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GLI1$^+$ progenitor cells in the adrenal capsule of the adult mouse give rise to heterotopic gonadal-like tissue

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ABSTRACT

As certain strains of mice age, hyperplastic lesions resembling gonadal tissue accumulate beneath the adrenal capsule. Gonadectomy (GDX) accelerates this heterotopic differentiation, resulting in the formation of wedge-shaped adrenocortical neoplasms that produce sex steroids. Stem/progenitor cells that reside in the adrenal capsule and retain properties of the adrenogonadal primordium are thought to be the source of this heterotopic tissue. Here, we demonstrate that GLI1$^+$ progenitors in the adrenal capsule give rise to gonadal-like cells that accumulate in the subcapsular region. A tamoxifen-inducible Cre driver (*Gli1*-creER^{T2}) and two reporters (*R26R*-lacZ, *R26R*-confetti) were used to track the fate of GLI1$^+$ cells in the adrenal glands of B6D2F2 mice, a strain that develops both GDX-induced adrenocortical neoplasms and age-dependent subcapsular cell hyperplasia. In gonadectomized B6D2F2 mice GLI1$^+$ progenitors contributed to long-lived adrenal capsule cells and to adrenocortical neoplasms that expressed *Gata4* and *Foxl2*, two prototypical gonadal markers. *Pdgfra*, a gene expressed in adrenocortical stromal cells, was upregulated in the GDX-induced neoplasms. In aged non-gonadectomized B6D2F2 mice GLI1$^+$ progenitors gave rise to patches of subcapsular cell hyperplasia. Treatment with GANT61, a small-molecule GLI antagonist, attenuated the upregulation of gonadal-like markers (*Gata4*, *Amhr2*, *Foxl2*) in response to GDX. These findings support the premise that GLI1$^+$ progenitor cells in the adrenal capsule of the adult mouse give rise to heterotopic tissue.

1. Introduction

As inbred mice age, patches of tissue resembling gonadal stroma appear beneath the adrenal capsule, a phenomenon known as subcapsular cell hyperplasia (Frith et al., 1983; Ward et al., 2002; Yates et al., 2013; Petterino et al., 2015). This process of ectopic gonadal-like differentiation is strain-dependent and accelerated by prepubertal gonadectomy (GDX) (Bernichtein et al., 2009). The resultant wedge-shaped lesions, termed GDX-induced adrenocortical neoplasms, are composed of two principal cell types: spindle-shaped type A cells that have limited steroidogenic capacity, and sex steroid-producing type B cells that accumulate later within patches of type A cells (Bielinska et al., 2006). The accumulation of type A or B cells is also a hallmark of adrenal neoplasia in several genetically-engineered mouse models (Looyenga and Hammer, 2006; Doghman et al., 2007; Berthon et al., 2010; Lee et al., 2011; Drelon et al., 2012; Hughes et al., 2012; Bandiera et al., 2013; Latre de Late et al., 2014).

Recent studies have shed light on the progenitors that give rise to heterotopic tissue in the adrenal cortex of mice [reviewed in (Bandiera et al., 2015; Röhrig et al., 2015)]. Fate mapping experiments have shown that the adrenal capsule contains long-lived stem/progenitor cells that retain properties of the adrenogonadal primordium (AGP), specialized cells in the genital ridge that are the

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precursors of steroidogenic cells in the adrenal cortex, testis, and ovary (Bandiera et al., 2013). Under basal conditions, these AGP-like cells in the capsule can give rise to normal steroidogenic cells in the adrenal cortex. GDX activates these capsular progenitors and triggers their differentiation into gonadal-like tissue. *Wt1* is expressed in these AGP-like cells, and downregulation of this transcription factor gene is required for their differentiation into adrenocortical cells (Bandiera et al., 2013). WT1 directly activates *Gli1*, a downstream effector of hedgehog signaling, a pathway implicated in steroidogenic cell differentiation in both the adrenal cortex and gonads [reviewed in (Finco et al., 2015)]. Activation of *Gli1* expression by WT1 is thought to inhibit the differentiation of these progenitors into adrenocortical cells and promote their differentiation into gonadal-like cells (Bandiera et al., 2013).

Although *Gli1* has been implicated as a key regulator of heterotopic differentiation in the adrenal cortex of mice, there have been no direct studies tracking the fate of GLI1$^+$ cells during this process. Here, we investigate the fate of GLI1$^+$ progenitors in B6D2F2 mice, a strain that develops both GDX-induced adrenocortical neoplasia and aged-related subcapsular cell hyperplasia (Bernichtein et al., 2007). We demonstrate that GLI1$^+$ capsule cells give rise to GDX-induced neoplasms and to patches of spontaneous subcapsular cell hyperplasia. Additionally, we show that treatment with a small-molecule GLI inhibitor [GLI antagonist 61 (GANT61)] impairs the accumulation of gonadal-like markers in the adrenal glands of gonadectomized mice.

2. Materials and methods

2.1. Experimental animals

Procedures involving mice were approved by an institutional committee for laboratory animal care and were conducted in accordance with NIH guidelines for the care and use of experimental animals. C57Bl/6J mice harboring *Gli1*-creER^{T2} [*Gli1*$^{tm3(cre/ESR1)Alj}$/J] (Ahn and Joyner, 2004), *Rosa26* loxP-stop-loxP lacZ [*R26R*-lacZ; *Gt(ROSA)26Sor*^{tm1Sor}/J] (Pangas et al., 2007), or *R26R*-confetti [B6.Cg-*Gt(ROSA)26Sor*$^{tm1(CAG-Brainbow2.1)Cle}$/J] (Snippert et al., 2010) transgenes were obtained from the Jackson Laboratory (Bar Harbor, ME). These strains were crossed with DBA/2J mice (Jackson Laboratory) to generate B6D2F1 mice carrying the transgenes. B6D2F1 *Gli1*-creER^{T2} mice were crossed with B6D2F1 *R26R*-lacZ or B6D2F1 *R26R*-confetti mice to generate B6D2F2 offspring for analysis. Mice were anesthetized and gonadectomized at 3 weeks of age (Bielinska et al., 2005). Mice carrying the *Gli1*-creER^{T2} transgene were injected subcutaneously with tamoxifen (Sigma; St. Louis, MO; 200 mg/kg in corn oil) at varying times (see figure legends), and adrenal tissue was harvested for analysis 2–5 mo later. *Sf1-cre* mice [FVB-Tg(Nr5a1-cre)2Lowl/J] were obtained from the Jackson Laboratory and genotyped as described (Dhillon et al., 2006; Sodhi et al., 2006). *Sf1-cre* mice were crossed with B6D2F1 *R26R*-confetti mice to assess reporter expression in adrenocortical and gonadal steroidogenic cells. Dopachrome tautomerase-cre (*Dct*-cre) mice were generated as described previously (Guyonneau et al., 2002).

2.2. X-gal staining

Gli1-creER^{T2};*R26R*-lacZ mice were anesthetized and perfusion-fixed with 4% paraformaldehyde in PBS (Pihlajoki et al., 2013). Adrenal glands were harvested, incubated in 4% paraformaldehyde in PBS for an additional 30 min, permeabilized with 100 mM potassium phosphate pH 7.4, 0.02% NP-40 and 0.01% sodium deoxycholate for 5 min, and then stained with X-gal at 37 °C overnight (Narita et al., 1997). After staining, the glands were post-fixed in 4% paraformaldehyde in PBS for 1 h, frozen in OCT cyropreservation media (Tissue-Tek, Torrance, CA), sectioned (10 μm), counterstained with eosin, and examined by light microscopy. Alternatively, the X-gal stained tissue was post-fixed with Karnovsky solution, treated with 2% OsO_4, and embedded in epon (Bielinska et al., 2007). Sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (Bielinska et al., 2007).

