

# Hormonal and spatial control of SUMOylation in the human and mouse adrenal cortex

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**ABSTRACT:** SUMOylation is a highly conserved and dynamic post-translational mechanism primarily affecting nuclear programs for adapting organisms to stressful challenges. Alteration of SUMOylation cycles leads to severe developmental and homeostatic defects and malignancy, but signals coordinating SUMOylation are still unidentified. The adrenal cortex is a zonated endocrine gland that controls body homeostasis and stress response. Here, we show that in human and in mouse adrenals, SUMOylation follows a decreasing centripetal gradient that mirrors cortical differentiation flow and delimits highly and weakly SUMOylated steroidogenic compartments, overlapping glomerulosa, and fasciculata zones. Activation of PKA signaling by acute hormonal treatment, mouse genetic engineering, or in Carney complex results in repression of small ubiquitin-like modifier (SUMO) conjugation in the inner cortex by coordinating expression of SUMO pathway inducers and repressors. Conversely, genetic activation of canonical wingless-related integration site signaling maintains high SUMOylation potential in the outer neoplastic cortex. Thus, SUMOylation is tightly regulated by signaling pathways that orchestrate adrenal zonation and diseases.—Dumontet, T., Sahut-Barnola, I., Dufour, D., Lefrançois-Martinez, A.-M., Berthon, A., Montanier, N., Ragazzon, B., Djari, C., Pointud, J.-C., Roucher-Boulez, F., Batisse-Lignier, M., Tauveron, I., Bertherat, J., Val, P., Martinez, A. Hormonal and spatial control of SUMOylation in the human and mouse adrenal cortex. *FASEB J.* 33, 000–000 (2019). www.fasebj.org

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The adrenal cortex is divided into structurally and functionally distinct concentric zones (1). Under the capsule, the *zona glomerulosa* (zG) ensures production of aldosterone under the control of angiotensin II and potassium, mainly

through calcium signaling (2). The *zona fasciculata* (zF), located just below, forms a wide layer of cells responsible for the secretion of glucocorticoids in response to ACTH, acting through cAMP/PKA signaling pathway (3). The *zona reticularis* (zR), located in the inner part of the cortex close to the medulla, supports the secretion of adrenal androgen precursors in response to ACTH (4). In rodents, the adult cortex only consists of zG and zF (5). However, in mice, a third and transient zone termed the X-zone is located in the innermost part of the cortex and is considered to be a remnant of the fetal cortex (6, 7). By its capacity to rapidly release hormones acting both on blood pressure control and glucido-lipidic metabolism, the adrenal gland is considered to be the endocrine gland for stress response.

The zonal organization and cellular renewal of the adrenal cortex are maintained through the recruitment of progenitor cells from the periphery of the gland that migrate centripetally and differentiate successively into zG and zF cells (8, 9). Hormonal signals that control adrenal function also participate in the zonation of the

**ABBREVIATIONS:** AKR1B7, aldo-keto reductase 1B7; ATC1, adrenocortical tumor cell line 1; CNC, Carney complex; Cre, cyclization recombinase; CREB, cAMP responsive element binding protein; DESH, deSUMOylating isopeptidase 1; HPA, hypothalamic-pituitary-adrenal; PIAS, protein inhibitor of activated signal transducer; PPNAD, primary pigmented nodular adrenocortical disease; PRKARIA, R1α/ PKA-dependent type 1 regulatory subunit α; qRT-PCR, quantitative RT-PCR; SAE, SUMO-activating enzyme; SENP, SUMO/sentrin specific peptidase; SF-1, steroidogenic factor-1; SIK, salt-inducible kinase; SUMO, small ubiquitin-like modifier; UBE2L, ubiquitin-conjugating enzyme E2 L; WNT, wingless-related integration site; WT, wild type; zF, *zona fasciculata*; zG, *zona glomerulosa*; zR, *zona reticularis*

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cortex. The R-spondin/wingless-related integration site (WNT)/ $\beta$ -catenin signaling pathway contributes to the acquisition of zG identity (10, 11), whereas ACTH/cAMP/PKA signaling is essential for zF and zR differentiation (12, 13). Maintaining adequate zonation during the unidirectional renewal of the cortex is ensured at least by the antagonistic interactions between these 2 signaling pathways and through epigenetic programming of newly recruited cells (14–16).

PKA is a tetramer composed of 2 catalytic and 2 cAMP-binding regulatory subunits. When intracellular levels of cAMP increase, following activation of the G-protein-coupled ACTH receptor, the regulatory subunits release the catalytic subunits, which become active and phosphorylate downstream effectors (17). These targets include transcription factors stimulating the expression of steroidogenic genes. Inactivating mutations of the PKA-dependent type 1 regulatory subunit  $\alpha$  gene (*PRKARIA*) encoding the regulatory subunit R1 $\alpha$  leads to constitutive activation of PKA and development of Carney complex (CNC), a multiple endocrine neoplasia syndrome (18, 19). Primary pigmented nodular adrenocortical disease (PPNAD), the most frequent endocrine tumor in these patients, is a benign adrenal hyperplasia responsible for ACTH-independent hypercortisolism (Cushing syndrome) (20). We have shown that the adrenal inactivation of *Prkar1a* in developing mice is sufficient to induce ACTH-independent Cushing syndrome and formation of apoptosis-resistant hyperplasia in the inner zF (21, 22). Moreover, by tracing the lineage of *Prkar1a* mutant cells in the adult gland, we have established that PKA signaling is a driving force for sexually dimorphic cortex replenishment and for the conversion of zF into zR identity (13).

SUMOylation has emerged as a critical mechanism for modulating fundamental cellular and developmental processes whose dysregulation can lead to severe diseases. SUMOylation regulates the activity of various proteins involved in DNA replication and repair, chromosome dynamics, genome integrity, nuclear shuttling, cell signaling, and proliferation. Its involvement in tissue morphogenesis (23) has been demonstrated in enterocytes (24), keratinocytes (25), and adipocytes (26, 27). However, it also participates in human malignancy by favoring cancer cell stemness (28, 29). Post-translational modification of target proteins by the covalent addition of small ubiquitin-like modifier (SUMO) 1–3 peptides to specific lysine residues involves an enzymatic cascade requiring the sequential action of the E1 heterodimer SUMO-activating enzyme (SAE) 1/2 for the activation step, the unique E2 enzyme ubiquitin-conjugating enzyme E2 I (UBE2I or UBC9) for the conjugation step, and various E3 ligation enzymes including protein inhibitor of activated signal transducer and activator of transcription-1 (PIAS) 1–4, Ran GTPase binding protein 2, structural maintenance of chromosome 5–6 complex SUMO ligase, and chromobox 4 proteins that increases efficiency of the conjugation step (30). Preserving the balance between SUMOylated and unSUMOylated proteins is a critical point for maintaining cellular physiology. Therefore, the dynamics and reversibility of the reaction are ensured by SUMO/sentrin specific peptidase (SENPs) 1–7 and deSUMOylating

isopeptidase 1 (DESI1) proteases that cleave and recycle SUMO peptides from their substrates (31, 32).

One of the major roles of SUMOylation cycles is the control of transcription processes through the individual modification of *trans*-acting factors and chromatin remodeling (33–35). The properties of the transcription factors Wilms tumor protein 1, nuclear receptor related-1 protein (also known as NR4A2), and steroidogenic factor-1 (SF-1, also known as NR5A1) that are essential for development and function of adrenal cortex are also regulated by SUMOylation (36–40). However, physiologic evidence of the impact of SUMOylation on adrenal cortex function remains scarce but was elegantly brought into light by a knock-in experiment from Lee *et al.* (41). Indeed, the complete lack of SUMO-conjugated SF-1 in these mice was shown to affect adrenal cortex homeostasis, disturbing cell identities and resulting in mild endocrine hypofunction. This highlighted the importance of SF-1 SUMOylation in fine-tuning the genetic programs of endocrine differentiation, but many other potential SUMO targets should influence adrenal function. As a first step in understanding this vast question, it requires a better knowledge of the overall SUMOylation patterns in the adrenal gland and the exploration of their sensitivity to hormonal regulation. Interestingly, increased cAMP levels were shown to influence the expression of members of the SUMO pathway in ovarian granulosa cells (42).

