

RESEARCH ARTICLE

Timing of adrenal regression controlled by synergistic interaction between Sf1 SUMOylation and Dax1

Yewei Xing¹, Ken-ichirou Morohashi², Holly A. Ingraham³ and Gary D. Hammer^{1,*}

ABSTRACT

The nuclear receptor steroidogenic factor 1 (Sf1, Nr5a1, Ad4bp) is crucial for formation, development and function of steroidogenic tissues. A fetal adrenal enhancer (FAde) in the *Sf1* gene was previously identified to direct *Sf1* expression exclusively in the fetal adrenal cortex and is bound by both Sf1 and Dax1. Here, we have examined the function of Sf1 SUMOylation and its interaction with Dax1 on FAde function. A diffused prolonged pattern of FAde expression and delayed regression of the postnatal fetal cortex (X-zone) were detected in both the SUMOylation-deficient-*Sf1*^{2KRI/2KR} and *Dax1* knockout mouse lines, with FAde expression/activity retained in the postnatal 20 α HSD-positive postnatal X-zone cells. *In vitro* studies indicated that Sf1 SUMOylation, although not directly influencing DNA binding, actually increased binding of Dax1 to Sf1 to further enhance transcriptional repression of FAde. Taken together, these studies define a crucial repressor function of Sf1 SUMOylation and Dax1 in the physiological cessation of FAde-mediated Sf1 expression and the resultant regression of the postnatal fetal cortex (X-zone).

KEY WORDS: Nuclear receptors, SUMOylation, X-zone, Adrenal gland

INTRODUCTION

Although the fetal adrenal zone was originally described as a transient inner-most layer of cells within the adrenal gland of the mouse by Masui and Tamura (1926), recent developmental and linear-tracing studies define the adrenal fetal zone as the adrenal primordium, which is derived from the adrenogonadal primordia prior to separation into the adrenal cortex and bipotential gonad. Specifically, once encapsulated and infiltrated by neural crest cells, the catecholamine-producing cells of the adrenal medulla, the adrenal primordia, establish the stem/progenitor cell niche of the definitive cortex. Four events are needed for this process: (1) Sf1 expression must be extinguished in the fetal cortex; (2) capsular stem cells must be established from fetal cells (Wood and Hammer, 2011); (3) Sf1-expressing cells must be established in the definitive cortex from these capsular stem cells (Zubair et al., 2006, 2008; Morohashi and Zubair, 2011); and (4) the remnant fetal zone itself must regress and decrease in size. All the above events happen with

strict timing to ensure proper development and normal function of the adrenal cortex.

Referred to postnatally as the X-zone in mice [defined by Howard-Miller (Howard-Miller, 1928)], the fetal cortex regresses at puberty in males or at first pregnancy in female mice. The first recognizable signs of the onset of degeneration are narrowing of the X-zone and development of a well-defined peripheral edge. At this stage, collagen fibers become evident between inner and outer borders of the zone, and gradually form a complete fiber layer around the medulla. The fibers become more evident with continued degeneration and narrowing (Holmes and Dickson, 1971; Tanaka and Matsuzawa, 1993). Although steroid hormones, including androgens, progesterone and oestradiol benzoate, have been shown to produce significant degenerative effects on the fetal cells (Ohno, 1962; Delost et al., 1971; Asari et al., 1979), little is known about how this zone is maintained after birth or what factors regulate the timing of its regression.

The nuclear receptor steroidogenic factor 1 or adrenal 4 binding protein (Sf1/Ad4bp/Nr5a1) is a member of the nuclear receptor superfamily. In mice, it is expressed in the pituitary, hypothalamus, gonads and adrenal glands (Ozsisik et al., 2003; Kohler and Achermann, 2010). Sf1 is a crucial factor in the formation of the adrenal gland, and central to the expression of steroidogenic genes such as cholesterol side chain cleavage (*Cyp11a1*), aromatase (*Cyp19a1*), steroidogenic acute regulating protein (*Star*) and melanocortin 2 receptor (*Mc2r*) (Breckwoldt et al., 1996; Chung et al., 1997; Buaas et al., 2012). Given its functional importance, great emphasis has been placed on studying mechanisms that regulate Sf1 activity in adrenal development and steroidogenesis.