2.3. Direct and indirect fluorescence microscopy

Sf1-cre;*R26R*-confetti and *Gli1*-creER^{T2};*R26R*-confetti mice were anesthetized and perfusion-fixed with 4% paraformaldehyde in PBS. Adrenal glands or gonads were harvested and fixed an additional 2 h in 4% paraformaldehyde in PBS. Following fixation, the glands were incubated overnight in 30% sucrose. Tissues then were embedded in OCT and cryosectioned (10 μm). Indirect immunofluorescence was performed (Bielinska et al., 2007) using the primary and secondary antibodies listed in Table 1. The analyses included negative control studies in which the primary or secondary antibodies were omitted. FITC-streptavidin staining of endogenous biotin in zG and zF cells was performed as described (Paul and Laufer, 2011).

2.4. Real time RT-PCR (RT-qPCR)

Total RNA was isolated and subjected to RT-qPCR analysis as described (Pihlajoki et al., 2013). Expression was normalized to the housekeeping genes *Actb* and *Gapdh*. Primer pairs are listed in Supplementary Table 1.

2.5. Pharmacological inhibition of GLI1/2 with GANT61

GANT61 (Cayman Chemical, Ann Arbor, MI) was dissolved in ethanol (50 mg/mL), sonicated on ice, and stored in aliquots at −80 °C. The ethanol stock was warmed and diluted in corn oil (1:4) before injection (50 mg/kg sc daily x 14 days) into gonadectomized weanling female DBA/2J mice (Lauth and Toftgard, 2007;

Table 1
Antibodies used for immunostaining.

Antibody	Primary antibody	Secondary antibody
GATA4	Goat anti-mouse GATA4 (sc-1237, Santa Cruz Biotech, 1:200 dilution)	Donkey anti-goat biotinylated IgG (Jackson Immunoresearch, West Grove, PA) at a 1:1000 dilution) followed by FITC-streptavidin (AlexaFluor-488, S11223, Invitrogen, 1:1000 dilution). Alternatively, the avidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit, Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine were used to visualize the bound antibody.
PDGFRα	Rabbit anti-mouse PDGFRα (sc-338, Santa Cruz Biotech, 1:100 dilution)	FITC-goat anti-rabbit IgG (111-095-003, Jackson Immunoresearch, 1:1000 dilution)
FOXL2	Goat anti-FOXL2 (IMG-3228; Imgenex, San Diego, CA; 1:400 dilution).	Donkey anti-goat biotinylated IgG (Jackson Immunoresearch) at a 1:1000 dilution) followed by FITC-streptavidin (AlexaFluor-488, S11223, Invitrogen, 1:1000 dilution). Alternatively, the avidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit, Vector Laboratories, Inc.) and diaminobenzidine were used to visualize the bound antibody.

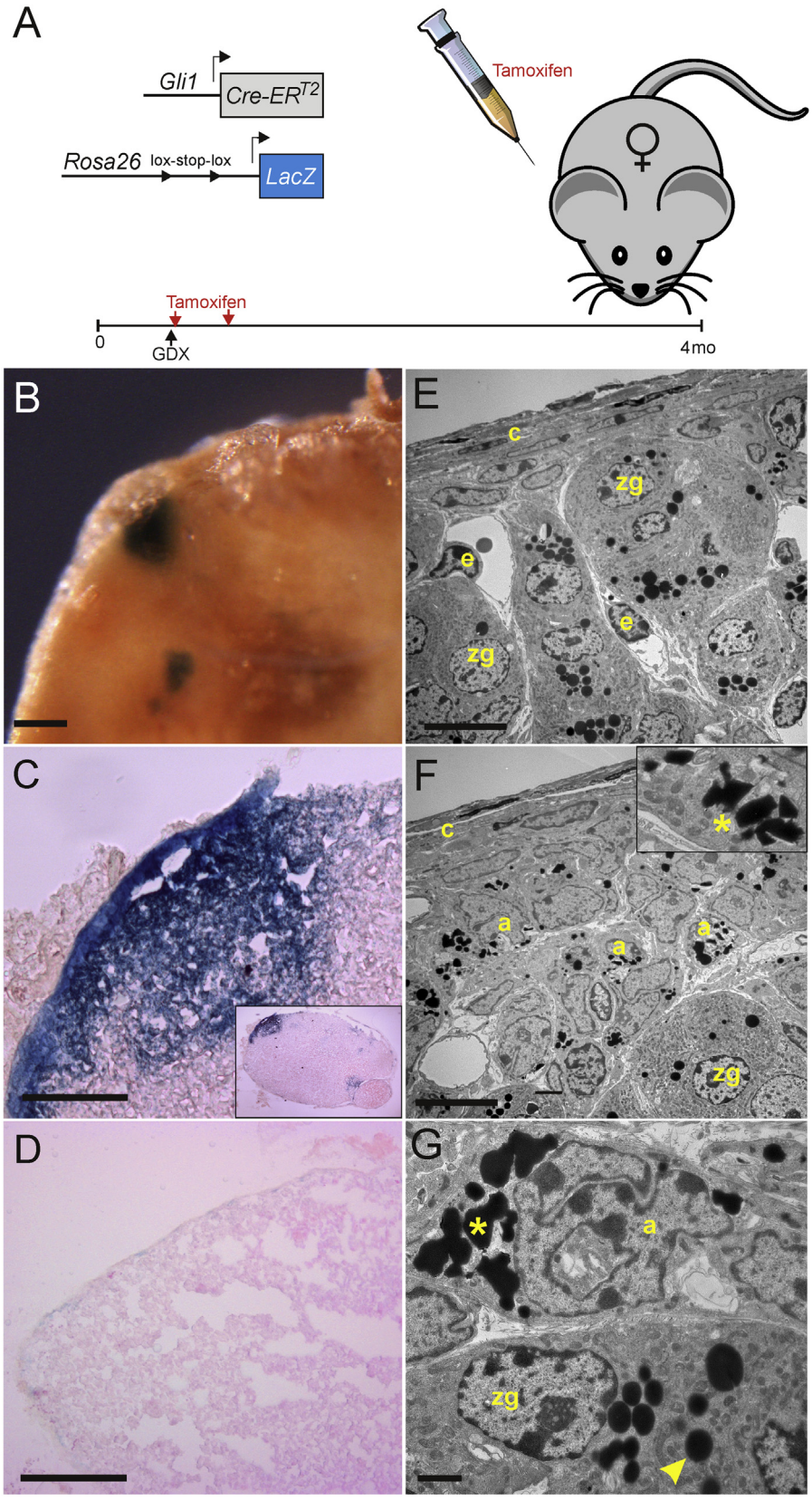

Fig. 1. GLI1^{+} progenitor cells contribute to GDX-induced neoplasms in the adrenal glands of *Gli1*-CreER^{T2};*R26R*-lacZ mice. **A)** Female B6D2F2 *Gli1*-CreER^{T2};*R26R*-lacZ mice were gonadectomized or subjected to sham surgery and then injected with tamoxifen at the indicated times. Adrenal glands were harvested at 4 mo of age. **B)** Whole-mount X-gal staining highlights patches of neoplastic cells. **C)** A wedge-shaped, β-gal^{+} neoplasm in a cryosection of an X-gal stained adrenal. The inset shows a lower magnification view of the same tissue section. **D**) Cryosection of an X-gal stained adrenal gland from a non-gonadectomized, tamoxifen-treated *Gli1*-CreER^{T2};*R26R*-lacZ mouse. Rare, small patches of β-gal^{+} cells are seen in the capsule but not the underlying cortex. **E,F)** Electron photomicrographs of normal and neoplastic adrenal cortex, respectively, from a *Gli1*-CreER^{T2};*R26R*-lacZ mouse. Note the accumulation of ovoid type A cells in the neoplastic cortex. The inset in panel F shows irregularly shaped X-gal crystalloids (*) in a type A cell. **G**) Ultrastructural comparison of a type A cell and zG cell. Type A cells have a polymorphic nucleus, scant smooth endoplasmic reticulum, few mitochondria, and other characteristic features (see the accompanying text). The asterisk indicates irregularly-shaped X-gal crystalloids. The arrowhead highlights a round lipid droplet. Abbreviations: a, type A cell; c, capsule cell; e, endothelial cell; zg, zona glomerulosa cell. Bars: (B) 150 μm, (C,D) 100 μm, (E,F) 10 μm, (G) 1 μm.

