State-dependent signaling by Ca_{\text{v}}1.2 regulates hair follicle stem cell function

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The signals regulating stem cell activation during tissue regeneration remain poorly understood. We investigated the baldness associated with mutations in the voltage-gated calcium channel (VGCC) Ca_{\text{v}}1.2 underlying Timothy syndrome (TS). While hair follicle stem cells express Ca_{\text{v}}1.2, they lack detectable voltage-dependent calcium currents. Ca_{\text{v}}1.2^{TS} acts in a dominant-negative manner to markedly delay anagen, while L-type channel blockers act through Ca_{\text{v}}1.2 to induce anagen and overcome the TS phenotype. Ca_{\text{v}}1.2 regulates production of the bulge-derived BMP inhibitor follistatin-like1 (Fstll), derepressing stem cell quiescence. Our findings show how channels act in nonexcitable tissues to regulate stem cells and may lead to novel therapeutics for tissue regeneration.

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Multipotent adult stem cells like those in the hair follicle possess the capacity for programmed organ replacement and carry the promise of induced organ repair in response to injury or damage (Li and Clevers 2010; Birmingham-McDonogh and Reh 2011). In many tissues, multipotent stem cells are found within specific tissue niches of support cells. These niches contain specific extrinsic and intrinsic cues and act to provide regulatory signals that help proliferation or differentiation (Fuchs and Segre 2000). While the relationship between stem cells and niches are not ubiquitously expressed throughout the tissue, but rather is highly expressed in the bulge stem cells as compared with interfollicular epithelium (Greco et al. 2009) and colocalizes with the outer bulge marker CD34 (Fig. 1A). As diversified channel properties arise from Ca_{\text{v}}1.2 alternate splicing (Gray et al. 2007), we compared the bulge Ca_{\text{v}}1.2 isoform with that found in excitable tissues such as neurons. RT–PCR of CACNA1C (Ca_{\text{v}}1.2) exons in FACS-sorted CD34^{+} bulge stem cells [B] (Nowak and Fuchs 2009) demonstrated a full-length channel isoform that also exists in P1 (postnatal day 1) mouse brains [Fig. 1B]. To determine whether these full-length isoforms carry calcium into cells, we performed electrophysiology to study FACS-isolated bulge stem cells from K15-EGFP mice [Morris et al. 2004]. We used whole-cell patch clamping to

While excitable cells respond to electric signals mediated by voltage-gated calcium channels [VGCCs] (Halling et al. 2006; Striessnig et al. 2010; Bidaud and Lory 2011), the role of VGCCs in nonexcitable cells such as adult stem cells remains poorly understood. VGCCs employ three well-characterized states in regulating calcium in response to strong membrane depolarization (Catterall 2000): a nonconducting closed state, a conducting open state that allows calcium entry, and a closed inactivated state that follows the open state (Supplemental Fig. S1A). VGCCs enter the inactivated state following prolonged depolarization or elevations of intracellular calcium or by binding to channel antagonists such as the dihydropyridine or phenylalkylamine class of L-type channel inhibitors (Catterall 2000). The glycine-to-arginine mutation at position 406 that causes Timothy syndrome (TS) dramatically reduces L-type channel inactivation (Splawski et al. 2004) and therefore provides an opportunity to investigate the role of channel inactivation in various cellular processes.

Here, we investigated how VGCCs such as Cav1.2 regulate the production of the bulge-derived BMP inhibitor follistatin-like1 (Fstll), derepressing stem cell quiescence. Our findings show how channels act in nonexcitable tissues to regulate stem cells and may lead to novel therapeutics for tissue regeneration.