Importantly, the fetal and definitive adrenal cells use unique enhancer sequences that mediate restricted Sf1 expression in each cell type. Although the fetal adrenal enhancer (FAde) of Sf1 has been defined, the definitive adrenal enhancer (DAde) of Sf1 remains unknown. In fetal adrenal cells, Sf1 maintains its own expression through autoregulation of the FAde (Zubair et al., 2006, 2009) until E14.5 when the definitive cells emerge below the adrenal capsule. However, mechanisms responsible for cessation of Sf1 expression (FAde) in the fetal cells and re-establishment of Sf1 expression (DAde) in the definitive cells with later regression of the fetal adrenal are unknown.

Post-translational modifications and the specificity of co-factor recruitment are important mechanisms that modify transcriptional activation and repression of Sf1. Moreover, the post-translational modifications, phosphorylation and SUMOylation of Sf1 play opposing roles in regulating Sf1 activity both *in vivo* and *in vitro* (Lee et al., 2011; Hammer et al., 1999; Sasaki et al., 2014; Yan et al., 2014). Of note, SUMOylation of Sf1 inhibits its activity on a subset of target genes, and mice with a SUMO-deficient form of Sf1 exhibit enhanced activation of a subset of target genes (Lee et al., 2011).

Dax1 (Nr0b1) is an unusual member of the nuclear receptor superfamily in that it lacks the conventional DBD (DNA-binding

¹Department of Internal Medicine, Division of Metabolism, Endocrinology and Diabetes, University of Michigan Health System, Ann Arbor, MI 48109-2200, USA. ²Department of Molecular Biology, Graduate School of Medical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan. ³Department of Cellular Molecular Pharmacology, School of Medicine, University of California, San Francisco, CA 94158, USA.

*Author for correspondence (ghammer@umich.edu)

 G.D.H., 0000-0001-6843-3628

domain), modulator domain and hinge region. Instead, Dax1 contains a novel N-terminal structure consisting of 3.5 alanine/glycine-rich repeats, each 65–70 amino acids long (Iyer and McCabe, 2004). Dax1 has been shown to function as a nuclear receptor co-repressor that interacts with Sfl to inhibit a number of genes involved in both adrenal development and steroidogenesis. The inhibition mechanism is thought to involve direct protein-protein interactions between Dax1 and Sfl, which lead to subsequent recruitment of co-repressors to the promoters of target genes. It is also possible that Dax1 competes with Sfl transcriptional co-activators, including CREB-binding protein (CBP)/p300, glutamate receptor interacting protein 1 (*Grip1*) and tyrosine-protein kinase (*Src*), to promote transcriptional repression of Sfl target genes (Zhou et al., 2008; Ferraz-de-Souza et al., 2009; Li et al., 2011). However, to date, it is unclear how Sfl post-translational modulation might modify Dax1-mediated repression of Sfl activity.

In the current study, we used two loss-of-function mouse models and *in vitro* assays to examine the contribution of SUMOylation of Sfl and the recruitment of Dax1 to the inhibition of FAdE-mediated Sfl expression in the fetal adrenal cortex. Our results demonstrate that the inability of Sfl to be SUMOylated and the genetic loss of Dax1 both cause extended activation of the FAdE and delayed regression of the postnatal fetal cortex (X-zone) in mouse adrenals. Finally, we define the molecular mechanisms of a synergistic repression of FAdE by SUMOylated Sfl and Dax1 that appear to be essential for repressing Sfl-mediated autoregulation of the FAdE enhancer and for ensuring proper timing of fetal adrenal (X-zone) regression.

RESULTS

Both Sfl SUMO-deficiency and genetic Dax1 loss result in delayed fetal adrenal regression *in vivo*

The fetal adrenal cortex is the inner-most zone of the adrenal cortex and is composed of remnant fetal cells. In mice, the remnant fetal zone cells (postnatally referred to as X-zone cells) regress at puberty in the male and during the first pregnancy in the female (Zubair et al., 2006, 2008, 2009; Morohashi and Zubair, 2011). In 3-week-old prepubertal *Sfl*^{2KR/2KR} mice, compared with wild-type mice, a markedly expanded X-zone with increased number of 20 α HSD-expressing cells is observed (Fig. 1A). In addition, male *Sfl*^{2KR/2KR} mice continue to retain a distinct zone of 20 α HSD-expressing fetal cells (X-zone) post-pubertally, at the age of 7 weeks (Fig. 1B). This zone only regresses after 15 weeks of age in the *Sfl*^{2KR/2KR} male mice (Fig. 1C). Although virgin wild-type females maintain some 20 α HSD-expressing X-zone cells at 15 weeks, a much broader expression of 20 α HSD is observed in 15-week-old *Sfl*^{2KR/2KR} mice (Fig. 1C). These data are in accordance with findings previously reported by some of us (Lee et al., 2011) and suggest that mutations that prevent Sfl SUMOylation lead to expansion of prenatal fetal zone cells and delayed postnatal X-zone regression.