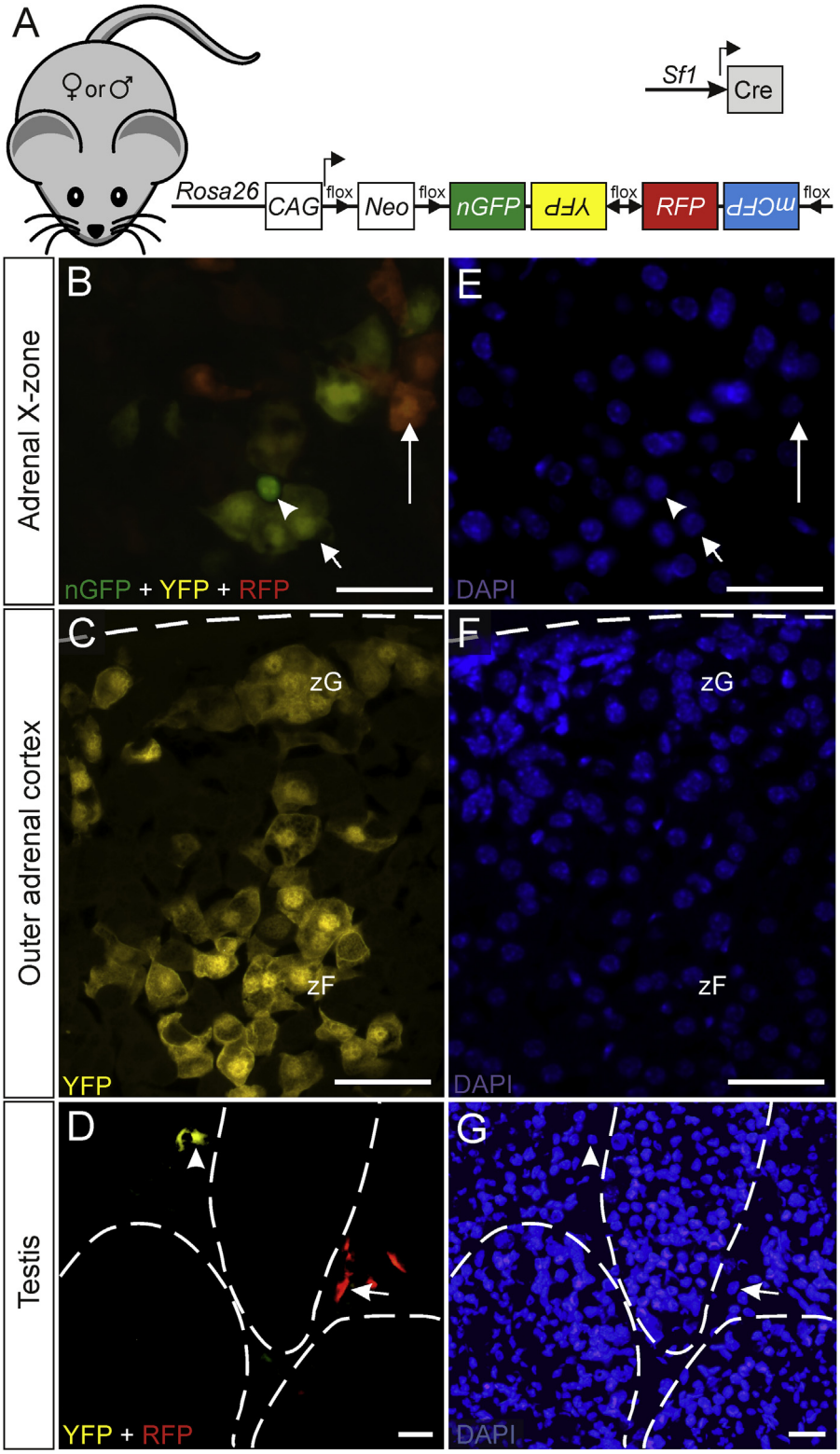

Fig. 2. Labeling of steroidogenic cells in the adrenal cortex and testis of *Sf1*-Cre;*R26R*-confetti mice. A) Adrenal glands or testis were harvested from 2-mo-old *Sf1*-Cre;*R26R*-confetti mice. Tissues were cryosectioned and examined by fluorescence microscopy. **B)** Clusters of nGFP$^+$ (arrowhead), YFP$^+$ (small arrow), and RFP$^+$ (large arrow) cells in the adrenal X-zone of a virgin female. In this image, green in the nGFP signal was selectively enhanced using software, so as to distinguish nGFP$^+$ from YPF$^+$ cells. **C)** A clone of YFP$^+$ zG and zF cells of the outer adrenal cortex of a female. Dashed lines denote the capsule surface. **D)** YFP$^+$ (arrowhead) and RFP$^+$ (arrow) testicular interstitial cells, presumed to be Leydig cells. Dashed lines denote seminiferous tubules. **E-G)** Corresponding DAPI stains. Bars: 30 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