Keywords: hair follicle stem cells, bulge; calcium channel; VGCC
results indicate that bulge Cav1.2 does not generate significant outward, voltage-gated potassium currents (Fig. 1E). These of isolation did not disturb the integrity of the ion channel (SOC) (Parekh and Putney 2005). To ensure that the process delayed rectifier, consistent with store-operated channels current observed appears to consist of a slowly inactivating found no detectable barium currents in response to mem-

measure voltage-gated currents using Ba2+ as the charge carrier in bulge cells 4–6 h after isolation (Fig. 1C). We found no detectable barium currents in response to mem-

branepolarization [Fig. 1D]. Interestingly, most of the current observed appears to consist of a slowly inactivating delayed rectifier, consistent with store-operated channels (SOC) (Parekh and Putney 2005). To ensure that the process of isolation did not disturb the integrity of the ion channel proteins at the membrane, we confirmed the presence of outward, voltage-gated potassium currents [Fig. 1E]. These results indicate that bulge Ca1.2 does not generate significant inward currents in bulge stem cells. Consistent with this result, we performed ratiometric calcium imaging in CD34+/CD6 cells. Depolarization of bulge stem cells with 65 mM KCl did not elic-

cation did not disturb the integrity of the ion channel proteins at the membrane, we confirmed the presence of outward, voltage-gated potassium currents [Fig. 1E]. These results indicate that bulge Ca1.2 does not generate significant inward currents in bulge stem cells. Consistent with this result, we performed ratiometric calcium imaging in CD34+/CD6 cells. Depolarization of bulge stem cells with 65 mM KCl did not elicit a voltage-dependent calcium elevation, consistent with a lack of functional VGCCs. In contrast, treatment with thapsigargin, a sarco/endoplasmic reticulum Ca2+ ATPase (SERCA) pump inhibitor, to deplete intracellular calcium stores activated SOCs and caused a significant calcium rise [Fig. 1F–H].

Previous studies have shown that the presence of the RGK, small GTPase proteins, GEM, REM, and SOC activators inhibits calcium influx through L-type VGCCs (Balijepalli et al. 2004; Park et al. 2010). We found high expression of STIM1, STIM2, REM2, and GEM in the bulge stem cells (Supplemental Fig. S1B), suggesting that these proteins may act to prevent calcium influx through Ca1.2 in the bulge stem cells. Taken together, we conclude that a full-length Ca1.2 is highly enriched in bulge stem cells but does not act as a functional VGCC.

Since Ca1.2 failed to act like a classical VGCC in freshly isolated bulge stem cells, we examined its function in vivo. TS patients are born bald and display a severe delay in the onset of the first postnatal hair growth (Splawski et al. 2004). To model this dominant disease, we conditionally expressed a Ca1.2 channel carrying the TS mutation (Supplemental Fig. S2A) under the control of the Rosa26/lax-stop-lox system (Pasca et al. 2011). Ca1.2TSfx/fx; K15CrePR mice that are treated with RU486 from day 21 to day 28 (Fig. 2A) to express the mutant protein specifically in bulge stem cells demonstrate late entry to anagen by 5–8 d (n = 6) (Fig. 2B, right), reflecting the phenotype observed in TS patients. Skin sections confirmed the telogen hair follicles in Ca1.2TS mice (Fig. 2D) compared with anagen hair follicles in the control mice (Fig. 2C). We characterized the bulge to ensure that Ca1.2TS specifically disrupts stem cell timing and not the bulge structure. To examine whether the bulge stem cells are quiescent, we fluorescently stained Ca1.2TS skin with Nfatc1 (Fig. 2E). Ca1.2TS skin showed nuclear Nfatc1 staining in the bulge stem cells, indicating quiescence (Horsley et al. 2008). Skin from control mice showed cytoplasmic Nfatc1 staining [Fig. 2E]. BrdU pulse-chase experiments were performed, and almost no BrdU incorporation was observed in the bulge stem cells. These findings suggest that Ca1.2 is not involved in bulge stem cell quiescence but rather in hair follicle maturation. As a result, the absence of functional Ca1.2 leads to the delay in hair follicle anagen.
Cav1.2TS mutant bulge, although BrdU incorporation was observed in the bulge and hair germ of control hair follicles (Fig. 2F). Similarly, Ki67 staining demonstrated that control tissue is in anagen, but Cav1.2TS mutant skin is still in telogen (Fig. 2G; Supplemental Fig. S2F). Together, these results suggest that overexpression of a Cav1.2TS mutant channel in the bulge stem cells of the skin inhibits anagen and extends quiescence without affecting hair follicle or epidermal differentiation (Supplemental Fig. S3).