Here, we hypothesized that SUMOylation, cortex architecture, and hormonal regulation of adrenocortical function should be coordinated. To test this hypothesis, we analyzed the influence of ACTH/PKA signaling on the SUMOylation process in cell culture, wild-type (WT) mice, genetic mouse models of adrenal diseases, and in human PPNAD tissues. This enabled us to propose that cell signalings critical for adrenal cortex homeostasis and stress response tightly coordinate the SUMOylation pathway, which in turn is found altered in cortical diseases.

## MATERIALS AND METHODS

### Adrenocortical cell cultures

Adrenocortical tumor cell line 1 (ATC1) cells were established from an adrenal tumor derived from a mouse expressing the Simian Virus 40 large T (SV40 T) antigen under the control of the aldo-keto reductase 1b7 (*Akr1b7*) gene promoter specific to the adrenal cortex (43, 44). The primary cultures were obtained from adrenals of 3-wk-old female mice following the protocol previously described (45). The cells were cultured on poly-D-lysine-coated dishes (MilliporeSigma, Burlington, MA, USA) in a DMEM-F12 medium (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in the presence of 5% CO<sub>2</sub>, insulin (10  $\mu$ g/ml), transferrin (5.5  $\mu$ g/ml), selenium (6.7 ng/ml) (Thermo Fisher Scientific), L-glutamine (2 mM), penicillin 0.1 U/ml, streptomycin (0.1  $\mu$ g/ml), 2.5% horse serum, and 2.5% fetal calf serum (43). For PKA induction experiments, cells were seeded in 6-well plates at a density of 2.10<sup>5</sup> cells per well, cultured to subconfluence, and then weaned 12 h (serum-free medium) prior to the addition of forskolin, ACTH, cycloheximide, and actinomycin at the times and concentrations indicated in the legends of the figures.

## Animals and treatments

Two-month-old CD-1 mice received an intraperitoneal injection of immediate ACTH (0.05 mg/30 g, Synacthene 0.25 mg/ml; Novartis, Basel, Switzerland) 2 or 4 h before adrenal sampling. Others received an i.m. injection of long-acting ACTH (12 µg/30 g, Synacthene Retard 1 mg/ml; Novartis), and the adrenals were dissected 6 or 24 h later. For the 24 h point, the mice received a second injection 12 h before sampling. Immediate ACTH, with a short half-life, allows the observation of the early effects of stimulation. The delayed ACTH is stabilized because, complexed with zinc salts, the diffusion is slowed, which allows the observation of long-term effects. Alternatively, 6-mo-old females were injected s.c. with dexamethasone 21-acetate for 5 d in order to deplete endogenous ACTH production (75 µg twice daily in corn oil) and were injected with immediate ACTH for 2 or 6 h. The female *Sf-1-Cre::Prkar1a<sup>fl/fl</sup>* and  $\Delta$ Cat [*Akr1b7-Cre::Catnb<sup>lox(ex3)</sup>*] mice used (where Cre is cyclization recombinase, fl or lox is loxP site, ex3 is exon3 of *Ctnnb1* gene encoding beta-catenin, *Catnblox(ex3)* is the exon3 floxed allele of *Ctnnb1* gene and *deltaCat* is the name of the transgenic mouse line expressing exon3-deleted form of *Ctnnb1* gene) were 2.5- and 18-mo-old, respectively (11, 14). The mice were euthanized by decapitation, and the adrenals were immediately frozen in liquid nitrogen.

## Protein extraction and Western blot

Cell and tissue samples were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented extemporaneously with phosphatase inhibitors (1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.5 mM NaF), protease inhibitors (Roche, Basel, Switzerland), and SUMO inhibitor proteases N-ethylmaleimide (MilliporeSigma) (3.13 mg/ml). The cell debris was removed by centrifugation at 13,000 rpm for 15 min at 4°C. The protein concentration was determined by the Bradford method (Bio-Rad, Hercules, CA, USA), and 50 µg of proteins was denatured for 5 min at 95°C in Laemmli (50 mM Tris, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 12% glycerol). The proteins were then separated on a 7% SDS-PAGE or precast gels 4–15% (Bio-Rad) (Supplemental Fig. 3D) and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Little Chalfont, United Kingdom) overnight at 4°C at 50 V followed by 1 h at 120 V. Nonspecific protein binding sites were saturated for 1 h at room temperature by incubation in Tris-buffered saline and Tween 20 [50 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.1% Tween 20] containing 5% Regilait. Incubation with the appropriate primary antibody (Supplemental Table 1A) was carried out in the same buffer overnight at 4°C with gentle stirring. After rinsing in Tris-buffered saline and Tween 20, the membranes were incubated for 1 h at room temperature with the appropriate secondary antibody conjugated with peroxidase. The specific complexes were revealed by chemiluminescence (ECL) system (Amersham Biosciences). The fluorescence signals were quantified using the MultiGauge v.3.2 software (Fujifilm, Tokyo, Japan).

## Quantitative RT-PCR analyses

The cell samples were lysed in Trizol (Thermo Fisher Scientific), and the total RNAs were extracted according to the manufacturer's recommendations. Tissue samples were ground in RNA II lysis buffer (Thermo Fisher Scientific), and total RNAs were extracted using the NucleoSpin RNA II kit as recommended by the manufacturer. Five hundred nanograms of mRNA were reverse-transcribed for 1 h at 37°C with 5 pmol (reverse transcriptase), 2.5 mM deoxynucleoside triphosphate, and 20 U of RNase inhibitors (recombinant RNasin, N2615; Promega,

Madison, WI, USA) in a final volume of 25 µl. The cDNAs obtained were used (at one-fourth dilution) as template for quantitative RT-PCR (qRT-PCR) analysis using the qPCR Sybr (TAKRR820W; Takara Bio, Kyoto, Japan) premix under standard conditions (40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s). The relative accumulation of mRNAs was determined from the average of duplicates by the  $\Delta\Delta C_t$  method (46) using the actin household gene. The primers are listed in Supplemental Table 1B.

## Immunohistochemistry

After collection, the adrenals were fixed in 4% paraformaldehyde for 24 h, dehydrated in an increasing gradient of ethanol, and then placed in HistoClear (HS200; National Diagnostics, Atlanta, GA, USA) for 2 h before being included in paraffin. Five-micrometer-thick sections were made using a microtome (HM 340E; Thermo Fisher Scientific). The tissue sections were then dewaxed by passing through the HistoClear and progressively rehydrated in an increasing gradient of ethanol. Immunohistochemical analyses were performed according to the conditions described in Supplemental Table 1C. The tissues were photographed using an Axioplan2 imaging microscope (Carl Zeiss, Oberkochen, Germany). In order to evaluate the presence of non-SUMOylated nuclei, a counterstaining with hematoxylin was performed on the same sections previously used for SUMO1 immunohistochemistry. Quantification of SUMO signals were performed using Zen Software (Carl Zeiss).

## Patients

Adrenal tissue analysis and genetic diagnosis was obtained from patients after signed consent. The study was approved by an institutional review committee (Advisory Committee for the Protection of Persons in Biomedical Research, Cochin Hospital, Paris, France). PPNAD paraffin sections were performed from adrenal samples of patients with PPNAD who underwent bilateral adrenalectomy for ACTH-independent Cushing syndrome. Most patients were carriers of *PRKARIA* inactivating germinal mutations.

## Statistical analysis

Statistical analyses were performed using a Student's *t* test or Mann-Whitney test depending on Gaussian values distribution to compare 2 groups. For multiple comparisons, 1-way ANOVA (Gaussian values distribution) test or Kruskal-Wallis (non-Gaussian values distribution) test followed by Dunnett's, Dunn's, or Tukey's *post hoc* tests were performed. A value of  $P \leq 0.05$  was considered statistically significant (\* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ ).