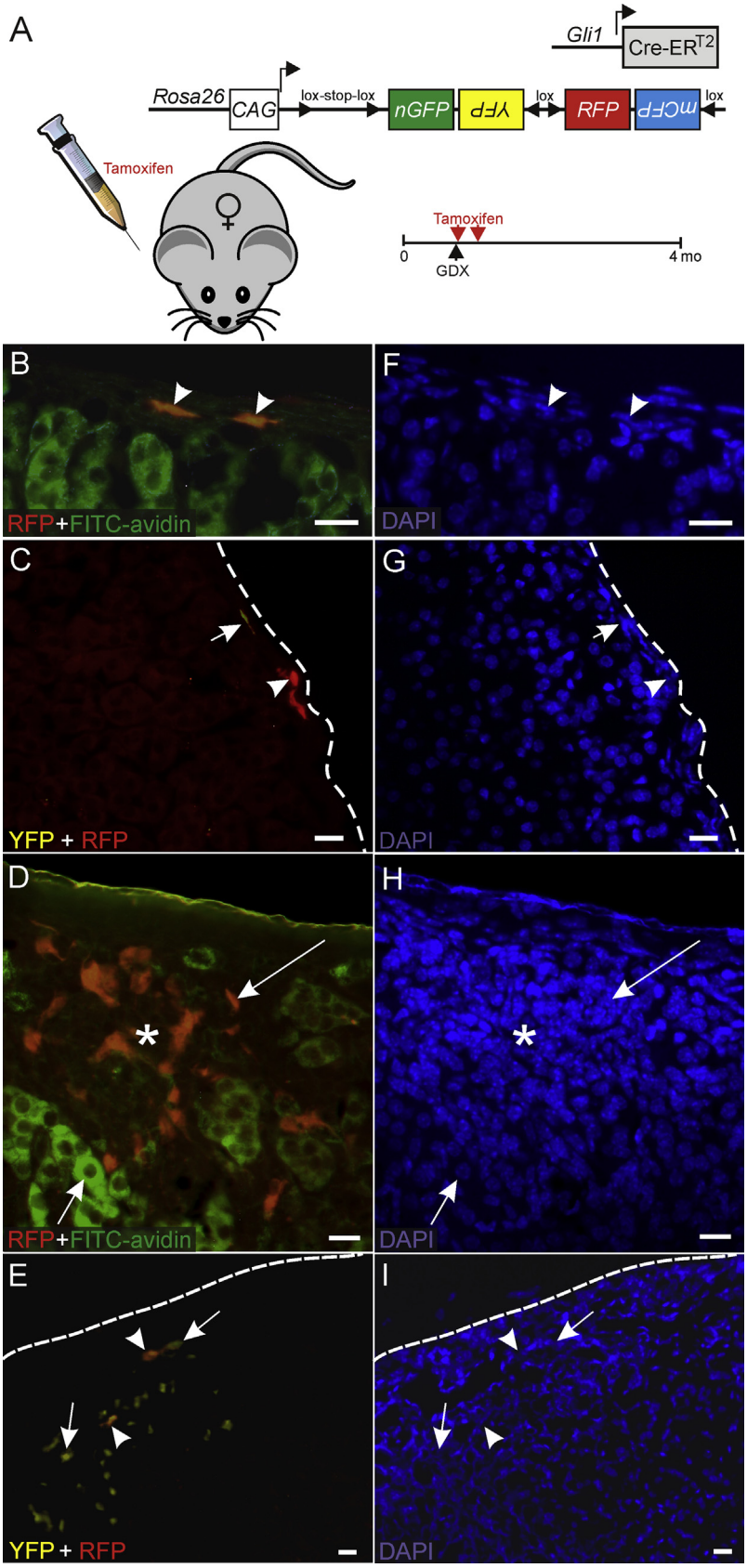

Fig. 3. GLI1^{+} progenitor cells contribute to long-lived capsule cells and to GDX-induced neoplasms in the adrenal glands of *Gli1*-CreER^{T2};*R26R*-confetti mice. A) Female B6D2F2 *Gli1*-CreER^{T2};*R26R*-confetti mice were gonadectomized and injected with tamoxifen at the indicated times. Adrenal glands were harvested, cryosectioned, and examined by fluorescence microscopy. Some of the sections were stained with FITC-streptavidin to detect endogenous biotin in normal steroidogenic cells. **B)** Arrowheads highlight two RFP^{+} capsule cells, presumably derived from a common GLI1^{+} progenitor. Biotin-rich steroidogenic cells are evident in the subjacent cortex. **C)** The arrowhead highlights a clone of RFP^{+}

Kramann et al., 2015). Control GDX DBA/2J mice were injected with vehicle alone (ethanol/corn oil, 1:4). At the indicated timepoints, adrenal tissue was harvested for RT-qPCR analysis or overnight fixation in 4% paraformaldehyde in PBS. The fixed tissue was embedded in paraffin, sectioned (4 μm), and subjected to immunoperoxidase staining (Anttonen et al., 2003; Bielinska et al., 2005; Schillebeeckx et al., 2015) using the primary and secondary antibodies listed in Table 1.

2.6. Isolation of neoplastic and normal tissue using laser capture microdissection (LCM)

Cryosections (10 μm) of mouse adrenal glands were collected on membrane slides (PEN-Membrane 2.0 μm; Leica), fixed in ethanol at −20° C, stained with crystal violet, and then dehydrated by passage through successively higher concentrations of ethanol followed by xylene (Pihlajoki et al., 2013; Schillebeeckx et al., 2013). LCM with a Leica LMD6000 microscope was used to isolate samples from neoplastic or normal tissue. Microdissectates were collected in RNA extraction buffer (RNeasy Mini Kit, Qiagen, Valencia, CA) or Arcturus Picopure RNA isolation kit (Applied Biosystems, Foster City, CA).

3. Results

3.1. Fate mapping in B6D2F2 Gli1-creER^{T2};R26R-lacZ mice

GLI1$^+$ progenitors in the adrenal capsule have been shown to contribute to steroidogenic cells during fetal and early postnatal development (King et al., 2009; Huang et al., 2010; Wood et al., 2013; Bandiera et al., 2015), but the contribution of GLI1$^+$ cells to adrenal homeostasis in adults has not been investigated in detail. We hypothesized that GLI1$^+$ cells are the precursors of GDX-induced adrenocortical neoplasms. To follow the fate of GLI1$^+$ progenitor cells in adult B6D2F2 mice, we used a tamoxifen-inducible cre-driver (*Gli1*-creER^{T2}) and a *Rosa26* flox-stop-flox-lacZ (*R26R*-lacZ) reporter allele. In the presence of tamoxifen, creER^{T2} excises the flox-stop-flox cassette, thereby indelibly activating reporter gene expression in a cell and its descendants (Ahn and Joyner, 2004; King et al., 2009). Whole mount X-gal staining of adrenal glands from gonadectomized, tamoxifen-treated *Gli1*-creER^{T2};*R26R*-lacZ mice demonstrated β-gal activity in discrete patches of cells (Fig. 1B). Sectioning of these adrenal glands showed that the patches of β-gal$^+$ cells corresponded to wedge-shaped subcapsular neoplasms (Fig. 1C). To optimize X-gal labeling of neoplastic cells, we varied the timing and frequency of tamoxifen administration in *Gli1*-creER^{T2};*R26R*-lacZ mice. Two doses of tamoxifen administered in the days following GDX (Fig. 1A) were sufficient to label adrenal capsular progenitors that subsequently gave rise to neoplasms in the mice. In contrast, administration of two doses of tamoxifen to non-gonadectomized *Gli1*-creER^{T2};*R26R*-lacZ mice resulted in only sparse labeling of cells in the adrenal capsule (Fig. 1D).

Transmission electron microscopy was used to further characterize the β-gal$^+$ cells in the adrenal glands of gonadectomized *Gli1*-creER^{T2};*R26R*-lacZ mice. Hydrolysis of X-gal by β-gal generates electron-dense, cytoplasmic crystalloids (Merchant-Larios and Moreno-Mendoza, 1998). Such crystalloids have irregular shapes that distinguish them from electron-dense lipid droplets (Fig. 1G, * *vs*. arrowhead). Crystalloid-laden neoplastic cells accumulated in the subcapsular region of gonadectomized *Gli1*-creER^{T2};*R26R*-lacZ mice and distorted the zonal architecture, displacing zG cells centripetally (Fig. 1F). The crystalloid-laden cells had the ultrastructural hallmarks of type A cells (Middlebrook et al., 2009), such as a polymorphic nucleus, thin cytoplasm, autophagocytic vacuoles, scant smooth endoplasmic reticulum, few mitochondria, and abnormal collagen deposits between cells (Fig. 1G). No crystalloids were observed in zG cells, endothelial cells, or other normal cell types adjacent to the neoplastic cells (Fig. 1E); nor did we detect β-gal$^+$ type B cells in our electron microscopic analysis. It is unclear whether the absence of type B cell labeling reflects a low efficiency of contribution of GLI1$^+$ progenitors to this cell population or inadequate sampling.