Previous studies of the Cav1.2TS mutant channel suggest that it acts dominantly in excitable cells through a loss of voltage-dependent inactivation and prolonged influx of calcium (Sławska et al. 2004). We reasoned that we might detect voltage-gated calcium currents in Cav1.2TS-expressing bulge stem cells. However, calcium imaging analysis indicated that even in Cav1.2TS mutant bulge stem cells, there is no detectable calcium current in response to polarization with KCl (Supplemental Fig. S1A). Since the Cav1.2TS mutation enhances the probability of channel loss using the loss-of-function pore mutant Cav1.214–15 (Supplemental Fig. S1A), we hypothesized that inducing the inactivated state of the channel by the addition of the L-type calcium channel blockers would have the opposite effect and stimulate anagen. Indeed, treatments of L-type channel blocker verapamil between day 21 and day 28 caused a precocious entry to anagen 5–7 d prior to vehicle-treated animals (n = 8) (Fig. 3A,B). Verapamil-treated tissue showed anagen hair follicles (Fig. 3D) compared with telogen hair follicles in the control mice (Fig. 3C) in hematoxylin and eosin-stained sections (Supplemental Fig. S4D). Additionally, other classes of channel blockers (Supplemental Fig. S4D) and the calcium channel pore blocker cadmium (Supplemental Fig. S8) had no effect on hair cycling, demonstrating the specificity of the response.

To confirm that Cav1.2 inhibitors are acting on bulge stem cells, we treated Cav1.214–15 mutant mice that lack the channel in the bulge with verapamil. In contrast to wild-type animals, drug-treated Cav1.214–15 mutant mice phenocopy the Cav1.214–15 loss-of-function mutant mice and go into anagen 4 d later (n = 5) (Fig. 3G, right). Hematoxylin and eosin staining of skin sections from treated mice showed anagen hair follicles compared with control skin with telogen hair follicles, and Ki67 staining confirmed this result (Fig. 3F,H,I; Supplemental Fig. S5E). Furthermore, Nfatc1 staining showed cytoplasmic staining in control bulge cells compared with nuclear staining in drug-treated Cav1.214–15 mutant bulge stem cells just like in untreated Cav1.214–15 mutant bulge stem cells (Supplemental Fig. S2D, S4E). These data provide strong support that L-type calcium channel blockers are acting on bulge stem cells to regulate hair cycling.

We next tested whether channel ligands that induce inactivation could overcome the dominant-negative activity of the Cav1.2TS channel. In excitable tissues, channel blockers act dominantly to induce the open channel into the inactivated state (Yazawa et al. 2011). Since Cav1.2 acts as a dominant negative, we reasoned that channel
blocks would relieve inhibition and induce early anagen. Application of verapamil to the bulge cells expressing $Cav_{1.2}^{T5}$ causes mice to enter anagen early ($n = 2$) [Supplemental Fig. S5A], as confirmed by hematoxylin and eosin staining and Ki67 quantitation [Supplemental Fig. S5B–E]. We conclude that channel blockers can revert dominant-negative activity of the $Cav_{1.2}^{T5}$ channel.

Intense investigation over the past decade has elucidated the major signaling regulators controlling the hair cycle. These include anagen-stimulating pathways such as Wnt and TGF-β pathways [Blanpain and Fuchs 2009; Woo and Oro 2011; Oshimori and Fuchs 2012] as well as the BMP and Nfatc1 pathways that are critical for maintaining quiescence [Kobielak et al. 2007; Horsley et al. 2008]. Staining for the quiescence enforcer Nfatc1 [Horsley et al. 2008] in treated versus untreated skin demonstrated similar levels of nuclear Nfatc1 [Supplemental Fig. S5F]. Nfatc1 is a transcription factor that works downstream from calcium signaling. As verapamil treatment did not change the localization of the nuclear Nfatc1 even though the tissue is anagen, $Cav_{1.2}$ cannot be upstream of the Nfatc1 pathway, and verapamil's effects are not mediated through repression of nuclear Nfatc1. In contrast, we detected significant alterations in the BMP pathway in verapamil-treated versus untreated skin samples. Nuclear phospho-Smad [pSmad] 1,5,8 staining marks BMP signaling and quiescence in early telogen [Kobielak et al. 2003; Kandyba et al. 2013]. Verapamil-treated bulge stem cells express significantly less nuclear pSmad 1,5,8 in early telogen, indicating that drug treatment alters BMP-mediated quiescence [Supplemental Fig. S5G].