Dax1 (NR0B1) is a unique nuclear receptor that functions primarily as a negative regulator of Sfl-mediated transcriptional regulation. Dax1 is expressed strongly in the prenatal fetal adrenal as early as E11.5 (Fig. S1) and postnatally in the outer zona glomerulosa with additional weaker scattered expression in the zona fasciculata of the definitive cortex (Mukai et al., 2002; Zubair et al., 2009). Genetic loss of Dax1 leads to adrenal failure in individuals with cytomegalic adrenal hypoplasia and in aging mice, highlighting the crucial role of Dax1 in homeostasis of the definitive cortex (Scheys et al., 2011). To delineate the role of Dax1 in the fetal adrenal cortex, we examined the fetal adrenal phenotype of Dax1 knockout mice (*Dax1*^{-/-} or Dax1 KO) and found similar defects as

observed in male *Sfl*^{2KR/2KR} mice. Not only were increased X-zone cell numbers observed in young male mice adrenals (Fig. 1A), but delayed X-zone regression was also detected in pubertal male mice (Fig. 1B). Real-time PCR was also used to quantify the expression levels of X-zone marker genes in those mice. In accordance with the observed expansion/retention of the X-zone in 2KR and *Dax1*^{-/-} models, we observe both: (1) a significant increase in peak gene expression; and (2) a temporal delay in extinguishment of gene expression of the X-zone markers 20 α HSD, Pik3c2g and Thr β in 2KR and *Dax1*^{-/-} adrenal glands compared with their wild-type littermates (Fig. 1D,E). Importantly however, loss of Sfl SUMOylation or loss of Dax1 expression was insufficient to prevent the ultimate regression of the X-zone in the adult adrenal gland, suggesting that while additional intrinsic or compensatory mechanisms must contribute to elimination of remnant fetal adrenal cells (X-zone), Sfl SUMOylation and Dax1 are both crucial for the proper timing of this event.

FAdE expression is retained in postnatal X-zone (remnant fetal zone) cells of *Sfl*^{2KR/2KR} and Dax1 KO mice

As reported previously, expression of Sfl in the fetal adrenal is regulated by a fetal adrenal enhancer (FAdE) in the *Sfl* gene locus and this enhancer is autoregulated by Sfl (Fig. 4). At early developmental stages, the FAdE is active in all fetal zone cells but is gradually repressed in cells located in the outer part of the cortical region of the adrenal after E14.5. Following birth, FAdE-positive cells can be found only in the innermost 20 α -positive X-zone (remnant fetal zone) cells. FAdE-mediated transcription ceases coincident with X-zone regression during puberty in the male mouse and during first pregnancy in the female mouse. Our observation of an expanded and retained X-zone in both *Sfl*^{2KR/2KR} and Dax1 KO mouse lines led us to investigate whether elevated/retained FAdE activity might contribute to this phenotype. First, *Sfl*^{2KR/2KR} mouse lines were crossed into the *FAdE-Ad-LacZ* line, and FAdE activity was assessed by *LacZ* expression. As expected, a broad expression of *LacZ* was detected in adrenals of young (3-week-old) *Sfl*^{2KR/2KR} mice, colocalizing with the same cells that express the X-zone marker 20 α HSD (Fig. 2). Few *LacZ*- or 20 α HSD-positive cells were observed in wild-type males at this age. This result indicates that disturbing the SUMOylation cycle of Sfl, FAdE remains active in both prenatal fetal zone cells and corresponding postnatal X-zone cells. Moreover, the prolonged FAdE expression correlates with retention of the X-zone in the pubertal adrenal cortex. Last, the FAdE-positive X-zone cells do not express the ZF marker, *Cyp11B1* (Fig. 2), supporting the conclusion that those cells are descendants of fetal zone cells rather than peripheral adult cortical cells. Although it remains possible that *FAdE-LacZ* expression observed in 20 α HSD-expressing cells reflects ectopic expression in a cell not derived from the FAdE-activated fetal cell, this is extremely unlikely based on previous lineage-tracing studies (Zubair et al., 2006; Freedman et al., 2013). Prior lineage data from Zubair et al. (2008) that used a fetal zone-specific driver (FAdE-Ad4BP-Cre-ERT2) reveal retained *LacZ* expression in the X-zone after 2 months of tracing, indicating that the postnatal X-zone is (derived from) the prenatal fetal zone. In addition, lineage data from Freedman et al. (2013) that used a peripheral zona glomerulosa driver (AS-Cre) indicate that X-zone cells do not originate from the peripheral cortex of the adult zone. These data indicate that the X-zone precedes the development of the adult cortex and is the postnatal fetal zone.

