDS inhibits 35% of I_{Best1}, while 30 μM DCPIB blocks 45%. 1 mM furosemide blocks nearly 100% of the I_{Best1}. We conclude that dBest1 expression results in a Drosophila-like I_{Cl\text{swell}}; it cannot be attributed to endogenous HEK I_{Cl\text{swell}} upregulation.

dBest1 Swell Activation can be Conferred on the Swell-insensitive mBest2

The structural domains necessary for swell-induced channel activation are unknown. Although dBest1 has a long poorly conserved C-terminus (Figure 4C), it is not necessary for swell activation. dBest1 remains swell-sensitive despite the removal of up to 330 of its C-terminal amino acid residues (Stop 303, Figure 4C; Figure 8A). Next we examined whether chimeras might reveal the domains underlying swell activation. dBest1’s closest mammalian homolog, mBest2, is not activated by hypo-osmotic solutions (Figure 8B). Chimera d64m (the first 64 residues are dBest1; the remaining residues are identical to those of mBest2; Figure 4C) expressed resulted in a constitutively active current that more than doubled with swelling (2.3 fold ± 0.3 increase; Student’s t-test, p<0.05; Figure 8B-D). The d64m chimera maintained the relative permeability and slope conductance of mBest2 (Figure 8E & F; Table 1 & 2), suggesting that the channel’s pore domain is downstream of residue 64. Two other groups have assessed mBest2 selectivity [44,45] and found greater permeability for SCN than we report here. Both groups used high intracellular calcium to activate I_{mBest2}; we report constitutive I_{mBest2} measured with high internal calcium buffering (i.e. <10 nM free calcium). Our HEK cell line was also screened for potentially contaminating I_{SCN} (data not shown) attributable to SLC1A family member expression [40]. The pharmacological profile of I_{d64m} noticeably diverged from both that of HEK I_{Cl\text{swell}} and I_{mBest2} (Figure 2). Furosemide blocked 75% of I_{d64m} while DIDS and DCPIB both blocked I_{d64m} to near completion (Figure 2D). We conclude that the dBest1 N-terminal domain is required for swell activation of the mBest2 channel. The reverse chimera (m64d) was nonfunctional; exogenous currents were not observed with swelling or in the presence of high intracellular Ca_{2+} (data not shown). We cannot conclude with this data however, that the N-terminus is a “swelling” domain as it lacks any predictive motifs. We hypothesize that it works in concert with domains present both in dBest1 and mBest2 to facilitate swell activation. The strong correlation between S2R+ I_{Cl\text{swell}} and I_{dBest1}, combined with the unique selectivity and pharmacology of the d64m chimera, support the conclusion that the Bestrophin protein itself forms the Cl_{swell} channel rather than functioning as an auxiliary subunit.

Discussion

Our study validates the H148Q-YFP fluorophore as a reliable reporter of Cl_{swell} channel activity in genome-wide RNAi screening studies. H148Q-YFP has been employed very effectively in the identification of novel chloride channel activators, modulators, and blockers of CFTR and Ca_{2+}-activated Cl- channels [18]. This is the first reported RNAi screen using an
Figure 6. The endogenous HEK cell $I_{\text{Cl,swell}}$ has characteristic mammalian $I_{\text{Cl,swell}}$ properties. (A) HEK cell $I_{\text{Cl,swell}}$ develops slowly, reaching steady state after 5 min exposure to 200 mOsm solution. Arrow indicates min in 200 mOsm. (B) Ramp protocol (inset) assessment of the HEK $I_{\text{Cl,swell}}$. Little $I_{\text{Cl,swell}}$ has developed after 2 min in 200 mOsm solution. The steady state $I_{\text{Cl,swell}}$ is outwardly rectifying and inactivating at positive potentials. No tail currents are apparent. (C) Step protocol (inset) assessment of the HEK $I_{\text{Cl,swell}}$. Rapid inactivation is observed at positive potentials. No tail currents are apparent. (D) Relative permeability and slope conductance sequences for the endogenous HEK $I_{\text{Cl,swell}}$ are SCN $=$ I $>$ Cl $>$ MES $>$ ASP vs SCN $=$ I $=$ Cl $>$ MES $>$ ASP.

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anion-sensitive fluorescent protein to assign molecular identity to a chloride channel. Our screen supports the findings of the Hartzell lab [15]: dBest1 RNAi eliminates *Drosophila* I\textsubscript{Cl\textsuperscript{swell}}.

We found that the RNAi effectiveness was essential for Cl\textsubscript{swell} candidate identification. Two separate dBest1-targeting RNAi’s were part of our initial screen: DRSC26457 and DRSC16909.