## Study approval

All animal work was conducted according to French and European directives for the use and care of animals for research purposes and was approved by the local ethics committee, C2E2A (Comité d'Ethique pour l'Expérimentation Animale en Auvergne).

## RESULTS

### SUMO conjugation is repressed by ACTH/PKA signaling in adrenocortical cells *in vitro*

To evaluate the role of PKA signaling on adrenocortical protein SUMOylation, we analyzed the effects of forskolin,

an adenylate cyclase activator, on the formation of SUMO conjugates in lysates of ATC1 mouse adrenocortical cells (43). Immunoblotting with either anti-SUMO1 (Fig. 1A) or anti-SUMO2/3 (Fig. 1B) showed that SUMOylated proteins in cell extracts were represented by a smear of bands of high molecular mass whose intensity decreased with forskolin, reaching a nadir after 6–12 h of treatment. Quantification of the Western blots showed that steady-state levels of Sumo1- and Sumo2/3-conjugated proteins were reduced by 54 and 40%, respectively, after 6 h of forskolin treatment.

To explore the mechanism leading to PKA-mediated inhibition of protein SUMOylation, the hormonal responsiveness of genes encoding SUMO modules and enzymes of the SUMO conjugation-deconjugation pathway (Fig. 1C) was assessed by qRT-PCR in an ATC1 cell line and primary adrenocortical cells (Fig. 1D). In both cell types, strong induction of *Akr1b7* mRNA levels, a well-characterized ACTH/PKA-responsive gene (43), validated treatments at all time points, including the expected more robust effect of forskolin compared with ACTH, the physiologic inducer (Supplemental Fig. S1A). In both cell culture systems, PKA stimulation down-regulated mRNA accumulation of *Sumo1*, *Sae1*, *Pias3*, and to a lesser extent, *Ube2i*, with a maximal effect occurring between 6 and 12 h (Fig. 1D). Conversely, these treatments induced a 200% increase in *Senp2* mRNA expression that culminated between 2 and 6 h. This positive PKA responsiveness was also observed at protein levels (Fig. 1E), suggesting that *Senp2* gene regulation by ACTH/PKA was mainly transcriptional, as confirmed by the sensitivity to actinomycin D and resistance to cycloheximide (Supplemental Fig. S1B). Mechanisms of PKA-dependent repression of genes involved in SUMO conjugation (*Sumo1*, *Ube2i*, *Sae1*, and *Pias3*) were less clear but presumably required *de novo* expression of a labile repressor as suggested by *Pias3* mRNA sensitivity to both actinomycin D and cycloheximide (Supplemental Fig. S1B). Interestingly, although *Ube2i* transcript levels were still elevated 6 h postinduction in ATC1 cells, protein levels declined between 2 and 6 h (Fig. 1E). Therefore, this further suggests that PKA-dependent repression might also be achieved by post-translational mechanisms. The responsiveness of the other effectors of SUMOylation appeared either unaffected by PKA stimulation or not reproduced under ACTH treatment (Supplemental Fig. S1C). Taken together, these results indicate that the PKA-induced overall decrease of SUMO-conjugated protein substrates in adrenocortical cells could be the consequence of 2 coordinated and convergent mechanisms: increased gene transcription of deSUMOylating enzyme (here, *Senp2*) and repression of genes encoding SUMOylating proteins including SUMO peptides.

### Acute and chronic effects of ACTH/PKA repress SUMOylation in mouse adrenal glands

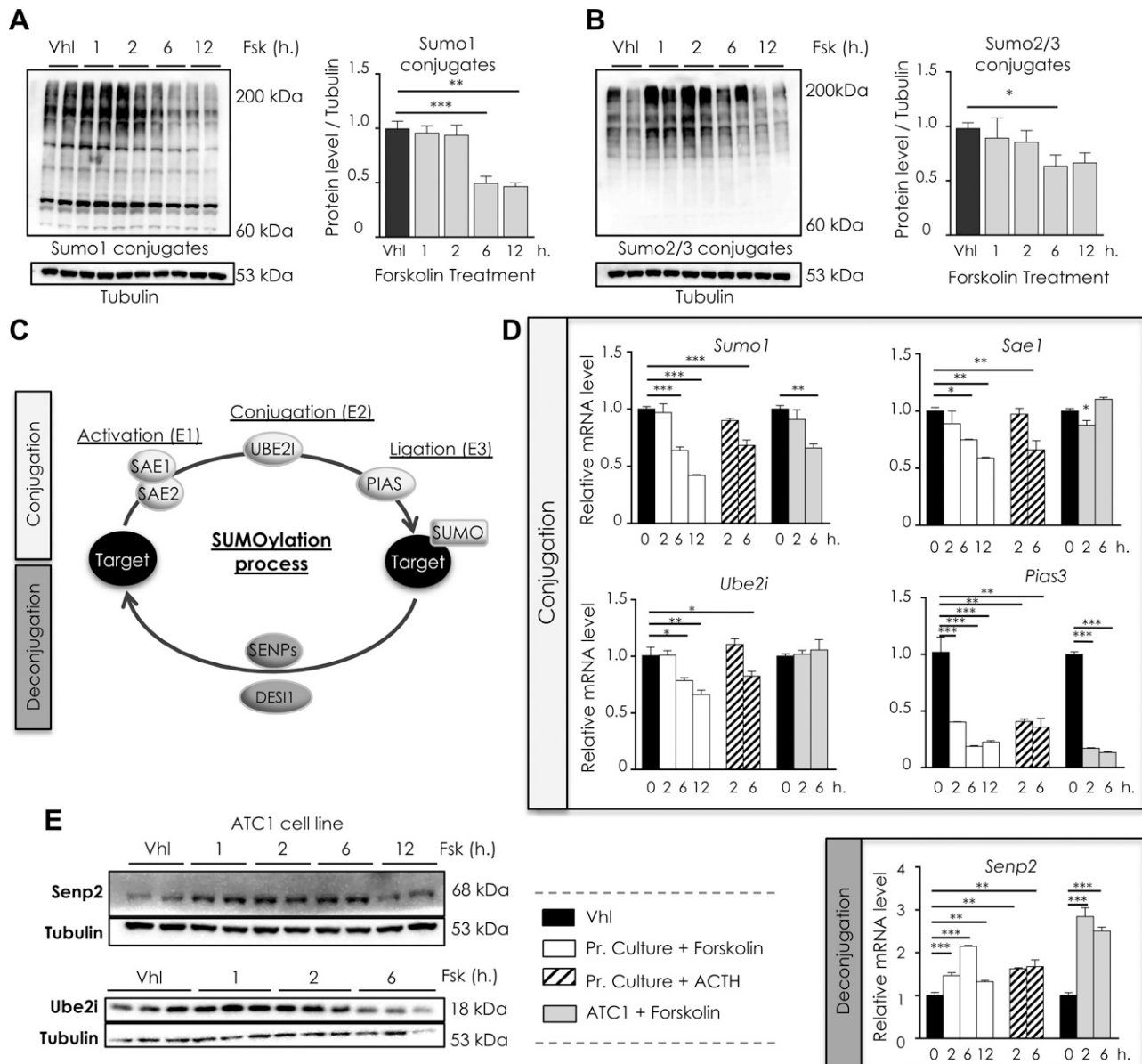
To evaluate *in vivo* the hormonal responsiveness of genes of the SUMO conjugation or -deconjugation pathways, WT young adult mice were injected with rapid- or long-acting