Next, we investigated FAdE activity in the *Dax1* KO male mice that express *FAdE-LacZ*. In contrast to adrenals from wild-type mice

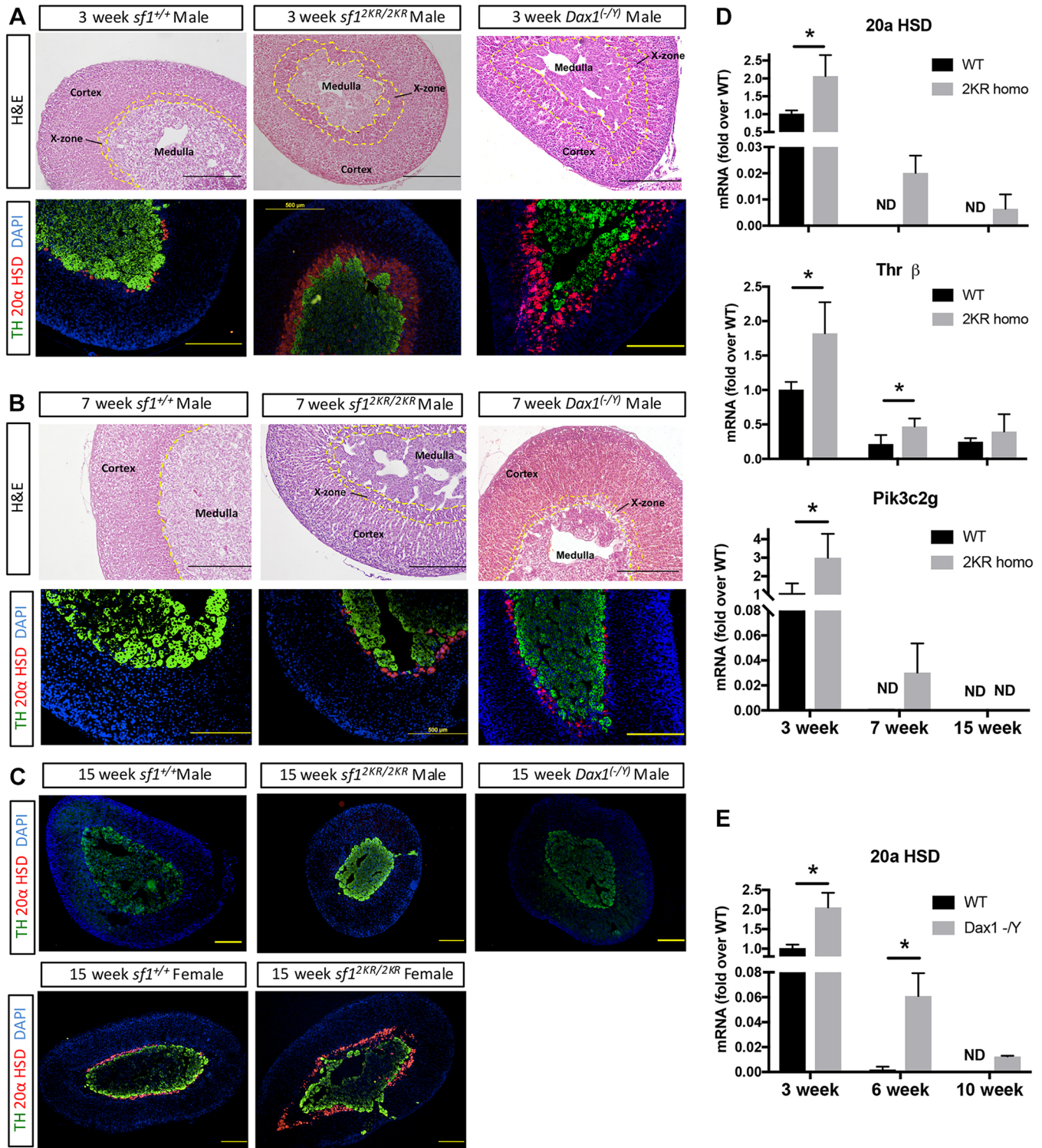


Fig. 1. The adrenal X-zone is expanded in SUMO-deficient and *Dax1* knockout male mice, and maintained after puberty. (A) Hematoxylin and Eosin (top panel) and immunostaining (bottom panel) of 3-week-old wild-type and *Sf1*^{2KR/2KR} male mouse adrenals. Staining with TH (green) marks the medulla and 20αHSD (red) marks X-zone cells. DAPI staining (nuclei) is in blue. (B) Hematoxylin and Eosin (top panel) and immunostaining (bottom panel) of 7-week-old wild-type and *Sf1*^{2KR/2KR} male mice adrenals. The inner yellow dotted line marks the margin between cortex and medulla, whereas the area between two dotted lines represents X-zone. *Sf1*^{2KR/2KR} and *Dax1*^{-/-} male mice have an expanded X-zone at a young age and this zone is retained after puberty. (C) Immunostaining of 15-week-old *Sf1*^{2KR/2KR} and *Dax1*^{-/-} male mouse adrenals (upper panel) and 15-week-old wild-type and *Sf1*^{2KR/2KR} virgin female mouse adrenals. The X-zone in male *Sf1*^{2KR/2KR} mice regresses at a later age, whereas the X-zone and 20αHSD expression are retained in virgin females of both wild-type and *Sf1*^{2KR/2KR} mice, the latter having an expanded fetal zone with an expansion of 20αHSD-expressing cells. Scale bars: 500 μm. (D) X-zone marker gene expression in *Sf1*^{2KR/2KR} male mouse adrenals. RNA were isolated from paraffin wax-embedded sections of 3-, 7- and 15-week-old male *Sf1*^{2KR/2KR} adrenal glands. Gene expression was quantified using real-time qPCR with primers designed for individual genes. *n*=4–8 per group, **P*<0.05. (E) X-zone marker 20αHSD gene expression in *Dax1*^{-/-} male mouse adrenals. RNA were isolated from paraffin wax-embedded sections of 3-, 6- and 10-week-old male *Dax1*^{-/-} adrenal glands. *n*=4–6 per group. **P*<0.05. ND, not detectable.