Figure 7. dBest1 overexpression in HEK cells produces a S2R+ cell-like I\textsubscript{Cl\textsuperscript{swell}}. (A) dBest1-gfp targets to the membrane of HEK cells. Confocal images of dBest1-gfp overexpressed in HEK-293 cells. The DIC image is on the left, GFP in the middle, overlapped images on the right. Scale bar indicates 10 μm. (B) I\textsubscript{dBest1} rapidly develops within the first 2 min of hypo-osmotic stimulation. (C) The developing I\textsubscript{dBest1} has the same “S” shape rectification as the endogenous S2R+ cell I\textsubscript{Cl\textsuperscript{swell}}. (D) Step protocol shows that I\textsubscript{dBest1} shares time-dependent activation and tail current properties with S2R+ cell I\textsubscript{Cl\textsuperscript{swell}}. (E) The constitutively active I\textsubscript{dBest1} and S2R+ cell I\textsubscript{Cl\textsuperscript{swell}} selectivity sequences are very similar. (F) I\textsubscript{dBest1} is clearly separable from the endogenous HEK cell I\textsubscript{Cl\textsuperscript{swell}}. I\textsubscript{dBest1} increases 15.8 fold ± 4.5 (n = 18; ** paired Student’s t-test, p < 0.005) in the first 2 min of hypo-osmotic stimulation; the endogenous HEK cell I\textsubscript{Cl\textsuperscript{swell}} increases 2.1 fold ± 0.4 (n = 29; * paired Student’s t-test, p < 0.05).

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(which corresponds to dB1S [15]), but only DRSC26457 was a hit. qPCR reported a 95% reduction in dBest1 mRNA with DRSC26457 treatment versus an 85% reduction with DRSC16909. Hartzell and colleagues found that S2 cell IClswell was significantly reduced following treatment with 0.4 μg of DRSC16909 [15], while in our screen each assay well had a standardized 0.25 μg of RNAi. Using more RNAi may have effected greater target knockdown and resulted in the detection of DRSC16909 as a hit in our screen. This result emphasizes the importance of RNAi effectiveness in hit identification.

Exogenously expressed I_cl_best1 and endogenous S2R+ IClswell share similar characteristics, including time-dependent activation, tail currents, relative permeability sequences, slope conductance sequences, and pharmacological profiles. The shared properties of Drosophila IClswell and I_cl_best1 suggest that the same protein forms the channel responsible for both. Bestrophin is a known ion channel modulator, altering voltage-gated calcium channel activity [46]. If

Figure 8. Chimeras between dBest1 and mBest2 confer swell activation on mBest2. (A) Truncation of dBest1 does not interfere with swell sensitivity. Constitutive currents are apparent with both dBest1 and Stop 383 overexpression in iso-osmotic solutions (320 mOsm). With swell, current increased dramatically for both constructs (*Student’s t-test, * p<0.05, ** p<0.005). (B) d94m current increases significantly within the first 2 min of swelling (* Student’s t-test, p<0.05). (C) Time course for d64m swell activation. (D) Current-voltage relations for constitutive d64m, and following 2 min 200 mOsm solution. (E) Selectivity sequence for the constitutively active d64m current. (F) Selectivity sequence for the constitutively active mBest2 currents.

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dBest1 expression simply modulated or upregulated the endogenous HEK IClswell channel expression, we would have expected the resulting IClswell to maintain the properties of HEK IClswell. Instead we observed that dBest1 introduced an exogenous Drosophila-like IClswell whose development preceded that of the endogenous HEK IClswell. IClswell matched the pharmacological profile of the S2R+ IClswell. Moreover, we found that the exogenous Drosophila-like IClswell permeability and conductance sequence could be transformed into that of mBest2 with the d64m chimera. The pharmacological profile of IClswell was again significantly different from the endogenous HEK IClswell. We conclude that dBest1 is the Drosophila IClswell channel.

Several Bestrophin mutations are associated with vitelliform macular dystrophy [36,37]. How these mutations are causally linked to the disease is not clear. Here we found that overexpression of the disease-linked W94C dBest1 mutant in S2R+ cells significantly suppressed the endogenous Drosophila IClswell. The W94C mutation occurs in the putative pore of dBest1 and thus may disrupt IClswell conductance. However, the fluorescently tagged W94C dBest1 protein appears to localize to intracellular compartments, consistent with mislocalization. Milenkovic et al have recently proposed that disease-associated Bestrophin mutations cause defects in intracellular trafficking [38]. Both scenarios may explain the dominant negative effect of dBest1 W94C on Drosophila IClswell: non-functional, pore-disrupting, mutant Bestrophin proteins complexing with wild-type dBest1 may be largely retained within the endoplasmic reticulum. The end result would be the elimination of endogenous Drosophila IClswell. Our experiments support the hypothesis that mutant Bestrophin W93C expression could significantly disrupt chloride flux and homeostasis in the human macula, contributing to the disease state.