On the basis of fate mapping studies with *R26R*-lacZ reporter, we conclude that GDX-induced adrenocortical neoplastic cells, specifically type A cells, are derived from GLI1$^+$ progenitors.

3.2. Fate mapping in B6D2F2 Gli1-creER^{T2};R26R-confetti mice

To refine the lineage tracing analysis, we turned to a fluorescent reporter, *Rosa26* flox-stop-flox-confetti (*R26R*-confetti) (Snippert et al., 2010). The *R26R*-confetti allele comprises two reporting cassettes flanked by LoxP sites; each cassette contains a pair of fluorescent reporters of differing colors in opposite reading frames (Fig. 2A). Cre recombination of this allele randomly labels a cell and its descendants with one of 4 markers: nuclear-localized green fluorescent protein (nGFP), cytoplasmic yellow fluorescent protein (YFP), cytoplasmic red fluorescent protein (RFP), or membrane-tethered cyan fluorescent protein (mCFP). An advantage of this multicolor reporter is that it can be used to sparsely label progenitor cells, facilitating the tracking of individual clones via direct fluorescence.

The sensitivity of a given reporter allele to Cre-mediated recombination may vary among tissue types (Alcolea and Jones, 2013). To ensure that the *R26R*-confetti reporter could label adrenocortical and gonadal(-like) cells, we examined adrenal and gonadal tissue from *Sf1*-cre;*R26R*-confetti mice, which express Cre constitutively in steroidogenic cells. Clusters of cells expressing a single color marker, presumed to reflect clonal populations, were evident in adrenal X-zone cells (Fig. 2B,E), the outer adrenal cortex (Fig. 2C,F), and testicular interstitial cells (Fig. 2D,G). Reporter-expressing cells were more abundant in the X-zone than in the outer cortex, consistent with the observation that *Sf1* is highly expressed in the fetal adrenal, the precursor of the X-zone (Zubair et al., 2008). In agreement with a prior study demonstrating direct lineage conversion of zG to zF cells (Freedman et al., 2013), we observed clonal columns of zG + zF cells expressing a single color marker (Fig. 2C,F), presumably derived from a common stem/progenitor cell.

Of note, we did not detect expression of the mCFP reporter in the adrenal cortex of *Sf1*-cre;*R26R*-confetti mice. This lack of detectable mCFP signal appears to be an idiosyncrasy of the *R26R*-confetti reporter in adrenocortical tissue, because mCFP was seen in heart tissue from a *Dct*-cre;*R26R*-confetti mouse that had been fixed and processed in the same manner (Supplementary Fig. 1).

Next, we examined cryosections of adrenal glands from tamoxifen-treated, gonadectomized *Gli1*-creER^{T2};*R26R*-confetti mice. To distinguish neoplastic from normal adrenocortical tissue in these sections, we relied on a combination of darkfield microscopy, DAPI staining, and FITC-streptavidin labeling. Unlike normal

capsule cells, while the arrow indicates a neighboring YFP$^+$ capsule cell. **D**) GLI1$^+$ derivatives marked with RFP contribute to neoplastic subcapsular cells (large arrow) but not to normal steroidogenic cells (small arrow). The asterisk highlights a region where neoplastic cells are crowded together. **E)** A neoplasm containing 2 distinct clones [YFP$^+$ (arrow) and RFP$^+$ (arrowhead)]. **F-G)** Corresponding DAPI stains. Dashed lines denote the capsule surface. Bars: 25 μm.

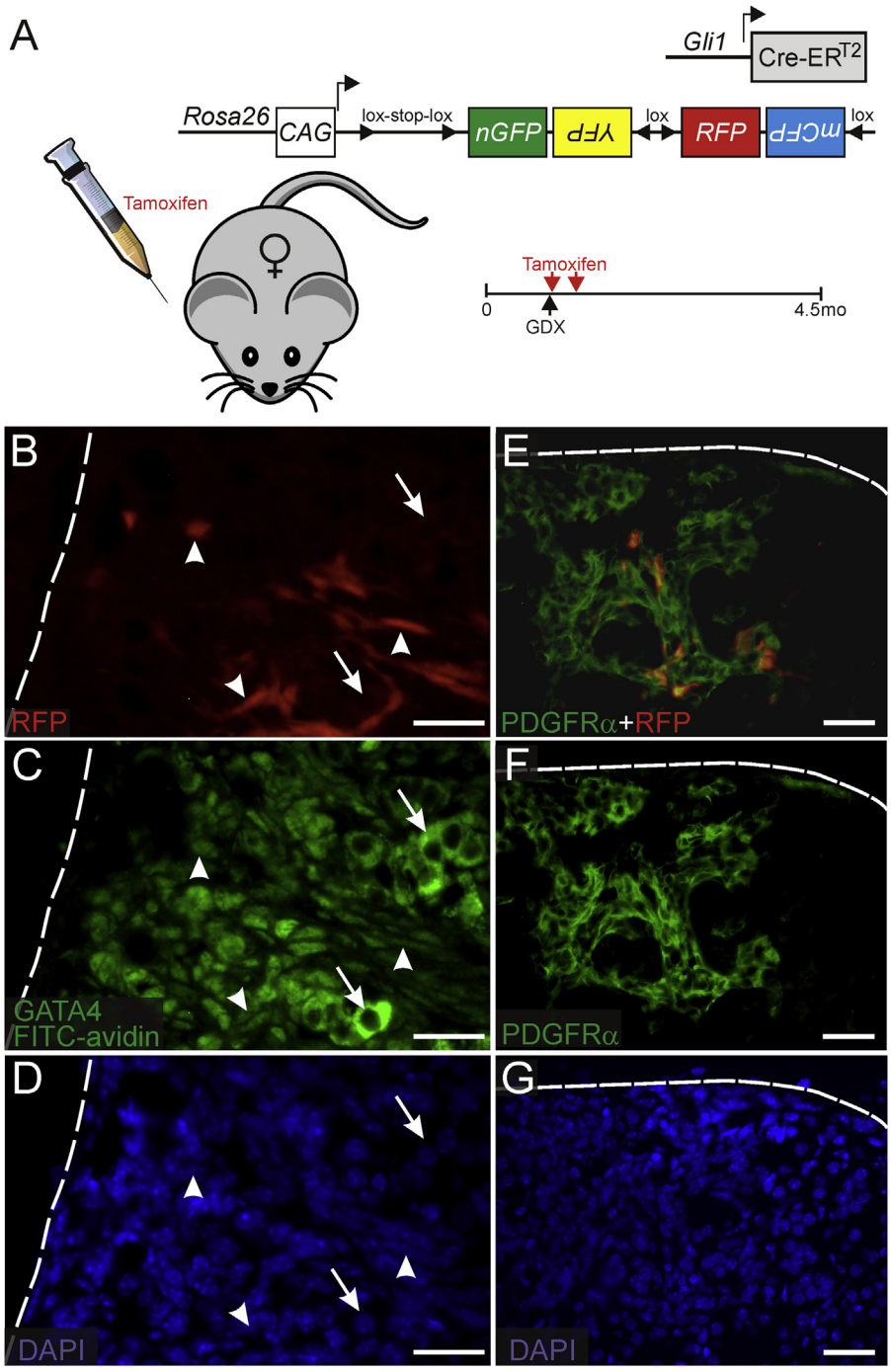

Fig. 4. GLI1^{+} progenitors contribute to GDX-induced adrenocortical neoplasms that express *Gata4* and *Pdgfra*. A) A B6D2F2 *Gli1*-CreER^{T2};*R26R*-confetti female mouse was gonadectomized and injected with tamoxifen at the indicated times. Adrenal glands were harvested, cryosectioned, and immunostained. **B-D)** GLI1^{+} derivatives marked with the RFP reporter contribute to ovoid- and spindle-shaped neoplastic cells exhibiting nuclear GATA4 immunoreactivity (arrowheads) but not to normal steroidogenic cells exhibiting cytoplasmic staining with FITC-streptavidin (arrows). **E-G)** GLI1^{+} derivatives marked with the RFP reporter contribute to cells that stain with PDGFRα antibody. Bars: 25 μm.

