Dlk1 Up-Regulates Gli1 Expression in Male Rat Adrenal Capsule Cells Through the Activation of β1 Integrin and ERK1/2

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The development and maintenance of the zones of the adrenal cortex and their steroidal output are extremely important in the control of gluconeogenesis, the stress response, and blood volume. Sonic Hedgehog (Shh) is expressed in the adrenal cortex and signals to capsular cells, which can respond by migrating into the cortex and converting into a steroidogenic phenotype. Delta-like homologue 1 (Dlk1), a member of the Notch/Delta/Serrate family of epidermal growth factor-like repeat-containing proteins, has a well-established role in inhibiting adipocyte differentiation. We demonstrate that Shh and Dlk1 are coexpressed in the outer undifferentiated zone of the male rat adrenal and that Dlk1 signals to the adrenal capsule, activating glioma-associated oncogene homolog 1 transcription in a β1 integrin- and Erk1/2-dependent fashion. Moreover, Shh and Dlk1 expression inversely correlates with the size of the zona glomerulosa in rats after manipulation of the renin-angiotensin system, suggesting a role in the homeostatic maintenance of the gland. **(*Endocrinology* 154: 4675–4684, 2013)**

The adult adrenal gland is a composite of 2 embryologically distinct tissues, the mesodermally-derived cortex and neural crest-derived medulla. The cortex is divided further into 3 distinct zones arranged concentrically around the medulla and within an outer stromal capsule: at the periphery of the gland is the zona glomerulosa (ZG), next lies the zona fasciculata (ZF) and then the zona reticularis (ZR), adjacent to the medulla (1). The adrenal cortex is the primary site of steroid synthesis in the body, producing mineralocorticoids from the ZG under the control of the renin-angiotensin system (RAS) and glucocorticoids from the ZF under the control of the hypothalamic-pituitary-adrenal axis. The ZR produces adrenal androgens in humans and some primates. Mineralocorticoids control blood volume and salt homeostasis, and hence influence blood pressure (2), whereas glucocorticoids control gluconeogenesis and mediate the mammalian stress response (3). The adrenal cortex therefore controls essential metabolic processes, and impaired adrenal function is often fatal.

Recent efforts in the identification of cell fate regulators coordinating adrenocortical stem/progenitor cell differentiation and specification towards the steroidogenic lineage have highlighted a crucial role for Sonic Hedgehog (Shh) (4–6; reviewed in Refs. 7, 8). Shh is expressed in relatively undifferentiated steroidogenic cells in the subcapsular region of the mouse (5, 9) and rat (10) adrenal cortex from embryonic day (e) 12.5 and e13.5, respectively, and throughout life. The Shh signal is transduced by cells in the mesenchymal capsule, and lineage-tracing studies showed that some of these cells delaminate into the cortex, lose their responsiveness to Shh, and become steroidogenic during organogenesis (5). Shh null adrenals have a hypoplastic adrenal cortex and a markedly thin capsule, and

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For News & Views see page 4446

Abbreviations: CYP11B1, 11-beta-hydroxylase; CYP11B2, aldosterone synthase; Dlk1, δ-like homologue 1; e, embryonic day; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gli-1, glioma-associated oncogene homolog 1; GST, glutathione S-transferase; IHC, immunohistochemistry; iZU, inner ZU; MEK, mitogen-activated protein kinase kinase; NR-ISH, nonradioactive in situ hybrization; oZU, outer layer of the undifferentiated zone; pERK1/2, phospho-Erk1/2; Q-PCR, quantitative real-time PCR; RAS, renin-angiotensin system; RGDS, Arg-Gly-Asp-Ser; SCC, side-chain cleavage; Shh, Sonic Hedgehog; siRNA, small interfering RNA; Sox9, SRY (sex determining region Y)-box 9; ZF, zona fasciculata; ZG, zona glomerulosa; ZR, zona reticularis;

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together, these data suggest that the glioma-associated oncogene homolog 1 (Gli-1)-positive capsular cells potentially represent adrenocortical stem cells during development (5). Shh signaling is therefore predicted to be crucial for adrenocortical maintenance and remodeling, whereby rapid changes of the sizes of the steroidogenic zones occur according to physiological demand for mineralocorticoid or glucocorticoid, as well as for adrenocortical development (8). However, mechanisms regulating Shh signaling in the adrenal cortex have not been described to date.

Delta-like homologue 1 (Dlk1)/preadipocyte factor-1 is a transmembrane and secreted protein encoded by an imprinted gene. Dlk1 is widely expressed during development but has a more restricted expression pattern in the adult, where it is mainly found in undifferentiated cells in fat (11), liver (12), and brain (13). In mouse preadipocytes, the Dlk1 extracellular domain is cleaved by TNF-α-converting enzyme to form a biologically active soluble form (14). This strongly inhibits their differentiation into mature adipocytes by binding to fibronectin (15), leading to the activation of the MAPK pathway, the phosphorylation of Erk1/2, and the subsequent up-regulation of SRY (sex determining region Y)-box 9 (Sox9), which represses the transcription of the proadipogenic factors CCAAT-enhancer-binding protein β and CCAAT-enhancer-binding protein δ (16, 17). The Dlk1 extracellular domain, despite containing 6 tandem epidermal growth factor (EGF)-like repeats, lacks key conserved amino acid residues required for binding to EGF receptors. Structurally, Dlk1 is similar to the Notch/δ/Serrate family, which is involved in cell fate determination decisions (18). However, Dlk1 lacks the DSL (δ/Serrate/lymphocyte-activation gene 2) domain, which is necessary for the interaction of classical Notch ligands with Notch and subsequent signaling, although it does contain other motifs that could mediate Notch binding. A physical interaction between Dlk1 and Notch has been demonstrated in vitro, which results in an inhibition of both Notch signaling and adipogenesis (19, 20), although others have found that Dlk1 neither interacts with nor requires Notch for its signaling in preadipocytes (15).

Dlk1 is also expressed in the subcapsular region of the developing and adult rat adrenal gland (21), and we demonstrate here that Dlk1 and Shh are coexpressed in the rat adrenal cortex and that Dlk1 activates Gli1 expression in capsular mesenchymal cells in a β1 integrin/phospho-Erk1/2 (pERK1/2)-dependent manner but can also inhibit activation of Gli1 expression by Shh through the same pathway. We further demonstrate that Dlk1 and Shh expression are coordinately regulated by modulation of the RAS, suggesting a role for both in controlling adrenal differentiation and thus the control of homeostasis and essential metabolic processes.