forms of ACTH and sacrificed after 2–4 or 6–24 h, respectively. qRT-PCR analyses in the adrenal cortex showed that activation of PKA signaling repressed mRNA levels of *Sumo1*, *Sumo2*, and *Sumo3* by 50, 25, and 30% after 4 h of treatment, respectively (Fig. 2A, B). Extended ACTH treatments showed that this decline was transient and could be maintained over 6 h. However, expression reincreased at 24 h (Supplemental Fig. S2A). A similar repression pattern was observed for *Pias3* mRNA levels that reached a nadir after 2–4 h of treatment (75% drop) and reincreased at 24 h (Fig. 2B and Supplemental Fig. S2A). In contrast, transcript levels for the SUMO protease *Senp2* were increased by 200% after 2 h, returning to pre-treatment values after 4 h (Fig. 2B). Remarkably, mRNA levels for SUMO protease *Desi1* remained stable for the first 6 h of treatment and were increased more than 150% after 24 h (Fig. 2B and Supplemental Fig. S2A). Regardless of the treatment duration, the expression of *Ube2i* transcripts was unaltered (Fig. 2B and Supplemental Fig. S2A). We next investigated the effect of chronic activation of PKA signaling on genes ensuring SUMO conjugation/deconjugation dynamics. For this, we used *Sfl1-Cre::Prkar1a<sup>fl/fl</sup>* mice with adrenal-specific deletion of PKA regulatory R1 $\alpha$  subunit (14). qRT-PCR analysis revealed a 38% reduction of *Pias3* expression compared with WT levels, whereas *Senp2* and *Desi1* expression was induced by 150 and 200%, respectively. *Senp3* and *Senp5* mRNA levels also showed a modest but significant increase (Fig. 2C). Interestingly, under these chronic stimulation conditions, there was no down-regulation of *Sumo* genes, and expression levels of all other conjugating/deconjugating genes were unaltered or only slightly increased (Supplemental Fig. S2B). In agreement with the repression of *Pias3* and simultaneous stimulation of genes for deSUMOylating isopeptidases, Western blot analysis of adrenal lysates showed that Sumo2/3 conjugation levels in mutant mice were ~30% of WT (Fig. 2D).

Taken together, these data suggest that ACTH stimulation *in vivo* limits the SUMOylation process in the adrenal gland through both acute ( $\leq 4$  h) and chronic ( $\geq 24$  h) transcriptional effects. These lead to the simultaneous and opposite regulation of a subset of PKA-responsive genes of the SUMOylation pathway, with repression of actors of the conjugation process (*Sumo1–3* and *Pias3*) and stimulation of deconjugation enzymes (*Senp2*, *Senp3*, *Senp5*, and *Desi1*).

### Regionalization of SUMOylation in the adrenal cortex is dependent on ACTH/PKA signaling

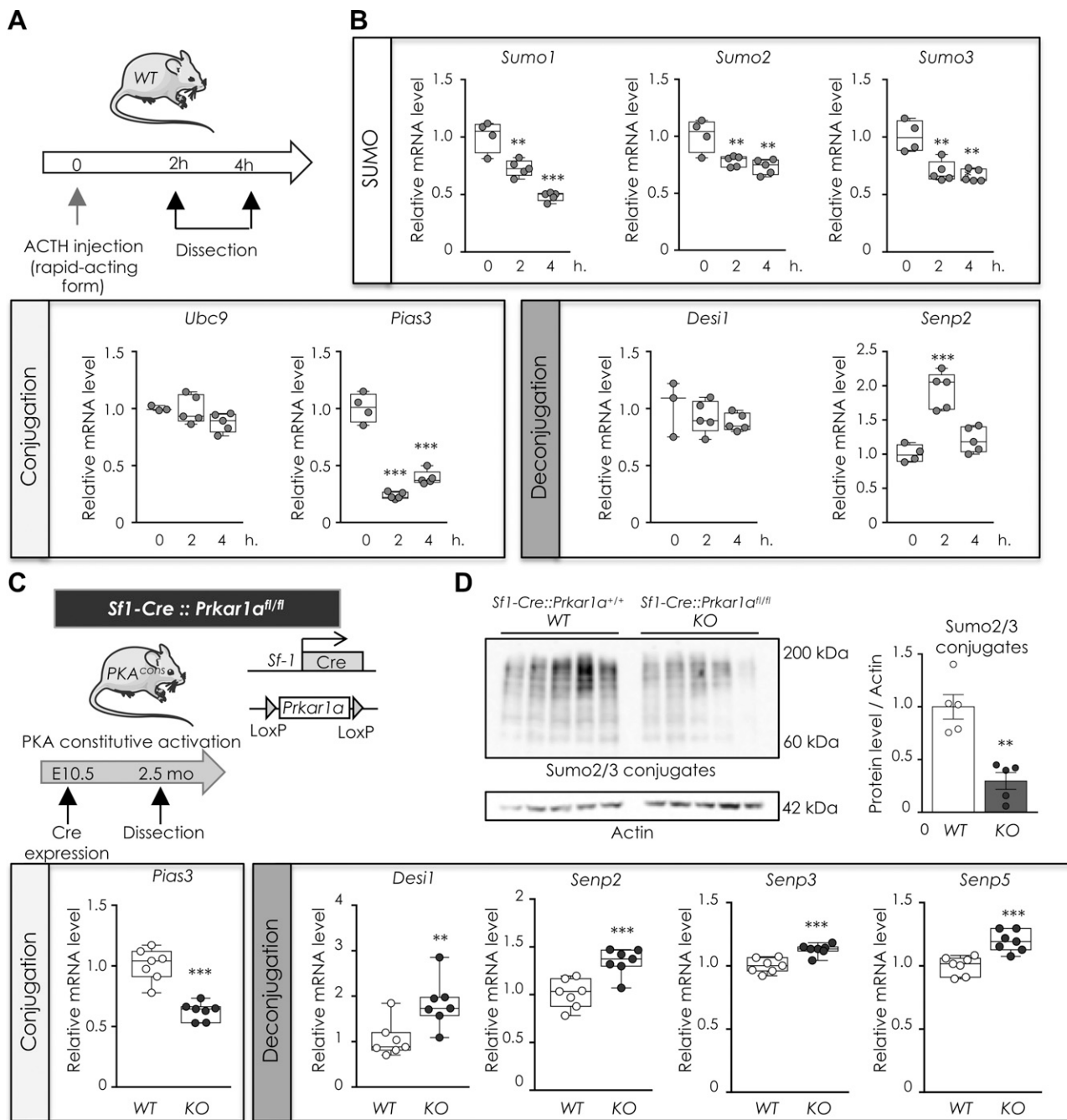
In the adrenal cortex, ACTH/PKA signaling is required for differentiation, maintenance, and function of the zF (12, 13). Active SUMOylation processes can be assessed in tissue sections by monitoring changes in the intensity of nuclear SUMO signal (47, 48). Immunohistochemical staining of mouse adrenal samples revealed a cortical gradient of Sumo1 and Sumo2/3 staining in steroidogenic cells, with high nuclear signal in the peripheral subcapsular/glomerulosa region, intermediate signal at the glomerulosa-fasciculata junction, and low labeling in



**Figure 1.** Activation of PKA signaling decreases global SUMOylation in adrenocortical cell cultures. *A, B*) Time course effects of PKA activation on the accumulation of adrenocortical proteins conjugated to Sumo1 (*A*) and Sumo2/3 (*B*). Murine adrenocortical ATC1 cell line was treated with forskolin ( $10^{-5}$  M) for the indicated time (h) or with vehicle (Veh) (DMSO) for the longest treatment time. The accumulation of Sumo1 and Sumo2/3 conjugates was analyzed by Western blot (representative figure) and quantified (histograms). Tubulin signal was used to normalize the quantification of SUMO signals. *C*) Schematic representation of the SUMOylation cycle. Sumo1, Sumo2, and Sumo3 peptides are conjugated to their substrate proteins by an enzymatic process involving E1, E2, and E3 enzymes. The dynamism of the SUMOylation is ensured by the proteases Senp and Des1, which allow the removal of the SUMO peptide from the targeted protein. *D*) Time course effects of PKA activation on the mRNA expression of SUMOylation pathway genes. Primary cultured cells from mouse adrenal cortex and ATC1 cell line were treated with forskolin ( $10^{-5}$  M) for the indicated time or with DMSO (Vhl) for the longest treatment time. Primary cultures were also treated with ACTH ( $10^{-9}$  M). qRT-PCR analyses were normalized with respect to actin gene expression. *E*) PKA responsiveness of Senp2 and Ube2i protein levels. ATC1 cells were treated with forskolin ( $10^{-5}$  M) for the indicated time (h) or with DMSO (Vhl) for the longest treatment time. Protein accumulation of Senp2 and Ube2i was analyzed by Western blot. Tubulin signal serves as loading control. Fsk, forskolin; Pr. Culture, primary culture. Quantification histograms represent means  $\pm$  SEM from 3 to 4 experiments. Statistical analyses were conducted by 1-way ANOVA followed by Dunett's test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ .

fasciculata cells (Fig. 3A, B and Supplemental Fig. S3A). Sumo1 and Sumo2/3 staining pattern was similar in both sexes, although the decreased SUMOylation levels between zG to zF appeared more pronounced in females (Supplemental Fig. S3B). Interestingly, SUMO labeling

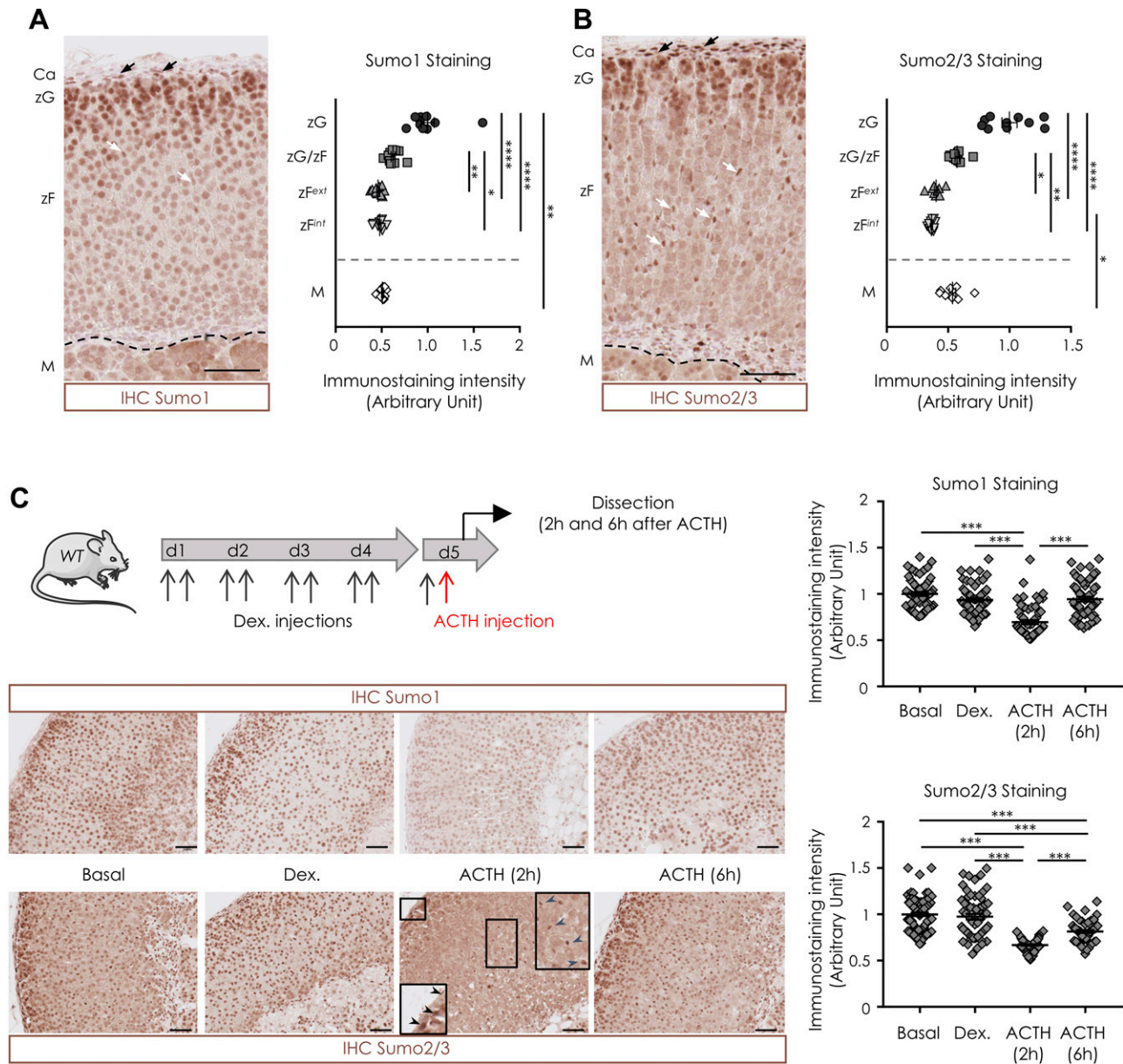
was not restricted to steroidogenic cells. Indeed, capsular as well as endothelial cell nuclei were strongly positive for Sumo2/3, whereas Sumo1 staining was much less pronounced in these cell populations (Fig. 3A, B and Supplemental Fig. S3B).



**Figure 2.** Acute hormonal induction or constitutive activation of PKA signaling affects protein SUMOylation and SUMO pathway gene expression in mouse adrenals. *A, B*) WT mice were injected with immediate ACTH, and adrenals were removed 2 or 4 h later; control adrenals were dissected from untreated mice (0 h) and subjected to RNA extraction: experimental setup (*A*); qRT-PCR analyses (*B*) of SUMOylation pathway gene expression normalized with respect to actin gene expression. Statistical analyses were conducted by 1-way ANOVA followed by Dunnett's test. *C*) CNC mouse model with constitutive activation of PKA following adrenal inactivation of *Prkar1a* (*Sf1-Cre::Prkar1a<sup>fl/fl</sup>* mice): experimental setup. Adrenals from *Sf1-Cre::Prkar1a<sup>+/+</sup>* (WT) and *Sf1-Cre::Prkar1a<sup>fl/fl</sup>* [knock-out (KO)] mice were removed and subjected to RNA and protein extraction. qRT-PCR analyses of SUMOylation pathway gene expression normalized with respect to actin gene expression; *D*) Western blot analyses of Sumo2/3 conjugates in adrenal protein extracts and quantification (histogram). Tubulin signal was used to normalize the quantification of SUMO signals. Statistical analyses were conducted by Student's *t* test. Quantification histograms represent means  $\pm$  SEM. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

In order to evaluate the consequences of changes in the hypothalamic-pituitary-adrenal (HPA) axis on SUMO staining profile, mice were either treated for 5 d with dexamethasone to block endogenous ACTH secretion or supplemented with (rapid-acting) ACTH injection on the

last day (Fig. 3C). Compared with basal conditions, HPA axis blockade had no significant effect on either Sumo1 or Sumo2/3 nuclear staining patterns. In contrast, ACTH supplementation resulted in a transient decrease in SUMO nuclear labeling that affected all cortical zones after 2 h and



**Figure 3.** SUMOylation is regionalized in mouse adrenal cortex and modulated by ACTH/PKA signaling. *A, B*) SUMOylation profile forms a decreasing gradient of intensity from the outer to the inner cortex. The accumulation of conjugated proteins to Sumo1 (*A*) and Sumo2/3 (*B*) was analyzed by immunohistochemistry (IHC) in adrenals of 6-mo-old female mice. The quantification graphs represent means  $\pm$  SEM of SUMO nuclear staining intensity in each histologic zone and interzone all along the cortex and in medulla for 3 adrenals from 3 different animals and 50–60 nuclei per adrenal. Black and white arrows: nuclear Sumo staining in capsular and endothelial cells, respectively. Statistical analyses were conducted by Kruskal-Wallis followed by Dunn's test. Ca, capsule; M, medulla; zF<sup>ext</sup>, external zF; zF<sup>int</sup>, internal zF. *C*) Stimulation of the corticotropic axis changes the cortical SUMOylation profile. Six-month-old female mice were treated with dexamethasone (Dex.) for 5 d, injected or not the last morning with ACTH, and euthanized 2 or 6 h later. Basal conditions correspond to untreated mice. The accumulation of conjugated nuclear proteins to Sumo1 and Sumo2/3 was analyzed by IHC. The quantification graphs represent means  $\pm$  SEM of SUMO nuclear staining intensity in zF cells for 6 adrenals from 6 different animals and 10 nuclei per adrenal. Insets, black and gray arrows: nuclear Sumo staining in capsular and endothelial cells, respectively. Statistical analyses were conducted by 1-way ANOVA followed by Tukey's *post hoc* test. Scale bars, 50  $\mu$ m. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001, \*\*\*\* $P$  < 0.0001.