zG and zF cells, GDX-induced adrenocortical neoplasms contain scant lipid (Schillebeeckx et al., 2013) and therefore exhibit decreased birefringence when viewed under darkfield optics. When visualized by DAPI staining, the nuclei of normal steroidogenic cells are large, round, and well separated from one another, whereas the nuclei of neoplastic cells are small, oblong, and closely packed (*, Fig. 3D,H). Due to high endogenous biotin content, the cytoplasm of steroidogenic cells in the normal cortex stains directly with FITC-streptavidin (Paul and Laufer, 2011) (Fig. 3B,D,F,H). In contrast, non-steroidogenic (type A) cells in GDX-induced neoplasms do not stain directly with FITC-streptavidin (Fig. 3D,H).

In tamoxifen-treated, gonadectomized *Gli1*-creER^{T2};*R26R*-confetti mice, we observed clusters of capsule cells that expressed a single color reporter, presumed to be descendants of a common GLI1^{+} progenitor (Fig. 3B,C,F,G). These labeled cells persisted in the adrenal capsule for more than 6 mo after tamoxifen treatment, suggesting that they are long-lived. In the subcapsular region of adrenal glands from tamoxifen-treated, gonadectomized *Gli1*-

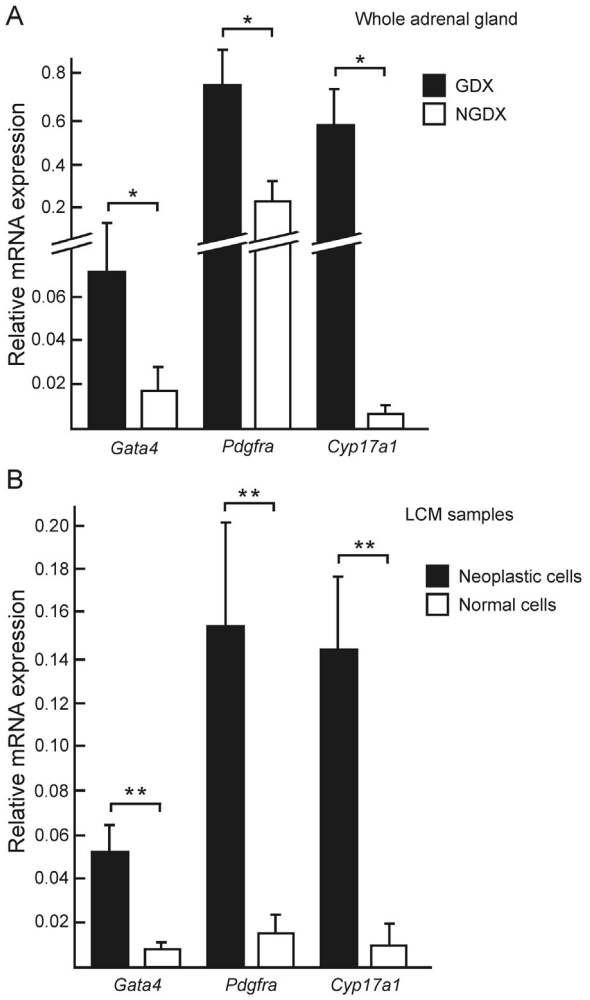

Fig. 5. Expression of gonadal-like differentiation markers in the adrenal glands of 4-mo-old gonadectomized *vs.* intact female DBA/2J mice. A) RNA was isolated from whole adrenal glands of gonadectomized (GDX) or non-gonadectomized (NGDX) mice and subjected to RT-qPCR (n = 3 per group). **B**) RNA was isolated from neoplastic tissue or normal cortical tissue (zG + zF) using laser capture microdissection (LCM) and subjected to RT-qPCR (n = 3 per group). Results were normalized to *Actb* expression. Comparable results were obtained when results were normalized to *Gapdh* expression. *$P < 0.05$, **, $P < 0.01$.

creER^{T2};*R26R*-confetti mice, we observed clones of marked neoplastic cells (Fig. 3D,E,H,I). These reporter-expressing neoplastic cells displaced zF and zG cells centripetally, distorting the cortical architecture. Neoplastic cells expressing the confetti reporter contained scant lipid, and only a small percentage of these cells were classified as steroidogenic on the basis of FITC-streptavidin staining [7 of 365 RFP$^+$ cells (2%) stained directly with FITC-streptavidin at 4 mo post-GDX, n = 6 adrenals]. Importantly, some wedge-shaped tumors in tamoxifen-treated, gonadectomized *Gli1*-creER^{T2};*R26R*-confetti mice contained separate clones of neoplastic cells derived from independent recombination events involving different reporter cassettes (e.g., YFP and RFP; Fig. 3E,I). This suggests that individual tumors can arise from patches of neoplasia-ready GLI1$^+$ progenitor cells in the capsule.

Immunofluorescence microscopy was used to characterize the marked cells in the adrenal glands of tamoxifen-treated, gonadectomized *Gli1*-creER^{T2};*R26R*-confetti mice. We focused our analysis on neoplasms expressing a single color reporter (RFP) and performed indirect immunofluorescence using FITC-conjugated secondary antibodies or biotin-labeled secondary antibody followed by FITC-streptavidin. We observed ovoid- or spindle-shaped neoplastic cells that expressed both RFP and nuclear GATA4, a marker of type A cells (Fig. 4B–D). In contrast to normal steroidogenic cells (arrows, Fig. 4B–D), the RFP$^+$/GATA4$^+$ neoplastic cells did not stain directly with FITC-streptavidin (arrowheads, Fig. 4B–D). Another marker known to be expressed in type A cells, FOXL2 (Schillebeeckx et al., 2015), was detected in RFP$^+$ neoplastic cells (Sup Fig. 2B–D). We also saw neoplastic cells that co-expressed RFP and the platelet derived growth factor receptor PDGFRα (Fig. 4E–G), a marker previously shown to be present in fibroblastic stromal cells of normal adrenal cortex (Wood et al., 2013).

To independently verify that *Pdgfra* was expressed in GDX-induced adrenocortical neoplasms, we performed RT-qPCR analysis on RNA from whole adrenal extracts from gonadectomized *vs.* intact mice (Fig. 5A) and on RNA from laser capture microdissectates of neoplastic *vs.* non-neoplastic adrenocortical tissue (Fig. 5B). For these RT-qPCR experiments, we used DBA/2J mice, a strain that is highly susceptible to GDX-induced adrenocortical neoplasia (Bielinska et al., 2003). Like *Gata4* and *Cyp17a1*, two well-established markers of GDX-induced adrenocortical neoplasia [reviewed in (Röhrig et al., 2015)], expression of *Pdgfra* mRNA was significantly increased in whole adrenal extracts and microdissected neoplastic tissue from gonadectomized mice.