Materials and Methods

Animals

Rats were housed in rooms with controlled light and temperature and treated under the United Kingdom Home Office Animals (Scientific Procedures) Act 1986. For captopril and dietary sodium studies, rats were treated as reported (22). Animals were killed by CO_2 asphyxiation, and tissue was collected and processed as previously reported (10).

Nonradioactive in situ hybrization (NR-ISH) and immunohistochemistry (IHC)

Probes specific for rat *Scc* and *Shh* were described previously (10). A rat Dlk1 cDNA fragment was PCR amplified using the following primers: forward, 5′- CCTCTTGCTCCTGCTGGC-3′ and reverse, 5′-TATCCTCATCACCAGCCTCC-3′ (1118 bp) and cloned into pGEM-T easy (Promega). NR-ISH, IHC, and combined IHC-NR-ISH were performed as previously described (10). Antibodies are listed in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. For comparative expression analysis of *Dlk1* mRNA and Dlk1 protein, pERK1/2 and proliferating cell nuclear antigen proteins in rats assigned to different dietary and pharmacological regimes, all steps from tissue fixation through to image acquisition were performed in parallel. Relative quantification of pERK1/2 and Dlk1 staining in sections was performed using ImageJ software, using at least 5 representative fields per sample.

Glutathione S-transferase (GST)-DLK1 fusion protein production

The cDNA encoding amino acids 8–297 of DLK1 protein was PCR amplified (forward: CGA*GCGGCCGC*CTGCGCGTC-CTCTTGCTCC, *Not*I site italicized; reverse: *GCGGCCGC*-AGCGTAATCTGGAACATCGTATGGGTAGAGGAGAGG-GGTTTTCTTGTTGAG, *Not*I site italicized, hemagglutinin tag-encoding sequence underlined) and cloned in frame with *Gst* gene in pGEX4T3 vector (Pharmacia). GST and GST-DLK1 were expressed in JM109 cells and purified by affinity binding to glutathione Sepharose beads (Pharmacia). The proteins were eluted with 10mM reduced glutathione (Sigma) and dialyzed against PBS. Preparation purity was assessed by SDS-PAGE and Coomassie blue staining (SafeBlue; Invitrogen). Proteins were added to media, and filter was sterilized before addition to cell cultures.

Cells

3T3-L1 cells were cultured, differentiated, and stained with Oil Red O (Sigma) as reported (23). GST or GST-DLK1 proteins were added at 50nM on day 0 of differentiation and maintained at subsequent medium changes.

H295R cells were grown in DMEM/F12 (1:1), 100-U/mL penicillin, 100-μg/mL streptomycin (Sigma), 2% Ultroser G (Pall Europe), and 1× insulin-transferrin-selenium solution (10-μg/mL insulin, 5.5-μg/mL human transferrin, and 5-ng/mL sodium selenite; Sigma) (24).

For capsule cells primary cultures, 6 rat adrenals were enucleated by squashing between microscope slides, and the capsule fractions were pooled, washed in DMEM/F12 with 10% fetal bovine serum (FBS), and then treated with collagenase (2 mg/mL;

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Sigma) in DMEM/F12 at 37°C for 1.5 hours. Cells were then collected by centrifugation at 800*g*, resuspended in 10-mL DMEM/F12/10% FBS, and strained through a 40-μL cell strainer (BD Biosciences). The strainer was washed twice with 5-mL DMEM/F12/10% FBS, and cells in the flow through were pelleted by centrifugation at 500*g*. After a 3-minute treatment with Red Blood Cell lysis buffer (Sigma), cells were collected by centrifugation at 500*g* for 5 minutes and resuspended in culture medium (as for H295R cells). Cells were used within 2 weeks of preparation.

All cells were incubated in a humid atmosphere of 5% CO2, at 37°C.

Cell stimulations

H295R and primary cultures from rat adrenal capsule were serum starved overnight before the addition of agonists or inhibitors. Arg-Gly-Asp-Ser (RGDS) peptides were from Sigma, recombinant SHH (ShhN) from R&D Systems, and U0126 from Promega.

Western blotting

H295R cells were lysed with 2× Novex buffer (Invitrogen). Samples were size separated through a NuPAGE Novex Bis-Tris 4%–12% gradient gel (Invitrogen), blotted onto nitrocellulose membranes (Protran), blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20, and incubated with anti-ERK1/2, anti-pERK1/2, anti-DLK1 (all at 1:1000 dilution), or antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000) After washes, membranes were incubated with goat antimouse IRDye800 or goat antirabbit IRDye680 (1:10 000 dilution; Invitrogen). Immunoblots were scanned using the Odyssey Infrared Imaging System. See also Supplemental Table 1.

Quantitative real-time PCR (Q-PCR)

RNA extraction (RNeasy Mini kit; QIAGEN) and cDNA synthesis (Moloney murine leukemia virus reverse transcriptase; Promega) were performed according to the manufacturers' instructions. Q-PCR was performed in a 10-μL reaction containing: 2-μL cDNA template, 5-μL 2× $SYBR_{GREEN}$ I Master Mix (KAPA Biosystems), 0.2-μL low ROX (KAPA Biosystems), 0.5-μL primers (10μM forward + reverse), and 2.3-μL nuclease-free H_2O. *GAPDH* was used as the endogenous housekeeping gene. The real-time PCR was performed using an Mx3000 Thermocycler (Stratagene) using primers and conditions reported in Supplemental Table 1. Q-PCR analysis of *CYP11B1* and *CYP11B2* expression was done with TaqMan probes. Data were analyzed with MxPro software (Stratagene). See also Supplemental Table 1.

Statistical analysis

The data reported are the mean ± SEM of at least 3 independent experiments, performed in triplicate. Statistical comparison was performed using the unpaired two-tailed Student's *t* test, and *P* values are indicated as $*P < .05$, $**P < .01$, and $***P < .001$.