almost returned to pretreatment labeling after 6 h. Interestingly, the negative effect of ACTH was restricted to steroidogenic cells because nuclear Sumo2/3 staining intensity was unaltered in capsular or endothelial cells. In agreement with histologic data, Western blotting analysis of the corresponding adrenal extracts showed a parallel change of Sumo2/3 and Sumo1 conjugate levels

(Supplemental Fig. S3D). General staining pattern for SUMO was also assessed in adrenal sections from *Sf-1-Cre::Prkar1a<sup>fl/fl</sup>* mice. This showed that chronic stimulation of PKA signaling in the cortex resulted in the relative loss of centripetal gradient of nuclear Sumo1 labeling typical of WT cortex (Supplemental Fig. S3C). Altogether, these results indicate that there is a centripetally decreasing

gradient of SUMOylation throughout adrenal cortical zones that relies on the activity of ACTH/PKA signaling, which maintains relative hypoSUMOylation context in steroidogenic cells of the zF.

### Differential SUMOylation pattern of adrenal cortex is conserved in human adrenal and is altered in primary pigmented nodular adrenal hyperplasia (PPNAD) from CNC patients

To assess whether our observations were transferable to humans, we analyzed by immunohistochemistry the SUMOylation pattern of adrenal samples from either healthy patients or from hypercortisolic patients suffering from pathologic activation of PKA due to CNC. The SUMO1 and SUMO2/3 immunolabeling performed on normal human adrenal sections revealed a centripetally decreasing gradient of steroidogenic cell nuclear staining intensity similar to that found in mice (Fig. 4A and Supplemental Fig. S4A). Accordingly, nuclear staining intensity was maximal in cells of the outer cortex (zG and zG/zF transition zone) and weaker in the zF and zR. Interestingly, SUMO1 immunolabeling of adrenal samples from patients with CNC ( $n = 3 + 3$ ) revealed obvious hypoSUMOylation of cells forming the hyperplastic nodules (as evidenced by hematoxylin counterstaining). This contrasted with the intense SUMO1 nuclear staining found in adjacent internodular tissue (Fig. 4B and Supplemental Fig. S4B). Reciprocally, the signal for SUMO protease SENP1 was more pronounced within nodules (Fig. 4C and Supplemental Fig. S4C). Altogether, these data suggest that the SUMOylation potential was inhibited in a context of constitutive PKA activity that is expected to occur through R1 $\alpha$  loss within PPNAD nodules (*PRKAR1A* loss of heterozygosity) and was maintained or even increased in a context of blunted PKA activity, as found in the atrophic internodular fasciculata tissue [due to repressed ACTH levels (49)]. We conclude that, as suggested by mouse studies, SUMOylation follows a centripetally decreasing gradient in human adrenal cortex that seems to negatively correlate with activation of PKA signaling in normal gland (zF) and in adrenal nodules from patients suffering from CNC.

### WNT/ $\beta$ -catenin signaling favors SUMOylation in the adrenal cortex

We have previously established that WNT and PKA signaling pathways exert complementary and antagonistic actions to maintain adrenal cortex zonation: WNT signaling is essential for zG identity, whereas PKA signaling drives identity of the inner cortex (zF and zR) (13, 14). Therefore, we hypothesized that relative hyperSUMOylation of the outer cortex could rely on WNT/ $\beta$ -catenin signaling. To evaluate this hypothesis, we analyzed changes in SUMO and  $\beta$ -catenin immunofluorescent staining in adrenal sections of  $\Delta$ Cat mice that present stochastic constitutive activation of  $\beta$ -catenin (encoded by *Ctnnb1* gene) due to *Ctnnb1* exon 3 excision (*Akr1b7-Cre::Ctnnb1<sup>fl(ex3)</sup>*) (11) (Fig. 5A). As previously described,

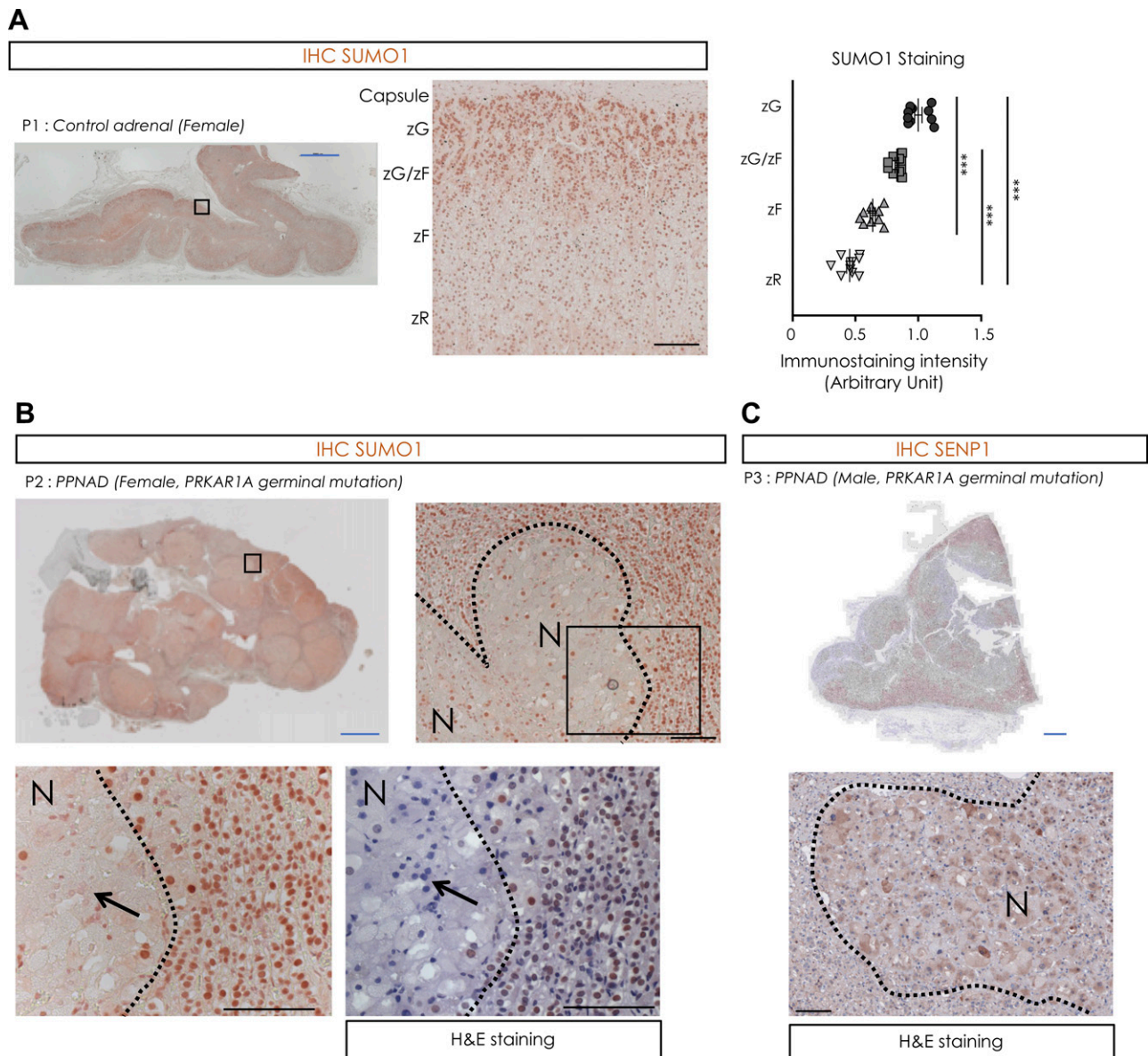
constitutive activation of WNT signaling resulted in expansion of the nucleo-cytoplasmic staining of  $\beta$ -catenin beyond zG and throughout the cortex. This resulted in the formation of large hyperplastic cells clusters (Fig. 5B) in which  $\beta$ -catenin staining intensity was massively increased compared with the adjacent nonrecombined tissue (Supplemental Fig. S5A). Coimmunofluorescent detection of either Sumo1 or Sumo2/3 revealed that hyperplastic clusters ( $\beta$ -catenin positive) and areas with highest Sumo nuclear labeling overlap. This was confirmed by quantifying Sumo nuclear signals in hyperplastic area ( $\beta$ -catenin high) and intact adjacent cortex ( $\beta$ -catenin low). This showed that increased SUMOylation correlated with  $\beta$ -catenin activation (Fig. 5C, D). We assessed possible concurrent changes in SUMO pathway gene expression in  $\Delta$ Cat adrenal glands using qRT-PCR. As illustrated in Fig. 5E, mRNA levels for genes involved in SUMO conjugation (*Sumo2*, *Sumo3*, *Pias1*, and *Pias2*) were induced, whereas mRNA expression of SUMO protease *Desi1* was reduced by 50% in adrenals from  $\Delta$ Cat mice. With the exception of genes for SUMO protease *Senp1* and *Senp6*, which were up-regulated, expression of all other tested genes was unaltered in  $\Delta$ Cat adrenals (Supplemental Fig. S5B). We conclude that WNT/ $\beta$ -catenin signaling pathway activation favors SUMOylation *in vivo* and can participate in establishing the centripetally decreasing gradient of SUMOylation in the adrenal cortex by maintaining high SUMOylation potential in the outer cortex.