In non-gonadectomized *Gli1*-creER^{T2};*R26R*-confetti mice, we observed long-lived confetti$^+$ cells in the capsule, but these labeled capsule cells did not contribute efficiently to steroidogenic cells in the subjacent cortex (data not shown). In 6-mo-old tamoxifen-treated, non-gonadectomized *Gli1*-creER^{T2};*R26R*-confetti mice, we saw patches of subcapsular cell hyperplasia expressing the confetti reporter (Fig. 6B,C), implying that these spindle-cell lesions, like GDX-induced adrenocortical neoplasms, are derived from GLI1$^+$ progenitors.

We conclude that: 1) the *R26R*-confetti reporter is useful for tracking cell fate in the adrenal cortex, 2) separate clones of GLI1$^+$ progenitor cells contribute individual GDX-induced neoplasms, 3) PDGFRα is a novel marker of GDX-induced adrenocortical neoplasia, and 4) long-lived GLI1$^+$ capsular progenitors give rise to patches of subcapsular cell hyperplasia in older, non-gonadectomized mice.

3.3. GANT61 treatment impairs the expression of gonadal-like markers in the adrenal glands of gonadectomized DBA/2J mice

Studies of mutant mice have shown that GLI1 is not required for hedgehog signaling, because GLI2 can rescue most GLI1 functions (Park et al., 2000; Bai et al., 2002). To garner evidence that GLI factors are crucial for GDX-induced adrenocortical neoplasia, we administered GANT61, an inhibitor of GLI1 and GLI2 (Lauth and Toftgard, 2007; Kramann et al., 2015), to gonadectomized mice (Fig. 7A). For these experiments we used the highly susceptible strain DBA/2J, because previous studies have shown that type A cells accumulate in the adrenal glands of these mice within 2 weeks of GDX (Bielinska et al., 2003). RT-qPCR analysis showed that GANT61 treatment reduced adrenal expression of *Gli1*, a downstream indicator of hedgehog signaling (Fig. 7B). GANT61 treatment also attenuated adrenal expression of 3 gonadal-like markers (*Gata4*, *Amhr2*, *Foxl2*) known to be expressed in type A cells (Röhrig et al., 2015). Immunohistochemical analysis confirmed reduced numbers of GATA4- and FOXL2-immunoreactive cells in the GANT61-treated specimens (Fig. 7C). These pharmacological inhibition studies suggest that GLI factors influence GDX-induced adrenocortical neoplasia.

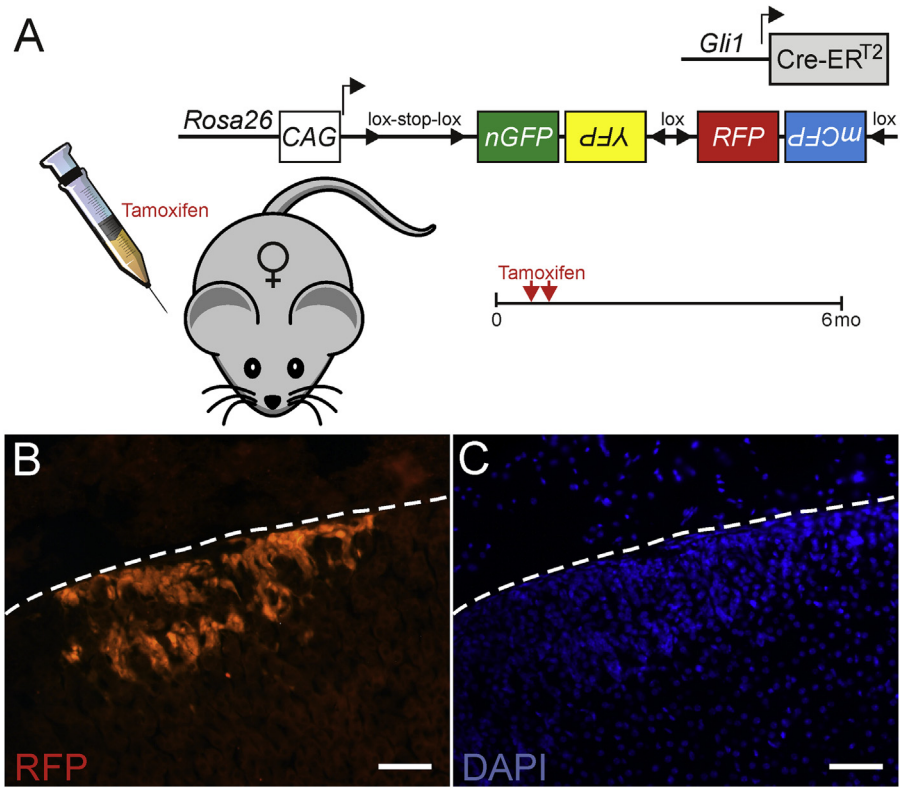

Fig. 6. GLI1^{+} progenitors contribute to subcapsular cell hyperplasia in an older, non-gonadectomized *Gli1*-CreER^{T2};*R26R*-confetti mouse. A) A female B6D2F2 *Gli1*-CreER^{T2};*R26R*-confetti mouse was injected with tamoxifen at the indicated times. Adrenal glands were harvested and cryosectioned. **B)** A clonal patch of subcapsular spindle-cell hyperplasia expressing RFP. **C)** Corresponding DAPI stain. The capsule surface is shown by the dashed lines. Bars: 100 μm.

4. Discussion

Lineage tracing is a powerful approach for understanding tissue development and homeostasis, particularly when it is combined with experimental manipulation of signals that regulate cell-fate decisions (Kretzschmar and Watt, 2012). Here, we have applied lineage tracing techniques to a classic model of altered cell fate: the GDX-induced accumulation of heterotopic tissue in the adrenal glands of mice (Röhrig et al., 2015). Our fate mapping studies with B6D2F2 mice support the premise that GLI1 is a key player in gonadal-like differentiation in the adrenal cortex. Long-lived GLI1^{+} capsular progenitor cells give rise to adrenocortical neoplasms in gonadectomized B6D2F2 mice and to patches of subcapsular cell hyperplasia in older, non-gonadectomized B6D2F2 mice. GANT61 treatment reduces the expression of gonadal-like markers (*Gata4*, *Amhr2*, *Foxl2*) in the adrenal glands of gonadectomized DBA/2J mice, supporting a role for GLI factors in GDX-induced adrenocortical neoplasia.

Our findings complement prior studies showing that distinct pools of stem/progenitor cells exist within the adrenal capsule and subjacent cortex [reviewed in (Pihlajoki et al., 2015; Walczak and Hammer, 2015)]. Progenitor cell populations characterized thus far include those expressing *Wt1*, *Gli1*, *Tcf21*, and *Shh* (Table 2). These progenitor populations may overlap to some degree. For example, WT1^{+} progenitors have been shown to co-express *Gli1* and *Tcf21*. Some of these progenitors give rise to differentiated cells only during specific developmental windows or in response to experimental manipulation. Prior lineage tracing studies with the *Gli1*-creER^{T2} driver have shown that GLI1^{+} progenitors in the adrenal capsule contribute to steroidogenic cells, particularly when tamoxifen induction is performed during fetal development; when tamoxifen is administered postnatally, the labeling of cortical steroidogenic cells appears less robust (King et al., 2009; Huang et al., 2010; Wood et al., 2013). Under the experimental conditions we used (2 doses of tamoxifen administered to weanling B6D2F2 mice) there was negligible contribution of GLI1^{+} cells to steroidogenic cells in the cortex. We surmise that mouse age, the timing of tamoxifen treatment, and other facets of experimental design impact lineage tracing results with the *Gli1*-creERT2 driver. Our findings, coupled with those of other investigators, suggest that the fate of GLI1^{+} cells in the adrenal capsule may differ in the fetus and adult.