Results

Dlk1 expression marks cells in the outer layer of the undifferentiated zone (oZU)

We have described the subcapsular localization of Shh-secreting cells and the capsular localization of Shh-receiving cells in the developing and adult mouse (5) and rat (10) adrenal gland. In the rat, *Shh* mRNA expression is restricted to cells in the oZU (10), and this expression pattern is similar to that reported for Dlk1 protein (21). We verified that *Dlk1* mRNA (and protein) was a marker of the oZU (Figure 1 and Supplemental Figure 1). Similarly to Shh, *Dlk1*-expressing cells were not 11-beta-hydroxylase (CYP11B1) positive (and separated from the ZF by the inner ZU [iZU]), but a minority of them were aldosterone synthase (CYP11B2) positive (yellow arrows in Figure 1D and its inset).

Given their similar subcapsular expression patterns, we sought to investigate whether *Shh* mRNA and Dlk1 protein were expressed in the same cells in the oZU by using combined NR-ISH (for *Shh*) and IHC (with an anti-Dlk1 antibody that specifically recognizes Dlk1-producing cells), and found that virtually all cells are double positive (Figure 1, F–H).

Dlk1 mRNA expression also marked cells of the ZU in the developing rat adrenal. Because of the absence of the ZG until late developmental stages, the embryonic ZU is located adjacent to the capsule at e17.5 and e19.5 (Supplemental Figure 1).

The iZU is partially committed towards a steroidogenic phenotype

Cells in the iZU express the transcription factor steroidogenic factor-1 but do not express the zonal-specific steroidogenic markers CYP11B1 and CYP11B2 (10) and neither Shh nor *Dlk1* (Figure 1). P-450 side-chain cleavage (SCC) is known to be expressed throughout the adrenal cortex, with higher levels in the ZF compared with the outer layers (25). When SCC-CYP11B1 double staining was performed on adrenal sections previously processed for *Dlk1* NR-ISH (Figure 2), we observed a 1- to 2-cell wide layer above the ZF that was intensely stained with SCC but was completely negative for CYP11B1. These cells did not express *Dlk1* mRNA and therefore represented the iZU. SCC immunoreactivity weakened in cells of the oZU/ZG and is completely absent in the capsule (Figure 2A). Based on these results, it appears that the iZU is more committed to a steroidogenic phenotype than the oZU.

Dlk1 activates the ERK1/2 pathway in H295R cells: pERK1/2-positive cells as markers for cells transducing Dlk1 signal in vivo

Although Shh-receiving cells, identified by their expression of patched1 and Gli1, have been found to be primarily located in the capsule in both mouse (4–6) and rat (10) adrenal glands, the identity of the Dlk1 receptor has not been determined, thus impeding identification of secreted

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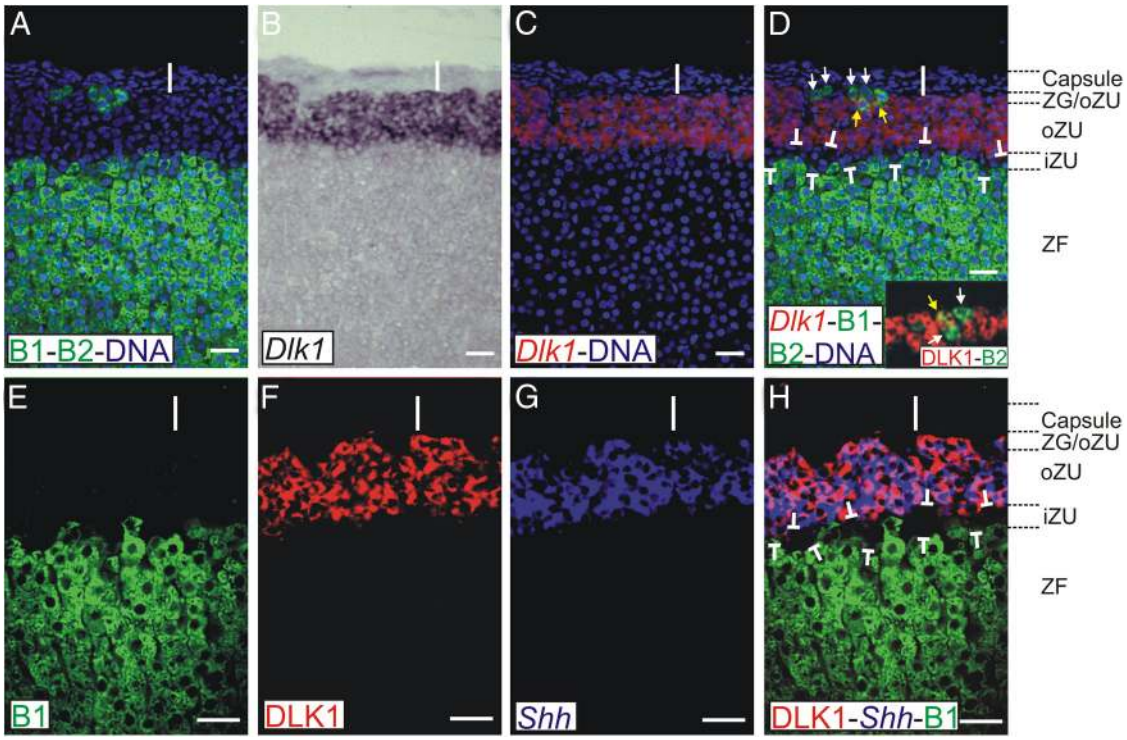

Figure 1. *Dlk1* mRNA and DLK1 protein is a marker of the oZU and is coexpressed with *Shh*. (A–D) Adult adrenal sections were processed for *Dlk1* NR-ISH (B) and then stained with antibodies to CYP11B1 (B1) and CYP11B2 (B2) (A, both green). Nuclei are stained with 4′,6-diamidino-2-phenylindole and indicated as DNA. The *Dlk1* signal was pseudocolored to red (C) and the merged image (D) assembled as reported (10). White arrows indicate CYP11B2-positive/*Dlk1*-negative cells, whereas the yellow arrows indicate a minority of double positive cells. The same result was obtained with an anti-Dlk1 antibody (inset). (E–H) Adult rat adrenal sections were processed for *Shh* NR-ISH (G, pseudocolored blue) and then incubated with antibodies to CYP11B1 (E, green) and to Dlk1 (F, red). The superimposed image (H) shows complete overlap between *Shh* mRNA and Dlk1 protein. The white brackets confine the iZU. The capsule thickness is indicated by the vertical white bars. Scale bar, 50 μm. See also Supplemental Figure 1.