The distinction of Bestrophin function in Drosophila versus mammalian cells is most clearly illustrated by Hartzell and colleagues [16]. IClswell measured in peritoneal mast cells isolated from mBest1−/−, mBest2−/−, and mBest1/2 double knockout mice was identical to wild-type IClswell. mBest1 and mBest2 are swell sensitive in that their currents are inhibited by hyperosmotic solutions. However, their activity does not increase with swell [12]. We confirm here that mBest2 activation does not increase when cells swell. Our d64m chimera contained only a small portion of dBest1, yet it responded to cellular swelling. The crucial N-terminal region contains no distinct association domains or predictive structures that might explain its coupling to changes in cell stretch, tension, or osmolality. We speculate that the N-terminus contributes to a required tertiary structure that enables swelling signaling events to activate the dBest1 channel.

Our genome-wide RNAi screen of S2R+ cells and follow-up study firmly establishes that the dBest1 protein forms the Drosophila Clswell channel. It further validates a live cell genetically engineered fluorescent screening platform to identify other mammalian chloride channels.

**Materials and Methods**

**Generation of the S2R+ YFP- H148Q Stable Cell Line**

The stable S2R+ cell line expressing a halide-sensitive YFP (H148Q-YFP, kindly provided by Dr. Alan Verkman, UCSF) was generated with a selection vector (pCoBlast). H148Q-YFP was subcloned into the pAc5.1/V5-HisA vector (Invitrogen, CA). The S2R+ cells were transfected by electroporation (Amaxa cell line nucleofector kit V; Lonza). Cells were placed under selective pressure with 25 µg/ml blasticidin for 2 weeks. Two rounds of fluorescence-activated cell sorting (FACS; DFCI Flow Cytometry Core Facility) normalized YFP fluorescence intensities. These S2R+ cells exhibited a robust IClswell as described in Figure 1. S2R+ cells were maintained in Schneider’s Drosophila medium (Invitrogen), with 10% heat inactivated fetal bovine serum (Invitrogen), and 1% penicillin/streptomycin (Sigma-Aldrich). For the primary screen, cells were spun down and resuspended in serum-free medium at a density of 9 × 10⁵ cells/ml. 10 µl of the cell suspension was added to each of the 304 wells using the Matrix Wellmate 8-channel microplate dispenser (ThermoScientific; DRSC). Cells were incubated for 30 min, then 30 µl of serum containing medium were added to each well. Cells were cultured for 5 d before the swell assay was performed (5 d RNAi treatment is necessary to sufficiently knock down proteins with slow turnover rates). For the secondary screen, cells were plated at a density of 40% in a 6 well dish. Once cells were adherent, the medium was replaced with 1 ml of serum-free medium containing 0.015 µg/µl dsRNA. Cells were incubated for 30 min at room temperature followed by addition of 3 ml of serum-containing medium to each well. Transfections were performed in duplicate; 1 well was used for functional studies and the other for qPCR analysis of knockdown at day 5.

**Screening Solutions**

Iso-osmotic (320 mOsm/kg; mOsm) solutions contained in mM: 105 NaCl or NaI, 2 CaCl₂, 1 MgCl₂, 5 KCl, 10 HEPES, 10 Glucose, 90 Mannitol (pH 7.4 NaOH). For hypo-osmotic (240 mOsm) solutions, mannitol was omitted. 1 mM furosemide (Sigma-Aldrich) blocked 100% of the S2R+ IClswell and prevented significant H148Q-YFP suppression. Furosemide is a specific blocker of Na-K-2Cl co-transporters (SLC12A2) at concentrations in the µM range.