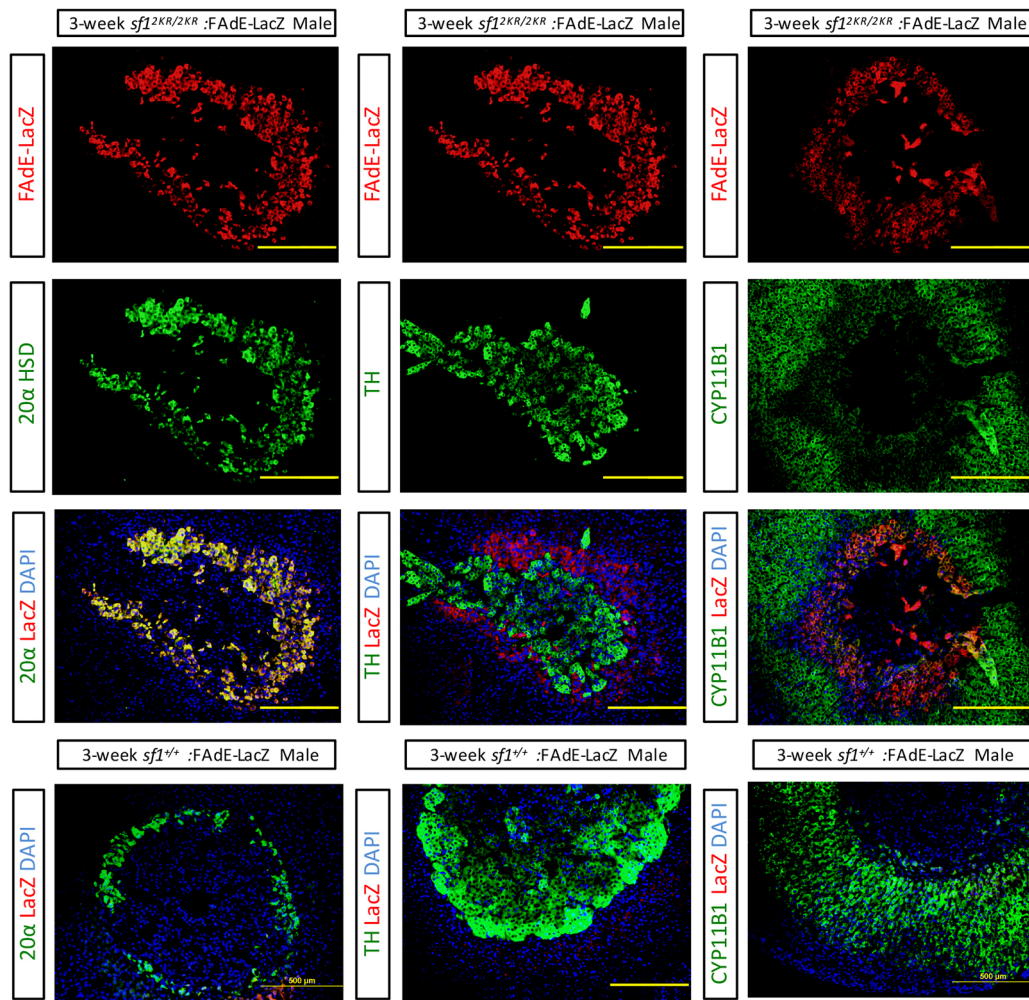


Fig. 2. FAdE expression is retained in the X-zone cells of *Sf1*^{2KR/2KR} mice. Immunostaining of 3-week-old *Sf1*^{2KR/2KR}; *FAdE-LacZ* male mice adrenals. Expression of *LacZ* is overlaid with either the fetal zone marker 20 α HSD (left panels), the medulla marker TH (middle panels) or zona fasciculata cell marker CYP11B1 (right panels). FAdE activity colocalizes with fetal zone cells. Scale bars: 500 μ m.

in which FAdE activity is largely extinguished by E17.5 (Zubair et al., 2009) (Fig. S2), *LacZ* expression was detected at as late as E17.5, P7 and P16 in *Dax1* KO mice (Fig. 3A,B). Interestingly, co-immunostaining with 20 α HSD demonstrated that *LacZ*-positive cells are present in the inner X-zone, but not observed in the peripheral X-zone or differentiated adult cortex. These data indicate that *Dax1* suppresses FAdE activity in fetal zone/X-zone cells, perhaps by repressing the activity of *Sf1*. Furthermore, similar to the *LacZ*-positive cells of the *Sf1*^{2KR/2KR} adrenals, those FAdE-expressing cells also expressed the X-zone marker 20 α HSD in young pre-pubertal *Dax1* KO mice (P7 and P16) (Fig. 3B). The *Dax1*-mediated effect on FAdE activity is sustained until at least P16 whereas in wild-type mice *LacZ*-positive cells are only present as a thin rim of remaining X-zone cells in the inner