Dlk1-receiving cells. In preadipocytes, Dlk1-mediated inhibition of adipocyte differentiation requires both a direct Dlk1/fibronectin interaction and subsequent integrin/ mitogen-activated protein kinase kinase (MEK)/ERK activation (15). We investigated whether a similar pathway was activated in adrenal cells and could be used to identify Dlk1 target cells. A GST-DLK1 fusion protein containing the soluble cleaved ectodomain of Dlk1 was able to inhibit the differentiation of 3T3-L1 cells into adipocytes (Supplemental Figure 2, A and B), demonstrating that it is biologically active. Addition of the fusion protein to human adrenocortical carcinoma H295R cells induced ERK1/2 phosphorylation in a time-dependent manner (Figure 3A). Furthermore, GST-DLK1-bound H295R cells were strongly pERK1/2 positive (Supplemental Figure 2C), suggesting that pERK1/2-positive cells in the adrenal could be responding to Dlk1. More data supporting this hypothesis are presented later (see figure 5 below).

To locate putative Dlk1 signal-receiving cells in the rat adrenal, we performed IHC to detect total and activated ERK1/2. We noted weak expression of ERK1/2 in the capsule and a stronger expression in the subcapsular region (Figure 3, B–D), consistent with *Erk1/2* mRNA expression (Supplemental Figure 2D) as well as previous studies (26).

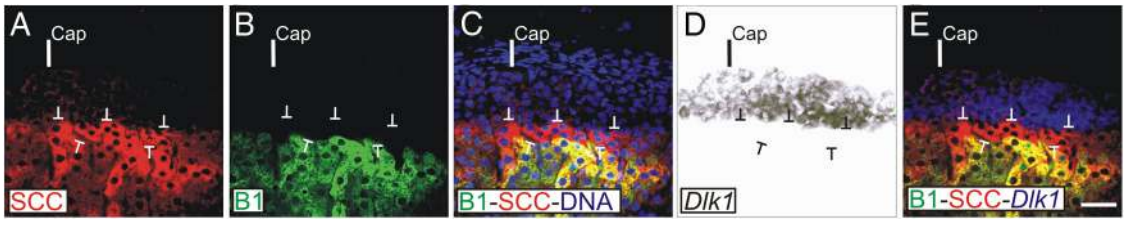

Figure 2. SCC is highly expressed in the iZU. Adult rat adrenal sections were first processed for *Dlk1* NR-ISH (D) and then double stained with antibodies to SCC (A) and CYP11B1 (B). Composite images of CYP11B1/SCC/4′,6-diamidino-2-phenylindole and CYP11B1/SCC/*Dlk1* are in C and E, respectively. Note that cells in the iZU (in between white brackets) are highly positive for SCC but completely lack CYP11B1 expression and are in direct contact with *Dlk1*-positive cells of the oZU. Cap, capsule. Scale bar, 50 μm.

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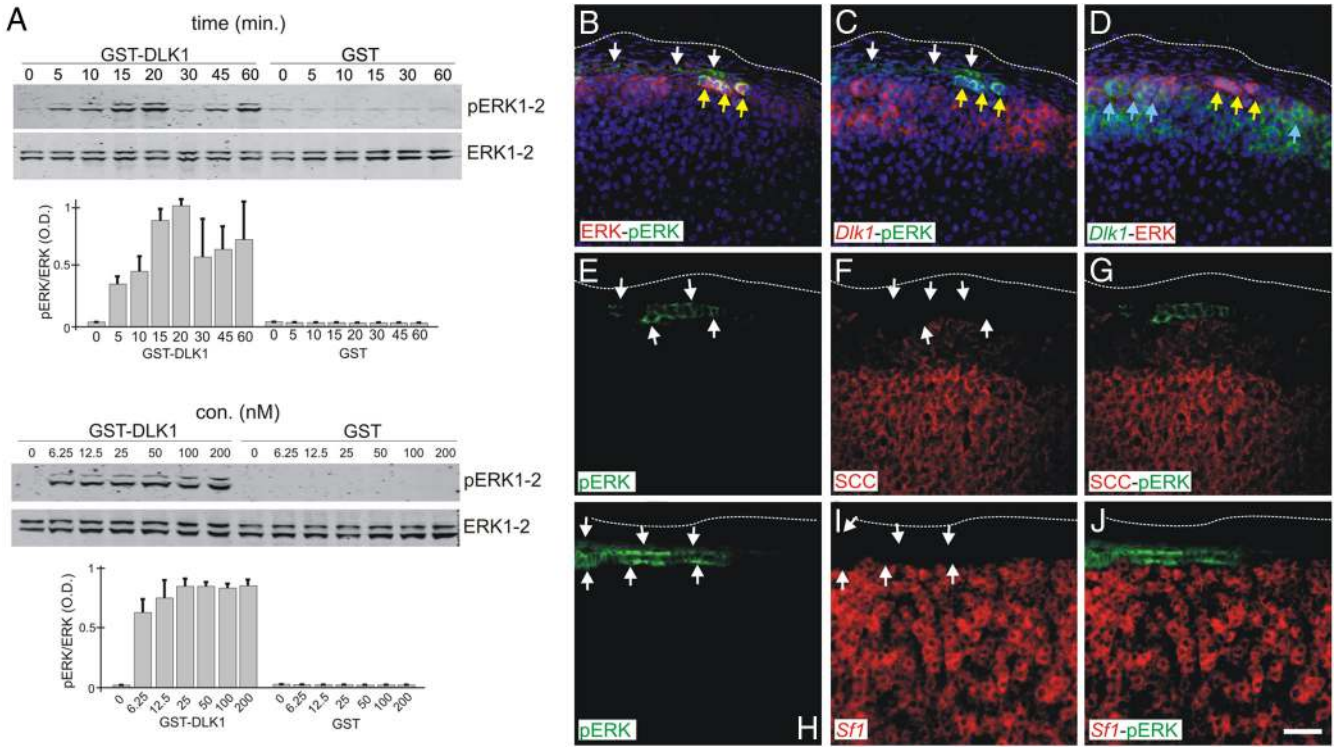