**The Primary Screen**

Our genome-wide screen was conducted at the Harvard/ HHMI Drosophila RNAi Screening Center using the DRSC 2.0 Genomewide RNAi Library. DRSC 2.0 is a collection of dsRNAs for genome-wide RNAi knockdown covering ~ 13,900 genes encoding proteins and non-coding RNAs while minimizing off-target effects due to sequence similarity to other genes. Each gene is targeted by 1.3 dsRNA/gene. The screen consisted of 66 384-well assay plates in duplicate. Each well of the 384-well plate contained 5 µl of 0.05 µg/µl dsRNA in water (0.25 µg dsRNA/well). Each plate contained control RNAi specific for Thread (Drosophila inhibitor of apoptosis protein), Rho (a small GTPase activator of the EGFR signaling pathway), and GFP. On day 5 of RNAi treatment, the cellular fluorescence of the H148Q-YFP probe was measured under several treatment conditions using the Analyst GT plate reader (Molecular Devices; DRSC). The probe was excited at 485 nm and emissions collected at 530 nm. Before fluorescence measurements were taken, the media was aspirated (384-well aspirator; VP Scientific) and the cells were equilibrated in serum-free medium at a density of 9 × 10⁵ cells/ml. After 10 min this solution was replaced with 1 ml of serum-free medium containing 0.015 µg/µl dsRNA. Wells with fluorescence or ratio fluorescence was determined by dividing the fluorescence in 240 mOsm NaCl by that in 240 mOsm NaI. Wells with fluorescence or ratio fluorescence was again significantly different from the endogenous HEK IClswell. Our genome-wide RNAi screen of S2R+ cells and follow-up study firmly establishes that the dBest1 protein forms the Drosophila Clswell channel. It further validates a live cell genetically engineered fluorescent screening platform to identify other mammalian chloride channels.
high internal pH as H148Q-YFP has a pKa of 6.7. Cell death was detected in control wells indicating effective RNAi treatment.

**Generation of dsRNA**
cDNA templates were generated by PCR amplification of genomic DNA using primers designed by the DRSC (SnapDragon tool). These primers had the 5' promoter sequence (TAAATACGACTCACTATAGGG) added to the 5' end of both primers. The templates generally corresponded to exons but occasionally sequences with two or more exons interrupted by introns were used. The PCR fragments were ~150–600 base pairs in length, and any complete 19-mer homology to other genes that could lead to non-specific dsRNA are reported. Individual RNAi sequences used here are found in the DRSC website (www.flyrnai.org). dsRNAs against *Drosophila* were synthesized with the MEGAscript in vitro transcription kit (Ambion). RNA was purified with the RNeasyPlus mini kit (Qiagen) and stored at −80°C.

**qPCR Analysis of RNAi Efficiency**
After 5 d RNAi treatment, RNA was prepared from the S2R+ cells using the RNeasyPlus mini kit (Qiagen). 2.5 μg RNA was used for each first-strand cDNA synthesis reaction (SuperScript Vilo cDNA Synthesis kit, Invitrogen). Primers for qPCR were designed on the NCBI/PrimerBlast site (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the following restrictions: PCR product size was between 70 and 300 bp, primer melting temperatures were between 57 and 63°C, primers spanned an exon-exon junction, and primers were specific to the intended PCR template as determined by BLAST analysis of the *Drosophila melanogaster* Refseq mRNA database. Primer sets were only used if the melting curve had a single peak. The RT² Real-Time SYBR Green/Rox PCR master mix (SABiosciences) was used for qPCR. qPCR reactions were set up in quadruplicate to minimize pipetting errors, and run on the Mastercycler ep Realplex real-time PCR system (Eppendorf). Average cycle numbers for each primer set were normalized to either dTaβ or dAct79b average cycle numbers.

**Secondary Screening of Candidates**
Comprehensive bioinformatics analysis of the hit list was performed to identify potential candidates for Clswell. Hits were limited to those with human homologs and at least a single transmembrane domain. Potential regulators of the Clswell activation pathway were left for future consideration. The effects of RNAi on fluorescence changes were confirmed by plate reader and imaging experiments. The specificity and effectiveness of the RNAi on fluorescence changes were confirmed by plate reader and imaging experiments. The specificity and effectiveness of the RNAi on fluorescence changes were confirmed by plate reader and imaging experiments.

**Electrophysiology**
Whole-cell patch clamp recordings were made at room temperature. Recordings were obtained using an Axopatch 200B amplifier, Digidata 1322A analog-to-digital converter, and pClamp 8.01 software (Molecular Devices, Union City, CA). Data were low-pass filtered at 2 kHz and digitized at 5 kHz. Fire-polished thin or thick wall borosilicate glass pipettes of 3–4 MΩ resistances were used for recordings; access resistance was compensated to >80%. Cells were held at −70 mV to clearly eliminate cells with leaky seals and voltage ramps (400 ms in duration) from −100 to +100 mV were applied every 2–5 s. Liquid junction potentials were corrected during analysis, and ramp data were plotted between −100 and +80 mV.