To understand the mechanism behind how $Cav_{1.2}$ blocks BMP-mediated quiescence, we performed microarray analysis on FACS-sorted verapamil-amd control-treated and also $Cav_{1.2}^{D14-15}$ mutant and control bulge stem cells [Fig. 4A, data not shown]. We identified factors that are oppositely regulated in mutant and drug-treated bulge cells. Out of this analysis, one factor clearly emerged: Fstl1 [Fig. 4A]. Fstl1 is a secreted glycoprotein that inhibits BMP signaling in lung and ureter development [Sylva et al. 2011; Xu et al. 2012]. Previous studies have identified other BMP and Wnt inhibitors and activators that affect hair cycling, such as Noggin and Bambi or the TGF-β-regulated Tmeff1; however, their expression is not significantly affected or up-regulated by alterations in $Cav_{1.2}$ [Fig. 4B]. Fstl1 mRNA is up-regulated twofold in verapamil-treated bulge stem cells compared with control bulge stem cells and is down-regulated more than fivefold in $Cav_{1.2}^{D14-15}$ mutant bulge stem cells compared with control bulge cells [Fig. 4C, D]. Immunofluorescence staining also indicates that Fstl1 protein levels are significantly elevated in the bulge and secondary germ of verapamil-treated skin and are reduced in $Cav_{1.2}^{D14-15}$ mutant and verapamil-treated $Cav_{1.2}^{D14-15}$ mutant bulge stem cells [Fig. 4E, data not shown], confirming the dependence of Fstl1 expression on $Cav_{1.2}$ and its ligand. To understand the effect of Fstl1 on hair cycling, we subcutaneously injected two different doses of Fstl1 protein-soaked beads into day 21–22 mice. Fstl1-treated mice went into anagen 4–7 d earlier than the bovine serum albumin (BSA)-injected control animals ($n = 8$) [Fig. 4F–I], with a dose-dependent increase in the size of the affected area [Fig. 4F]. Injection of beads containing follistatin (Fst), a highly related family member expressed in the bulge, failed to induce anagen [Supplemental Fig. S6A–C]. FACS analysis of bulge stem cells treated with Fstl1, but not Fst, showed decreased pSmad 1,5,8 staining, confirming that Fstl1 acts to inhibit BMP signaling in the bulge [Supplemental Fig. S7].

Through the study of the $Cav_{1.2}^{T5}$ protein in nonexcitable tissues, our genetic and biochemical studies uncovered the surprising role for VGCCs in hair follicle stem cell regulation. This is the first report that shows that a calcium-independent L-type channel regulates stem cell quiescence. We could not detect significant voltage-gated currents in the bulge and could not rule out the role for smaller, transient currents that fall below our detection methods. An alternative explanation is that $Cav_{1.2}$ and the calcium channel blockers act like a more traditional ligand–receptor pair. Upon binding to its receptor, verapamil induces allosteric changes in the channel that result in increased Fstl1 expression. Our efforts to understand the connections between $Cav_{1.2}$ and Fstl1 induction have been thwarted by the inability of bulge stem cells to maintain their properties in long-term culture. We are currently searching for...
other in vitro systems to study these nonvoltage channel properties of this versatile family of proteins. Hair follicle stem cell proliferation, like those in other tissues, requires tight regulation of competing activation and quiescence pathways. These pathways are in balance until one side wins over the other. Our study shows that Cav1.2 provides a key signal to break this symmetry and induce tissue regeneration through the novel BMP antagonist Fstl1 (Fig. 4J). Cav1.2 serves as a pacemaker in cardiac tissue, and its expression in many tissues and stem cell compartments suggests that a similar role might be played in nonexcitable tissues. As-yet-unidentified endogenous channel ligands promoting the inactivated state could affect stem cell and regenerative functions independent of calcium-gating activities. These unique ligands would have novel therapeutic applications for treating channelopathies like TS and in regenerative medicine.