Figure 3. (A) Effects of recombinant DLK1 on H295R cells. H295R cells were incubated with GST or GST-DLK1 for the indicated times (upper panels) or at the indicated concentrations (lower panels), then lysed, size separated, and blotted with anti-ERK1/2 and anti-pERK1/2. The ratio of pERK to total ERK (pERK/ERK) was quantified by densitometric evaluation of Western blot analyses (by measuring the OD), and the data represent the mean ± SEM of at least 4 independent experiments. (B–J) Localization of ERK1/2 and pERK1/2-positive cells in the adrenal. Adult rat adrenal sections were processed for *Dlk1* NR-ISH (C, pseudocolored in red; D, pseudocolored in green) and then stained with antibodies to ERK1/2 and pERK1/2. pERK1/2-positive cells are a subgroup of ERK1/2-positive cells and mainly localized in capsular mesenchymal cells closer to the cortex (white arrows in B and C) and more abundantly in groups of cells at the capsule/cortex boundary (yellow arrows in B–D) and are *Dlk1* negative, although located near *Dlk1*-expressing cells (C). Some ERK1/2-positive cells are *Dlk1* positive (blue arrows in D). Sections were also costained with pERK1/2 (white arrows in E and H) and either SCC (F) or *Sf1* NR-ISH (I, pseudocolored in red), showing that pERK1/2 are SCC (G) and *Sf1* (J) negative. The white dotted lines indicate the top of the capsule. Scale bar, 50 μm. See also Supplemental Figure 2.

Costaining with anti-pERK1/2 showed that only a subset of the ERK1/2 cells was pERK1/2 positive. These cells were principally located at the boundary between the capsule and the cortex. Most pERK1/2-positive cells (yellow arrows in D) were not *Dlk1* positive but were located adjacent to *Dlk1* mRNA-positive cells. These cells were also SCC (Figure 3, E–G) and *Sf1* negative (Figure 3, H–J).

Dlk1 activates *Gli1* transcription through a β1 integrin and ERK1/2-dependent mechanism in H295R cells and rat capsule cells

Interestingly, 47 ± 11% of pERK1/2-positive cells were *Gli1* positive (Figure 4A, a″), suggesting that there might be a functional interplay between Dlk1 and Shh in common target cells. To verify this, we used H295R cells, which not only respond to recombinant Dlk1 by activating ERK1/2 (Figure 3 and Supplemental Figure 2) but also express all the components of the Hedgehog pathway and are a mixed cell population with regard to Dlk1 expression (~40% Dlk1 positive) (Supplemental Figure 3). Q-PCR analysis showed that GST-DLK1 was able to induce *GLI1* mRNA transcription dose dependently (Figure 4B). Moreover, this effect was pERK1/2 dependent, because it was abolished by U0126 in a dose-dependent manner (Figure 4C).

Fibronectin activates integrin signaling in many cellular settings, and Dlk1 inhibits preadipocyte differentiation through activation of the fibronectin-β1 integrin-ERK1/2 axis (15). In H295R cells, membrane-bound GST-DLK1 and fibronectin showed a pattern of colocalization (Supplemental Figure 4, A–C) very similar to that described in 3T3-L1 cells (15). In the adrenal cortex, fibronectin was expressed exclusively in the capsule, partially overlapping with pERK1/2-positive cells (Figure 4D, d″). To determine whether this interaction could be involved in Dlk1 signaling in adrenal cells, H295R cells were incubated with a β1 integrin-activating antibody, TS2/16 (27), in the presence of GST-DLK1. TS2/16 alone was able to significantly increase *GLI1* mRNA levels, and this effect was potentiated by GST-DLK1 (Figure 4E). Moreover, RGDS peptides, which disrupt fibronectin-integrin interaction, were able

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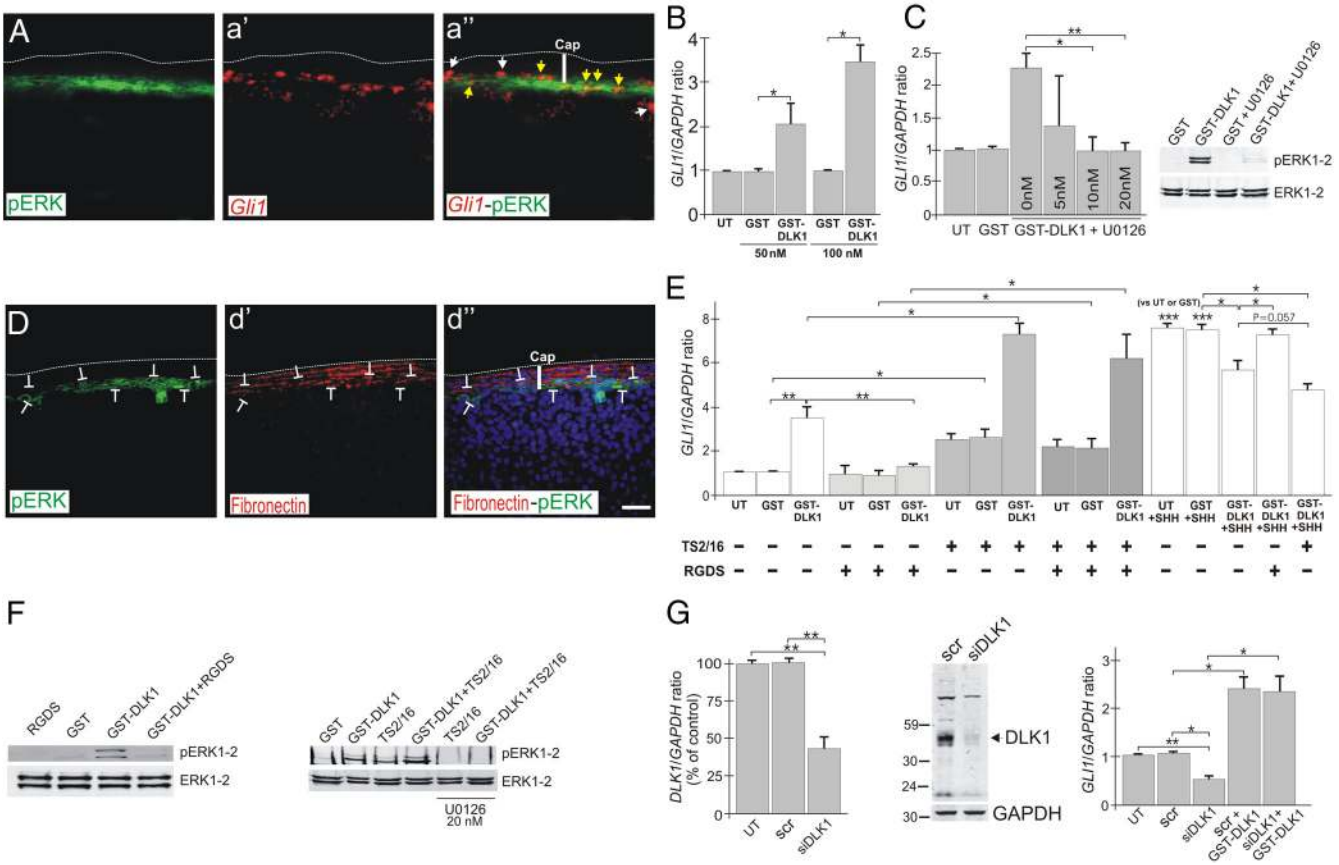