adrenal; *Dax1* KO mice exhibit increased numbers of *LacZ*-positive cells that are arranged in a broader and more diffused pattern (Fig. 3C). However, by P90, *LacZ*-positive cells disappeared in *Dax1* KO mice as in their wild-type littermates, supporting the hypothesis that additional inherent or compensatory mechanisms are used to fully extinguish FAdE activity and ultimately induce X-zone regression in male mice.

When comparing the two genetic models, 2KR adrenals exhibit a more profound but qualitatively similar phenotype when compared

with the *Dax1*^{-/-} adrenals. Specifically, *LacZ* expression (reflecting *FAdE-LacZ* activity) is maintained for a longer period of time and in a larger number of cells of the X-zone of the 2KR adrenals when compared to the adrenals of *Dax1*^{-/-} mice. This is consistent with a dominant role of *Sf1* as a transcription factor at the FAdE locus. By contrast, *Dax1* has indeed classically been defined as a transcriptional co-factor: a co-regulator of *Sf1*-mediated transcription that requires *Sf1* for its activity. More specifically, our data are consistent with the SUMOylation of *Sf1* playing a dominant role in the extinguishment of FAdE expression and the correlative regression of the X-zone. The data are also consistent with *Dax1* being a co-regulator of *Sf1*-mediated transcriptional regulation of FAdE.