**Recording Solutions**
Internal pipette solution contained (in mM): 160 CsASP, 10 Cs4BAPTA, 4 MgATP, 2 MgCl2, 8 NaCl, and 10 HEPEs (pH 7.4 with CsOH). 10 mM BAPTA was used to prevent activation of channels by calcium and to reduce the endogenous HEK cell IClswell, which is optimally activated with 100 mM Ca2+ [47]. 240 mOSM solution composition is detailed in ‘Screening solutions’. 200 mOSM solutions contained in mM: 82.5 NaCl, NaI, NaSCN, NaMES, or NaASP, 2 CaCl2, 1 MgCl2, 5 KCl, 10 HEPEs, and 10 Glucose (pH 7.4 with NaOH). 90 mM mannitol was added to bring osmolality to 320 mOSM. The relative permeabilities were estimated from the Goldman-Hodgkin-Katz equation. For our calculations, the [Cl]o was set to 0 mM. Cells were held at −70 mV during all recordings, rapidly depleting Cl−. Cation permeability was essentially nil, as replacement of 200 mM NaCl solution with 200 mM NMDG-Cl solution did not change the reversal potential (Erev, data not shown). Slope conductances were calculated for each anionic substitution between the Erev and +400 mV. For the S2R+ and HEK cells Erev and I80 ms were measured in 200 mOSM solutions. For exogenously expressed dBest1, mBest2, and d64m Erev and I80 ms were measured in 320 mOSM solutions to prevent contamination with the endogenous HEK cell IClswell. The HEK cell line chosen for over-expression studies had no constitutive IClswell (EClswell did not change when switching between Cl and ASP), the endogenous IClswell developed very slowly, and the cells did not have an endogenous IClswell (attributable to SLC1A family member expression [40]).

**Pharmacology**
Stock solutions of DIDS (0.1 M in DMSO; Sigma), DCPIB (50 mM in EtOH; Tocris), and furosemide (1M in DMSO; Sigma) were prepared and diluted in 320 mOSM or 200 mOSM NaCl solution to their final concentrations.

**Molecular Biology**
*dBest1* was a kind gift from Dr. Criss Hartzell (Emory University). All other constructs were either ordered from Open Biosystems or cloned from a *Drosophila* cDNA library or Human Brain (whole Marathon ready cDNA library; BD Biosciences). Candidate cDNAs were subcloned into pEGFP-N3 (C-terminal tag; BD Biosciences) and an engineered Red pTracer vector (untagged). We found that an N-terminal EGFP tag rendered dBest1 nonfunctional (data not shown). Using site-directed mutagenesis we introduced the W94C substitution between the Erev and Clswell. Using site-directed mutagenesis we introduced the W94C mutation into dBest1/pEGFP-N3 (GeneArt site-directed mutagenesis, Invitrogen, CA). dBest1 W94C-gfp was then subcloned into the pAc5.1 V5-HisA vector (Invitrogen, CA).

**Supporting Information**
Figure S1 The late activating component of S2R+ IClswell remains despite overexpression of dBest1 W94C-gfp. (A) The late activating component of S2R+ IClswell is isolated after dominant negative elimination of dBest1. Inset: Step protocol. (B) The late activating component of S2R+ IClswell is sharply rectifying (ramp protocol; inset). Red trace is 320 mOSM solution, blue trace is 80 s after the 200 mOSM solution change. The late activating IClswell develops after 36 s in 200 mOSM solution.

Table S1 Secondary screening identifies Best1 as the *Drosophila* Clswell channel. Candidates with transmembrane domains and human homologs were further studied to determine if they formed the Clswell channel. ✓ indicates a positive secondary screening result; X indicates a negative result. ♦ indicates that several qPCR primer sets consistently had more than 1 melting
point peak suggesting nonspecific primer binding. The effectiveness of RNAi knockdown, therefore, could not be determined by qPCR. ?? indicates that two cells overexpressing SLC1A2 had substantial $I_{\text{Clwell}}$ currents but small $I_{\text{Clwell}}$. Thus, SLC1A2 overexpression may upregulate endogenous HEK cell $I_{\text{Clwell}}$ in the majority of the population but does not form the channel itself. HeLa cells treated with SLC1A3 siRNA (which reduced SLC1A2 and SLC1A3 mRNA by 90% and 92% respectively) had unaltered $I_{\text{Clwell}}$ (data not shown).

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**Author Contributions**

Conceived and designed the experiments: SCS. Performed the experiments: SCS. Analyzed the data: SCS. Contributed reagents/materials/analysis tools: SCS DEC. Wrote the paper: SCS DEC.

**References**