Figure 4. Signaling pathway elicited by DLK1 in adrenal cells. (A, a′) Adult rat adrenal sections were processed for *Gli1* NR-ISH (a′, pseudocolored in red) and then stained with antibodies to pERK1/2 (A). Yellow arrows in a″ point to *Gli1*/pERK1/2-double positive cells, whereas white arrows point to *Gli1*-positive cells without pErk1/2 immunoreactivity. The white dotted lines indicate the top of the capsule. (B) H295R cells were incubated with or without GST or GST-DLK1 at the indicated concentrations for 24 hours. *GLI1* Q-PCR was then performed on cDNA obtained from each sample. (C) H295R cells were incubated with 50nM of GST or 50nM of GST-DLK1 and U0126 at the indicated concentrations for 24 hours. *GLI1* Q-PCR was then performed on cDNA obtained from each sample. Data are normalized to *GAPDH* expression and presented as fold increases to GST treatment (left panel). H295R cells were treated as indicated for 20 minutes, then lysed, size separated, and blotted with anti-ERK1/2 and pERK1/2 (right panel). (D, d″) Adult rat adrenal sections were stained with antibodies to pERK1/2 (D) and to fibronectin (d′). The boundary between the capsule and the cortex where both pERK1/2 and fibronectin immunoreactivity are present is within brackets. The white dotted lines indicate the top of the capsule. Scale bar, 50 μm (applies to all). (E) H295R cells were incubated with or without GST or GST-DLK1 in the absence or presence TS2/16-activating antibody (2.5 μg/mL), RGDS peptides (500 μg/mL), or a combination of the 2 for 24 hours. *GLI1* Q-PCR was then performed on cDNA obtained from each sample. Data are normalized to *GAPDH* expression and presented as fold increases to GST treatment (n = 3 independent experiments in triplicate). (F) H295R cells were treated as indicated for 20 minutes, then lysed, size separated, and blotted with anti-ERK1/2 and pERK1/2. (G) H295R cells were transfected with 3 siRNAs specific to *DLK1* sequence or with a scramble siRNA. After 24 hours, RNA and proteins were extracted and Dlk1 mRNA, and protein levels were assayed with Q-PCR (left panel, data normalized to *GAPDH* expression and presented as percentage reduction compared with scramble) and Western blotting (middle panel), respectively. *GLI1* Q-PCR on these samples is reported in the right panel. Cap, capsule; UT, untreated (not transfected) cells. See also Supplemental Figure 4.

to inhibit GST-DLK1-mediated, but not TS2/16-mediated, *GLI1* up-regulation (Figure 4E) as well as ERK activation (Figure 4F, left panel). Again, U0126 blocked both TS2/16 and TS2/16-GST-DLK1-induced ERK1/2 activation (Figure 4F, right panel). Interestingly, recombinant SHH-mediated *GLI1* mRNA induction was partially but significantly blocked by GST-DLK1, an effect that was abolished by RGDS (Figure 4E). We observed a trend towards potentiation of the Dlk1 inhibitory effect on Shh induction of *GLI1* by the further inclusion of TS2/16, but this did not reach significance (Figure 4E). Similar results were obtained on primary cultures established from the rat adrenal capsule, although the potentiation of GST-DLK1-mediated *Gli1* transcription by TS2/16 was not as marked (Supplemental Figure 4, F and G). We next used a pool of 3 human Dlk1 small interfering RNA (siRNAs) to achieve a significant down-regulation of both *DLK1* mRNA and Dlk1 protein expression in H295R (Figure 4G, left and middle panels, respectively): siRNA-transfected cells expressed lower levels of *GLI1*. However, GST-DLK1 up-regulated *GLI1* similarly in scrambled and Dlk1-knockdown cells (Figure 4G, right panel). Taken together, these data demonstrate that Dlk1 activates *GLI1* transcription through a β1 integrin- and ERK1/2-dependent mechanism

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and that in H295R cells Dlk1 uses a similar pathway to that described in preadipocytes (15).

Dlk1 and *Shh* mRNA expression inversely correlates with the expression of CYP11B2

In order to assess the regulation of *Dlk1* and *Shh* expression during adrenal remodeling, rats were assigned to 2 different protocols modulating the RAS, namely a low-sodium diet, which increases the size of the ZG upon activation of the RAS, or captopril (an angiotensin-converting enzyme inhibitor) treatment, which depletes the ZG by blocking the RAS (28). Zone-specific proliferation was measured and reported in Supplemental Figure 4, A and B. The expression of *Shh* and *Dlk1* was evaluated by NR-ISH and Q-PCR (Figure 5, A–C). We found that both *Shh* and *Dlk1* mRNAs were down-regulated in adrenals obtained from rats fed a low-sodium diet and up-regulated when treated with captopril. During remodeling, the coexpression of *Shh* mRNA and Dlk1 protein was maintained (Supplemental Figure 5C). Interestingly, the level of pERK1/2 expression paralleled that of Dlk1 protein, being reduced in the low-sodium diet group and increased in the captopril group (Figure 5D), further supporting the validity of using pERK1/2 to localize Dlk1 signal-receiving cells as well as for assessing the activity of secreted Dlk1. Angiotensin II-treated H295R cells, which differentiate towards a ZG phenotype (29), also showed reduced levels of Dlk1 protein and mRNA (Supplemental Figure 5, D and E). Interestingly, H295R cells treated with forskolin, which is known to induce differentiation towards glucocorticoid-producing cells (30), showed a significant up-regulation of both Dlk1 protein and mRNA (Supplemental Figure 5, C–E).