Materials and methods

Mice

All mouse studies were approved by and conformed to the policies and regulations of the Institutional Animal Care and Use Committees at Stanford University. Ca$_{1.2}^{D_{14-15}}$ mice conditionally expressing Ca$_{1.2}^{D_{14-15}}$ under the Rosa26 promoter in response to Cre recombinase were generated as previously described (Pasca et al. 2011). These mice were then crossed to K15CrePR (Ito et al. 2005) mice to be able to express the TS channel in the bulge stem cells. Ca$_{1.2}^{D_{14-15}}$ mice were a gift from Jean Pierre Kinet (Harvard University) (Jeon et al. 2010).

Immunostaining

Immunofluorescence was carried out overnight on frozen sections fixed in 4% paraformaldehyde and dropped in sucrose. Alternatively, skin samples were embedded in OCT directly and then post-fixed in 4% paraformaldehyde for 2–5 min. Antibodies used were Cav1.2 (1:200; Millipore, #AB5156), Nfatc1 (1:200; Santa Cruz Biotechnology, #sc-7294), CD34 (1:750; BD Pharmingen #553731), pSmad 1,5,8 (1:50; Cell Signaling, #9511), BrdU (1:500; Abcam, #ab6326), Ki67 (1:200; Neomarkers/LabVision, clone SP6), Fstl1 (1:200; Abcam, #71548), and Pcad (1:1000; R&D Systems, #MAB761).

FACS and RNA isolation

FACS isolation (Stanford Shared FACS Facility) of stem cells was performed as previously described (Nowak and Fuchs 2009). Antibodies used were PE rat anti-human CD49f (1:200; BD Biosciences, #555736) and biotin anti-mouse CD34 antibody (1:100; eBioscience, #13-0341-81). Alternatively, K15-EGFP mice from Jaks Laboratories were used for isolation of the bulge stem cells. RNA isolation was carried out using a Mirvana kit from Ambion or RNAeasy Mini Plus kit from Qiagen. cDNA synthesis was carried out by using High-Capacity RNA-to-cDNA Master Mix from Applied Biosystems. pSmad FACS analysis was reported elsewhere (Kandyba et al. 2013).

Real-time PCR

Total RNA or cDNA [see above] were used in a Stratagene Mx3000P using SYBR green quantitative RT–PCR master mix [Agilent] with technical and biological replicates.

Microarray analysis

Illumina MouseRef-8 version 2.0 expression BeadChip was used. Microarray analysis was performed by Stanford Functional Genomics Facility.
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SAMS analysis was performed for statistical analysis (Tusher et al. 2001). Data were submitted to Gene Expression Omnibus (accession no. 16773750).

Patch clamping

K15EGFP bulge cells that were FACS-isolated were plated on Matrigel-treated BD Biosciences coverslips and incubated for 4-6 h at 37°C prior to recording. Coverslips were mounted on the stage of an inverted Nikon fluorescence microscope (Ellipse TE2000) in a standard recording chamber at room temperature. Cells were visualized under a 40× air lens using GFP fluorescence. Whole-cell barium currents were recorded using EPC 10 amplifier [HEKA] with the following external solution: 5 mM BaCl2, 160 mM TEACl, and 10 mM HEPES (pH adjusted to 7.4 with TEAOH). The pipette solution contained 135 mM CsMeSO3, 5 mM CsCl, 0.5 mM EGTA, 1 mM MgCl2, 4 mM Mg-ATP, 0.5 mM Na-GTP, and 10 mM HEPES (pH adjusted to 7.4 with CsOH). Potassium currents were measured using the following external solution: 129 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 25 mM HEPES, and 30 mM sucrose (pH 7.4). The pipette solution contained 140 mM KCH3SO3, 2 mM MgCl2, 2 mM EGTA, 5 mM KCl, and 20 mM HEPES (pH 7.2). Recording pipettes made of borosilicate glass (Sutter Instruments, BF150-110-10) had a resistance of 4–6 MΩ when filled with intracellular solution. Leak and capacitance currents were subtracted. Data were filtered at 2 kHz and digitized at 5 kHz. Data were collected and initially analyzed with Patchmaster software [HEKA].

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References