## DISCUSSION

The results presented here show that there is a negative correlation between the activation of PKA signaling (genetically or hormonally induced) and the SUMOylation of adrenocortical proteins in cell culture and *in vivo*. This PKA-dependent hypoSUMOylation relies on the coordinated transcriptional stimulation of deSUMOylating isopeptidase genes (*Senp2*, *Desi1*, *SENP1*) and repression of genes encoding SUMO peptides and SUMO (*Pias3*) ligase. In agreement with this inhibitory effect, we show that in both human and mouse adrenal cortices, the SUMOylation pattern appears as a centripetally decreasing gradient. This is inversely correlated with PKA sensitivity of the cells, as emphasized by strong nuclear staining in zG and weaker staining in zF/zR. Activation of PKA signaling by ACTH injections leads to a transient decrease in SUMOylation in zF, which suggests a possible active role of this post-translational modification in zonation and/or stress response. Conversely, this gradient seems less sensitive to ACTH suppression, as dexamethasone treatment leads to a slight though nonsignificantly increased SUMOylation (Supplemental Fig. S3D). Moreover, the hypoSUMOylating effect of PKA signaling could participate in the etiology of PPNAD. Indeed, we show that global SUMOylation is reduced in the nodules of patients with PPNAD as well as in the adrenals of a mouse model of PPNAD (*Sf-1-Cre::Prkar1a<sup>fl/fl</sup>*).

In adrenocortical cells, kinetics of SUMO pathway gene expression in response to PKA stimulation are similar to that of steroidogenic genes (43). This observation suggests



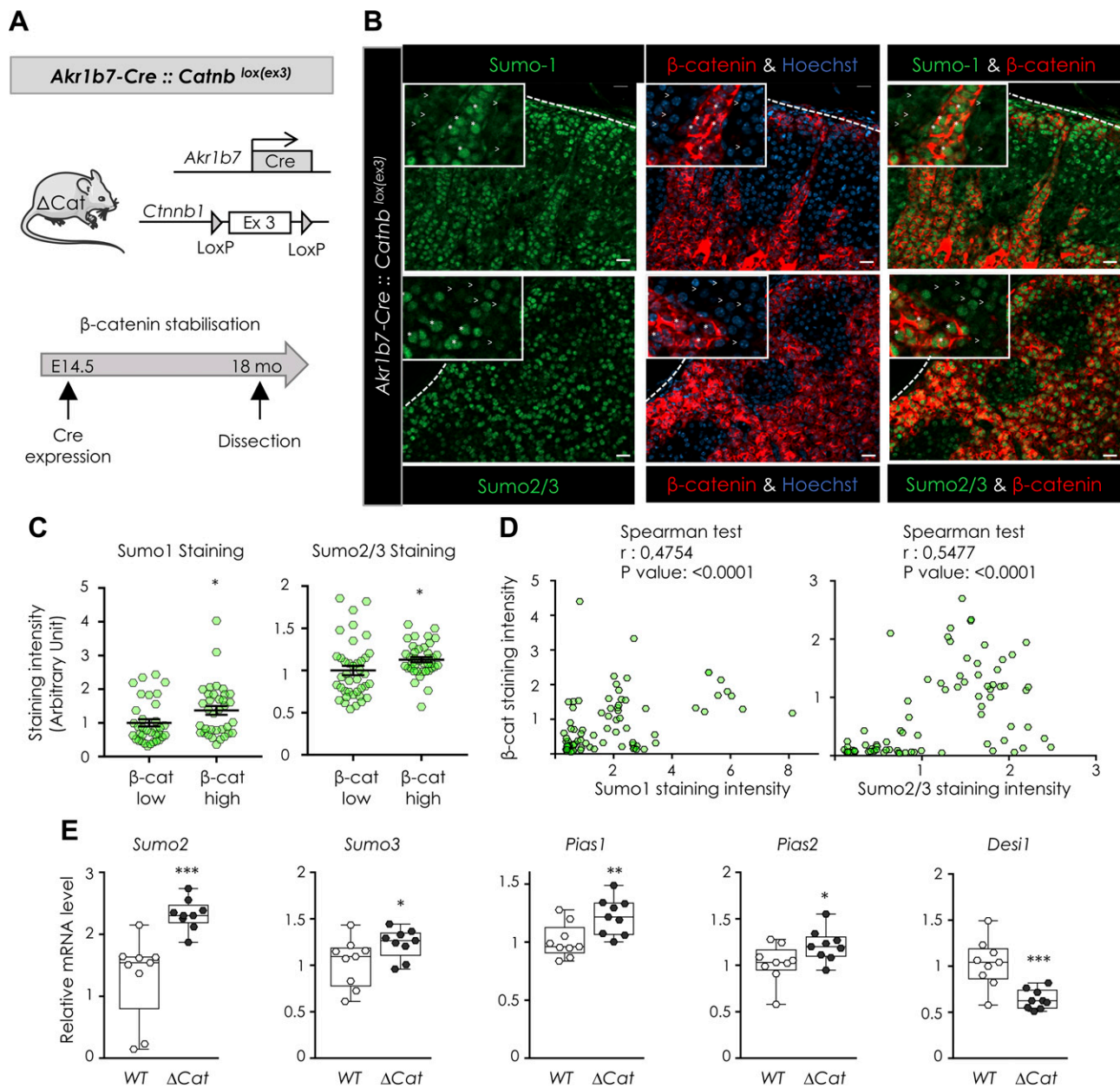


**Figure 4.** SUMOylation is regionalized in human adrenal cortex and altered in nodular hyperplasia from patients with CNC. *A*) SUMOylation profile forms a decreasing gradient of intensity from the outer to the inner cortex. The accumulation of conjugated proteins to SUMO1 was analyzed by immunohistochemistry in a control human adrenal. The quantification graph represents means  $\pm$  SEM of SUMO1 nuclear staining intensity in steroidogenic cells in each histologic zone and interzone all along the cortex. Statistical analyses were conducted by 1-way ANOVA followed by Dunett's test. \*\*\* $P < 0.001$ . *B*, *C*) HypoSUMOylation of PPNAD nodules. PPNAD sections from patients with *PRKAR1A* germinal mutation were immunostained for SUMO1 (*B*) or for SENP1 (*C*) and counterstained with hematoxylin (H&E). HypoSUMOylated nuclei (black arrow). N, nodule. Blue scale bars, 2 cm; black scale bars, 100  $\mu$ m.

that the SUMOylation process may be subordinated to the same regulatory mechanisms as steroidogenesis. Although PKA-mediated transcriptional activation is well documented, data on mechanisms relaying its repressive effects are scarce, even though they are quite prominent. Indeed, our transcriptome analyses of mice with adrenal *Prkar1a* gene inactivation with various Cre drivers (13, 14) showed similar numbers of down- and up-regulated genes following constitutive PKA activation. The only negative molecular mechanism identified to date in the adrenal gland mobilizes the oscillating salt-inducible kinase (SIK)-cAMP responsive element binding protein (CREB)-regulated transcription coactivator cascade, in

which SIK represses the activity of CREB in a transient PKA-dependent manner (50–52). The participation of SIK-CREB-regulated transcription coactivator in the transcriptional repression of *Sumo1–3* and *Pias3* genes will require further investigation.