Discussion

We have demonstrated that Dlk1 and Shh are coexpressed in the rat adrenal cortex in the oZU. In contrast to earlier studies (21), however, we do not see extensive coexpression of Dlk1 and CYP11B2, rather only a small number of cells at the outer boundary of the Dlk1-expressing population. Interestingly, our data also demonstrate that the Shh- and Dlk1-negative iZU has higher levels of SCC expression than the oZU, comparable with those of the ZF; this might indicate that the iZU represents a differentiating transitional zone, which has lost Shh and Dlk1 expression and is converting to a more steroidogenic phenotype, although it has yet to acquire CYP11B1 expression. The expression of the melanocortin receptor 2 and its accessory factor melanocortin receptor associated protein at high levels in the iZU (31) further support this hypothesis and may suggest a role for ACTH in the differentiation of these cells towards the ZF phenotype in vivo, as has been proposed in vitro (32).

The inhibitory role of Dlk1 in mesenchymal differentiation, and the pathways it activates, is well established (18). However. the molecular identity of the Dlk1 receptor is unknown. It has been suggested that it can bind to Notch (20, 33), but Wang et al (15) found no evidence for this and instead demonstrated an interaction with fibronectin, predicting that this would be in a complex with an as yet unidentified receptor. More recently, it has been demonstrated that soluble Dlk1 signals through membrane-bound Dlk1 in neural stem cells (13). We attempted to localize Dlk1-receiving cells in the adrenal gland by verifying that recombinant Dlk1 could elicit ERK1/2 activation in adrenal cells as has been demonstrated in preadi-

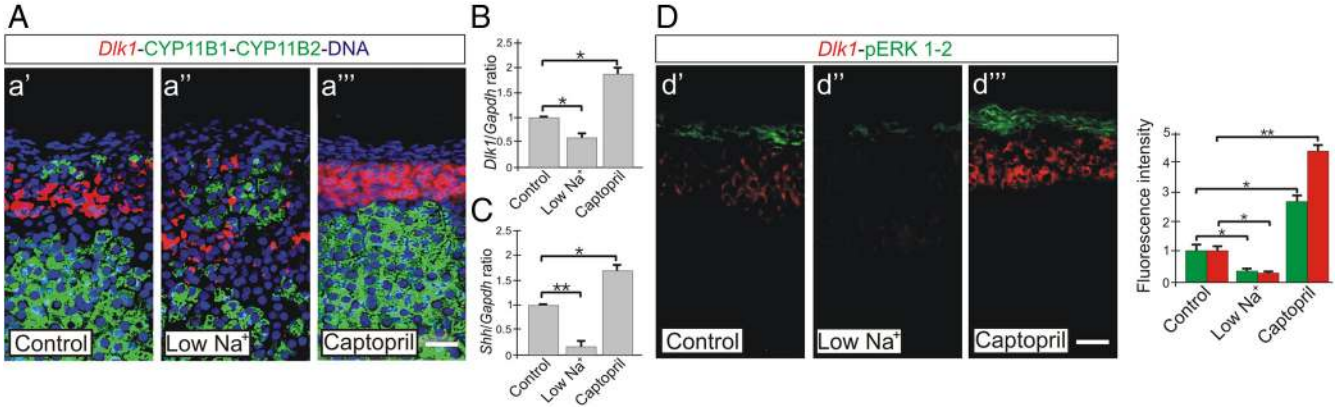

Figure 5. *Shh* and *Dlk1* expression inversely correlates to the growth of the ZG. (A) Adrenal sections from control rats (a'), from rats fed on a low-sodium diet (a'') or treated with captopril (a''') were processed for *Dlk1* NR-ISH and then stained with antibodies to CYP11B1 and CYP11B2. *Dlk1* mRNA (pseudocolored to red) was decreased in the low-sodium group and increased in the captopril group. (B and C) Q-PCR analysis of *Dlk1* (B) and *Shh* (C) expression in the contralateral adrenals from the same rats used for the experiments reported in A. (D) Adrenal sections from control rats (d'), from rats fed on a low-sodium diet (d'') or treated with captopril (d'''), were processed for *Dlk1* NR-ISH followed by pERK1/2 immunostaining. Scale bar, 50 μm. The quantification is reported in the panel on the right, where green and red columns refer to pERK1/2 and *Dlk1* staining, respectively. See also Supplemental Figure 5.

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pocytes (16). The pERK1/2-positive cells have a very specific expression pattern in the adrenal cortex, being restricted to the boundary between the capsule and the cortex. As most these cells are adjacent to Dlk1-expressing cells, it suggests that Dlk1 is acting on them in a paracrine manner, and given that they are not Dlk1 positive, Dlk1 cannot be activating the MEK/ERK pathway via interaction with its membrane-bound form, as in neural stem cells. However, these capsular cells do express fibronectin, and approximately half of the pERK1/2-positive cells express *Gli1*.

Although the H295R cell line is derived from an adrenocortical carcinoma, it expresses *GLI1* and *PTCH1*, whose expression is restricted to the capsule and subcapsular mesenchymal cells in the gland. Interestingly, it also has a variable expression of Dlk1, and this indicates that the cell line has a heterogeneous phenotype and potentially contains a population of Gli1-positive putative stem cells that responds to Shh and Dlk1 signals. This is in line with the observations that the H295R cell line has a relatively pluripotent phenotype and can differentiate into more mature steroidogenic cell types representative of all 3 adult zones under appropriate stimuli (reviewed in Ref. 34). The primary cells established from the rat adrenal capsule and the cell line gave us very similar results that allowed us to investigate Dlk1 signaling in the adrenal. We have shown that Dlk1 positively regulates *Gli1* transcription, in both H295R cells and rat adrenal capsule primary cells, in a manner dependent upon ERK1/2 activation and potentiated by β1 integrin activation. Our data are therefore consistent with the formation of a macromolecular complex at the plasma membrane of Dlk1 (and Shh)-receiving cells containing fibronectin, β1 integrin, Dlk1 ectodomain, and its putative receptor, in order to activate ERK1/2. It is formally possible that Dlk1 exerts its effects on *Gli1* transcription by up-regulating Shh through this pathway. However, Dlk1 also inhibits Shh-mediated Gli1 expression in the same manner, indicating that this may be unlikely and also suggesting that, as well as activating Shh target genes independently, Dlk1 fine tunes Shh signaling at the adrenal capsule and possibly other sites of coexpression, such as in the preadipocyte. The cooperation of Shh and Dlk1 to control capsule cell Gli1 expression is an interesting area for further study.