In the course of our fate mapping experiments we identified *Pdgfra* as a marker of GDX-induced adrenocortical neoplasia. A previous transcriptome-wide search (Schillebeeckx et al., 2015) designed to detect novel markers of GDX-induced adrenocortical neoplasia overlooked *Pdgfra*, because this gene was not represented on the microarray used in the analysis. Platelet derived growth factor (PDGF) signaling is known to control the differentiation of gonadal steroidogenic cells in both sexes (Schmahl et al., 2008), and PDGFRα is a marker of putative Leydig stem cells in rodents and humans (Landreh et al., 2013, 2014; Odeh et al., 2014). PDGFRα has been shown to be a marker of fibroblastic stromal cells in the non-neoplastic adrenal cortex (Wood et al., 2013). GLI1 has been shown to enhance expression of *Pdgfra* in C3H10T½ mouse mesenchymal cells (Xie et al., 2001).

To our knowledge, this is the first article describing the use of the *R26R*-confetti reporter in adrenocortical tissue. This reporter offers certain advantages compared to other widely used reporters. In particular, sparse multicolor labeling by the confetti reporter permits tracing of individual clones or subclones of cells. Our studies establish that GDX-induced adrenocortical neoplasms

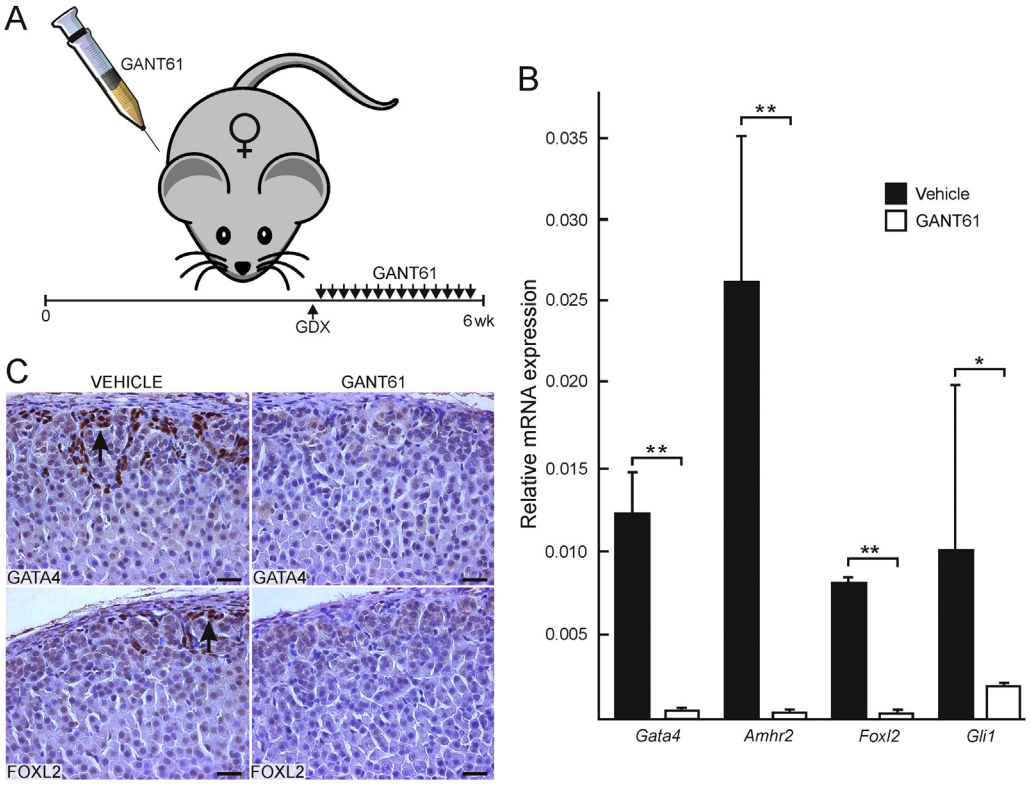

Fig. 7. Expression of gonadal-like differentiation markers in the adrenal glands of gonadectomized female DBA/2J mice treated with GANT61 or vehicle. A) Weanling female mice were gonadectomized and then injected daily with GANT61 (50 mg/kg) or vehicle control. **B)** After 14 days, RNA was isolated from whole adrenal glands and subjected to RT-qPCR (n = 4 per group). Results were normalized to *Actb* expression. Comparable results were obtained when results were normalized to *Gapdh* expression. $^{*}P < 0.05$, $^{**}P < 0.01$. **C)** Adrenal glands from vehicle- or GANT61-treated mice were subjected to immunoperoxidase staining for GATA4 or FOXL2. Nuclear immunoreactivity is evident in type A cells in the subcapsular region of the vehicle-treated adrenals (arrows). Bars: 50 μm.

Table 2
Stem/progenitor cell populations in the periphery of the adrenal gland as defined by fate mapping studies.

Stem/Progenitor population	Location	Comments	References
WT1^{+} progenitors	Capsule	Under basal conditions WT1^{+} capsule cells give rise to adrenocortical cells, but GDX triggers their differentiation into gonadal-like tissue.	(Bandiera et al., 2013)
GLI1^{+} progenitors	Capsule	These cells are descendants of *Sf1*-expressing fetal adrenocortical cells. GLI1^{+} progenitors differentiate into steroidogenic cells, particularly during fetal development. GDX triggers their differentiation into gonadal-like tissue.	(King et al., 2009; Huang et al., 2010; Wood et al., 2013) and this study
TCF21^{+} progenitors	Capsule	TCF21^{+} cells, which are descendants of the AGP rather than SF1^{+} fetal adrenocortical cells, give rise to non-steroidogenic stromal cells in the adrenal cortex.	(Wood et al., 2013)
SHH^{+} Progenitors	Subcapsular region	These cells give rise to steroidogenic cells in the zF and zG but not capsule cells.	(King et al., 2009; Huang et al., 2010)

are polyclonal in nature. Individual wedge-shaped tumors in *Gli1*-creER^{T2};*R26R*-confetti mice are composed of multiple marked clones, each derived from a separate GLI1^{+} capsular progenitor. This suggests that GDX-induced adrenocortical tumors arise from patches of "neoplasia-ready" progenitors in the capsule. Such neoplasia-ready cells may be defined in part by epigenetic modifications that impact the phenotypic plasticity of adrenocortical stem/progenitor cells, allowing some to respond to the hormonal changes associated with GDX (Bielinska et al., 2009; Schillebeeckx et al., 2013, 2015). Epigenetic variability among stem/progenitor cells may explain why GDX of susceptible mouse strains leads to discrete wedges of proliferating neoplastic cells in the adrenal cortex rather than uniform tumor formation along the periphery of the cortex. Interestingly, the level of *Wt1* expression has been shown to vary stochastically among AGP-like stem/progenitor cells in the adrenal capsule, and this variability in *Wt1* expression correlates with differentiation potential (Bandiera et al., 2013). WT1^{low} cells differentiate into cells that express *Sf1*, whereas WT1^{high} express *Gata4*, *Gli1*, and *Tcf21*, have low levels of SF1, and fail to differentiate into normal adrenocortical cells. Since WT1 can directly enhance *Gli1* expression in capsular progenitors (Bandiera et al., 2013) and GLI1^{+} capsular progenitors can give rise to other capsule cells (this study), one can envision a scenario wherein high *Wt1* expression in one stem/progenitor, owing to epigenetic effects, leads to formation of a patch of neoplasia-ready progenitors.

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Disclosure summary

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2016.08.043.

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