In contrast to SUMO-conjugating genes, and in agreement with the overall hypoSUMOylation induced by PKA, expression of deSUMOylases *Senp2* and *Des1* is stimulated by activation of PKA signaling in the same way as steroidogenic genes. The induction of *Senp2* mRNA expression by ACTH/PKA might rely on the recruitment of CREB to a functional cAMP responsive element located in the proximal promoter region that was described to



**Figure 5.** Adrenal cortex SUMOylation is enhanced by constitutive activation of WNT/ $\beta$ -catenin ( $\beta$ -cat) signaling. *A*) Experimental setup.  $\beta$ -cat (encoded by *Ctnnb1* gene) stabilization in adrenal cortex was allowed by deletion of exon 3 of *Ctnnb1* [*Catnb*<sup>lox(ex3)</sup> allele] using *Akr1b7-Cre* line (referred to as  $\Delta$ Cat mice). *B*) Coimmunofluorescent labeling of Sumo1 (upper) or Sumo2/3 (bottom) (green),  $\beta$ -cat (red) and merged signals in adrenal sections from  $\Delta$ Cat female mice of 18 mo. *Inset*, magnification view showing nuclei from cells with low (white arrowheads) or high (white asterisk)  $\beta$ -cat staining. Scale bar, 20  $\mu$ m. *C*) The quantification graphs represent means  $\pm$  SEM of SUMO nuclear Sumo1 and Sumo2/3 staining intensity in cells that express low or high levels of  $\beta$ -cat (3 adrenals from 3 different animals and 10–15 nuclei per adrenal). *D*) Sumo1 or Sumo2/3 nuclear staining is correlated with  $\beta$ -cat activation. Spearman correlation between staining intensity values of Sumo1 or Sumo2/3 nuclear signal and staining intensity of  $\beta$ -cat nucleocytoplasmic signal. *E*) Adrenals from *Akr1b7-Cre::Catnb*<sup>+/+</sup> (WT) and *Akr1b7-Cre::Catnb*<sup>lox(ex3)/+</sup> ( $\Delta$ Cat) mice were removed and subjected to RNA extraction. qRT-PCR analyses of SUMOylation pathway gene expression normalized with respect to actin gene expression. Statistical analyses were conducted by Student's *t* test. Quantification histograms represent means  $\pm$  SEM. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001.

mediate PKA responsiveness in 3T3L1 preadipocytes (53). No functional data are available for the *Desi1* regulatory regions. However, the kinetics of ACTH responsiveness *in vivo*, with fast up-regulation of *Senp2* transcripts (2 h) and slow up-regulation of *Desi1* (24 h), suggests that mechanisms contributing to their regulation by PKA may differ. In addition, *Senp2* and *Desi1* proteins are supposed to

diverge in their subcellular localization. Indeed, whereas *Senp2* is mainly nuclear (54) *Desi1* is cytoplasmic (32). This suggests that their target substrates may differ.

The centripetal decreasing gradient of SUMOylation within the adrenal cortex (zG > zF/zR) together with its sensitivity to the HPA axis is suggestive of a role for SUMOylation in the response to stress. Accordingly,

requirement of SUMO pathway for adaptative response to stress seems to have been conserved in evolution (55). Indeed, proteomic analyses have demonstrated the coordinated SUMOylation of protein groups involved in the same biochemical pathway after genotoxic stress in yeast (56). SENP2-dependent deSUMOylation is required to adapt cells to etoposide-induced genotoxic stress, whereas SENP1-mediated deSUMOylation is required to counteract colon tumor growth induced by genotoxic *E. coli* strains (57, 58). In agreement with this concept, the repressor effect of PKA signaling on the SUMOylation process could participate in the transcriptional response of adrenocortical cells following stress. The constitutive hypoSUMOylation in zF could exert a permissive action on the response to stress by lifting the tonic repression exerted by SUMOylation on transcription factors such as SF-1 and nuclear receptor related-1 protein (37, 59). Under stress conditions, ACTH-induced transient hypoSUMOylation would further facilitate steroidogenic response. Furthermore, activation of some phosphodiesterases (PDEs) following their SUMOylation (60) suggests that such an intracellular feedback mechanism would allow the adrenal cortex to sustain elevated intracellular levels of cAMP and promote activation of PKA. The hypoSUMOylation observed in PPNAD nodules suggests the involvement of this modification in the etiology of the disease. These results are consistent with the increasing number of studies involving SUMOylation in tumor mechanisms (61).

Finally, the decreasing gradient of SUMOylation in the adrenal cortex also suggests its possible involvement in the establishment and/or maintenance of cortical zonation. Increased SUMOylation consecutive to depletion of SENP1/2/7 proteins was reported in various models to activate the WNT/ $\beta$ -catenin pathway by increasing  $\beta$ -catenin stability (62, 63) and by promoting its nuclear translocation (64, 65). The ability of SUMOylation to activate the WNT pathway is likely to explain the intense staining observed in zG. Reciprocally, we report here that constitutive  $\beta$ -catenin activation in mouse adrenals not only induces zG tumor formation (11, 66) but also expands the hyperSUMOylation domain. This suggests that a positive feedback loop between SUMO-induced WNT pathway and  $\beta$ -catenin-dependent SUMOylation increase could allow maintaining higher SUMO staining in the zG. Conversely, the hypoSUMOylating effects of PKA signaling activation could participate (*e.g.*, through *Senp2/SENP1* up-regulated expression) in the antagonism between WNT/ $\beta$ -catenin and ACTH/PKA pathways, which is needed to establish proper functional zonation and to counteract  $\beta$ -catenin-induced tumor formation (13, 14).

In summary, our findings reveal that protein SUMOylation follows a conserved centripetally decreasing gradient within the adrenal cortex. This relies on antagonistic actions of ACTH/PKA and WNT/ $\beta$ -catenin signaling pathways: ACTH/PKA signaling acutely reduces SUMOylation in zF by coordinating the expression of both SUMO pathway inducers and repressors, whereas WNT/ $\beta$ -catenin induces hyperSUMOylation. Our data suggest that programming of the SUMOylation pattern could play a role in endocrine function and stress

response and maintenance of zonation and pathology (hypersecretion syndromes and cancer). FJ

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## AUTHOR CONTRIBUTIONS

T. Dumontet, I. Sahut-Barnola, D. Dufour, A.-M. Lefrançois-Martinez, P. Val, and A. Martinez conceived of and designed the experiments; T. Dumontet, I. Sahut-Barnola, D. Dufour, A.-M. Lefrançois-Martinez, A. Berthon, N. Montanier, C. Djari, J.-C. Pointud, and F. Roucher-Boulez performed the experiments; T. Dumontet, I. Sahut-Barnola, D. Dufour, A.-M. Lefrançois-Martinez, and A. Martinez analyzed the data; B. Ragazzon, M. Batisse-Lignier, I. Tauveron, and J. Bertherat provided human samples; A. Martinez supervised the project; T. Dumontet and A. Martinez wrote the manuscript; and P. Val and all authors edited the manuscript.

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