How Dlk1 causes activation of Gli1 transcription remains to be elucidated. In 3T3-L1 cells, activation of ERK1/2 induces Sox9 expression, which binds to and inhibits the promoters of key genes required for adipocyte differentiation (17). However, Sox9 expression has not been detected in the adrenal gland (35). Nevertheless, our data suggest that Dlk1 up-regulates Gli1 expression via a noncanonical, β1 integrin- and ERK-dependent pathway. Noncanonical activation of members of the Gli family of transcription factors has been described involving PI3K/AKT (36, 37), PKCδ (36), EGF receptor (38), ras (39), and TGFβ (40) pathways (reviewed in Ref. 41). TGFβ activates Gli1 expression via a small mothers against decapentaplegic 3-mediated induction of Gli2, whereas the ras pathway activates signaling via the MEK/ERK pathways, and both pathways operate to up-regulate Gli1 expression in pancreatic ductal adenocarcinoma cells (42). PKCδ was shown to activate a Gli luciferase reporter in 3T3-L1 cells via the MEK pathway, and the activity of overexpressed Gli1 in this assay was enhanced by cotransfection of an activated MEK construct (36). Interestingly, this enhancement was lost in an N-terminal deletion mutant of Gli1 lacking the first 130 amino acids. Furthermore, it has been demonstrated that the Gli transcription factors are bound and phosphorylated by Erk2, on Ser130 in the case of Gli1 (43). It is therefore possible that Dlk1 binds fibronectin and activates $\alpha 5\beta 1$ integrin signaling, which in turn activates ERK1/2. This may then lead to Gli1 and/or Gli2 phosphorylation and an increase in Gli1 expression levels in adrenal cells. In support of a role for integrins is the recent observation that β1 integrins regulate Gli1 expression via signaling through the IGF receptor (44).

As well as being expressed in the same cells in the cortex, Shh and Dlk1 expressions are inversely correlated to growth of the ZG. We have shown that during mouse adrenal development, both Shh- and Gli1-expressing cells can differentiate into CYP11B2-expressing cells (5), and the coexpression of Dlk1 (Figure 1) and Shh (10) with CYP11B2 in cells at the boundary of the ZU suggests that this may also take place in the adult rat under normal conditions. Maintenance of Shh expression in the adult gland suggests a role for Shh signaling beyond development, potentially in controlling zonation and remodeling. Activation of the RAS remodels the adrenal cortex by provoking a rapid expansion of the CYP11B2-expressing cell population, and this may, at least in part, be achieved by differentiation of steroidogenic cells from capsular mesenchymal progenitors. Dlk1 has a well-documented role as a negative regulator of cell differentiation in a number of settings (18), and Shh can also inhibit adipogenesis (45), perhaps in concert with Dlk1. It is possible therefore that both Shh and Dlk1 negatively regulate the differentiation of Gli1-expressing progenitor cells, and down-regulation of the expression of these molecules is a prerequisite for remodeling under these conditions. In this study, activation of the RAS by sodium restriction causes an increase in cell proliferation adjacent to the capsule, with the observation of columns of proliferating cells traversing the cortex from the capsule to the ZU (Supplemental Figure 5A). It is interesting to speculate that this indicates capsular

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cells converting to a steroidogenic phenotype during remodeling, as has been observed during development. In support of this role for Dlk1 is the observation that after surgery, in which the adrenals of rats are enucleated to leave only the fibrous capsule and adherent subcapsular cells, Dlk1 expression is down-regulated as the cortex regenerates until zonation is reestablished (21). If the activation of differentiation of Gli1-positive cells into steroidogenic CYP11B2-positive cells does occur during remodeling, it remains to be determined whether the differentiation is a direct process or occurs via a Shh/Dlk1-expressing intermediate.

An alternative hypothesis would be that RAS activation promotes the differentiation of the Shh/Dlk1-expressing progenitor cells, with their conversion to CYP11B2-expressing cells resulting in the rapid down-regulation of Shh and Dlk1 expression, in a similar manner to that seen for the progression of cells from the ZU to the ZF. In support of this model, it is interesting to note that some proliferating cell nuclear antigen/Dlk1-double positive cells can be observed at the oZU/ZG boundary, similar to the apparent conversion of Dlk1- and Shh-expressing cells into CY11B2-expressing cells under normal conditions. Furthermore, previous studies have observed the migration of 5-bromo-2′-deoxyuridine-labeled cells from the ZU into the ZG in the adrenals of rats placed on a low-sodium diet (46).

In either situation, it is envisaged that inhibition of the RAS, in this study by captopril, would cause dedifferentiation of the CYP11B2-positive cells to expand the Shh/Dlk1-positive progenitor cell population, as has been observed in adrenal regeneration after enucleation (25). It is envisaged that similar responses will be observed upon remodeling the ZF by manipulation of the hypothalamic-pituitary-adrenal axis after activation by ACTH or inhibition by dexamethasone administration, and it is noteworthy that differentiation of H295R cells towards a more ZF/ZR phenotype by prolonged treatment with forskolin increases Dlk1 expression (Supplemental Fig 5E). Whether this reflects the in vivo response to ACTH-induced remodeling is the focus of current studies, and the resolution of the mechanisms controlling remodeling and Dlk1/Shh expression awaits the use of genetic mouse models to activate or inactivate Shh expression at the time of remodeling and to follow the fate of Shh- and Gli1-expressing cells under the same conditions.

In conclusion, we have shown that Dlk1 is coexpressed with Shh in candidate adrenocortical progenitor cells. Both secreted molecules activate *Gli1* expression in mesenchymal cells in the adrenal capsule, with Dlk1 using a noncanonical pathway involving β1 integrin and pErk1/2. Dlk1 can inhibit Shh activation of *Gli1* in a similar manner, suggesting a potential control of Shh signaling by Dlk1. As well as being coexpressed, Dlk1 and Shh expression are equally affected by adrenal remodeling. These data suggest that Dlk1 and Shh act in concert to control adrenocortical zonation and remodeling, thus maintaining homeostasis.

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