Although the spatial and temporal extent of FAdE expression is correlated with the timing of X-zone regression in both 2KR and *Dax1*^{-/-} mice, whether or not the delay in X-zone regression in both 2KR and *Dax1*^{-/-} mice is mediated directly by prolonged FAdE activity in the X-zone cells of these mice is not known. It is feasible that *Sf1* SUMOylation and *Dax1* have transcriptional effects that influence X-zone regression independent of FAdE activity.

Besides its above-shown function in later adrenal development, *Dax1* has also been predicted to play an important role in early establishment of the adrenal gland. Consistent with previous

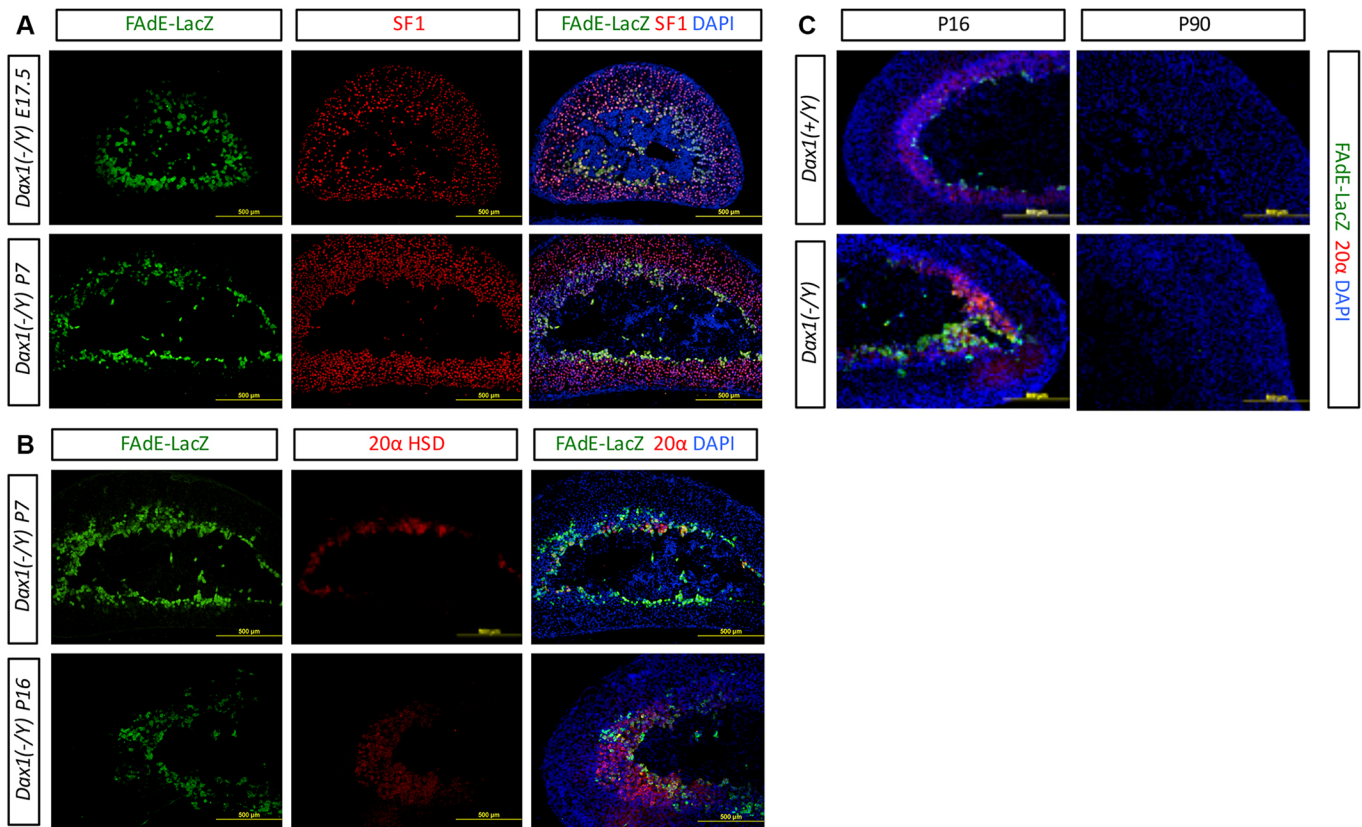


Fig. 3. Expansion and delayed regression of FAdE-expressing cells in adrenals of young *Dax1* knockout mice compared with wild type. (A) FAdE activity is maintained in *Dax1* KO mice during early developmental stages as shown by immunostaining for β -galactosidase to detect *LacZ* expression (upper panel, E17.5; lower panel, P7). (B) *LacZ* expression colocalizes with the fetal zone marker 20α HSD and is found in the inner rim of the 20α HSD-expressing zone at the age of P16 (lower panel). (C) Immunostaining with *LacZ* and 20α HSD in wild-type and *Dax1* KO male mice at P16 (left panel) and P90 (right panel). Although *Dax1* KO mice have more FAdE-positive cells at a young age, those cells and the fetal zone regress by 3 months of age. Scale bars: 500 μ m.

studies, immunohistochemistry in E11.5 mouse embryo showed that both Sfl and *Dax1* are expressed in the gonadal ridge and adrenal. Moreover, *Dax1* is also detected in wider areas, including medial celomic bay located in the posterior of adrenal region, in where Sfl cannot be detected (Fig. S1A). As shown by Zubair et al. (2009), Sfl is expressed weakly and instantly in a wider region in E11.5 embryos; however, this weak expression is immediately canceled by an unknown mechanism, and Sfl can be detected only in the genital ridge and adrenal region at later stages. Studies using transgenic assay revealed that fusion of Sfl promoter and FAdE drives reporters such as EGFP or Cre expression in the fetal adrenal, and instantly in the wider region of several lines of transgenic mice (Zubair et al., 2009), indicating that FAdE controls embryonic Sfl expression patterns. When *Dax1* KO mice are crossed with *FAdE-Ad-LacZ* mice and analyzed at different development stages, whole-mount *LacZ* staining indicated modified FAdE function in embryos from E11.5 and E13.5 (Fig. S1B,C). In the wild-type embryo at E11.5, *LacZ*-positive cells were detected clearly to be present in the fetal adrenal region. In contrast, in the *Dax1* KO embryo, *LacZ*-positive cells were observed in a larger area, and sporadically unorganized in the surrounding region (arrows). This shows that the number of *LacZ*-positive cells increase in the absence of *Dax1* at E11.5, supporting the notion that *Dax1* inhibits FAdE expression. However, at later stages (E13.5), *LacZ* expression becomes restricted to the adrenal gland and is almost indistinguishable in both genotypes. These data suggest that *Dax1* is the key repressor of the FAdE activity at E11.5.

SUMOylation of Sfl leads to increased recruitment of *Dax1* and an inhibition of FAdE-mediated transcriptional activity *in vitro*

To examine the molecular mechanisms of increased FAdE activity in *Sfl*^{2KR/2KR} and *Dax1* KO mice, we investigated the contributions of Sfl SUMOylation and *Dax1* to the inhibition of FAdE-mediated transcriptional activation in an adrenocortical cell culture system. As shown previously, there are four potential Sfl-binding sites in the FAdE region (Fig. 4A). Using ChIP assay, we confirmed that Ad4BP site #1 (Zubair et al., 2002) is the major binding site of Sfl in the FAdE region, with more than threefold higher affinity than site #2 and site #3/4 (Fig. 4B). Based on these data, we mainly focused on the Ad4 sites #1 and #2 for the remainder of our studies. Promoter reporter constructs were then engineered by fusing the -88 to +467 bp of the enhancer sequence with the minimal promoter of thymidine kinase of herpes simplex virus into pGL3-Basic (FAdE-Luc construct) and tested in HEK293K cells, used as a heterologous cell line not expressing endogenous Sfl or *Dax1*. Cells were transfected with FAdE-Luc and different constructs of SUMOylation-modified Sfl with or without pcDNA *Dax1*. Analysis of luciferase activity revealed that Sfl-2KR induced the highest stimulating activity on the FAdE-Luc construct. That SUMOylation of Sfl decreases the ability of Sfl to activate FAdE is shown most dramatically using a construct in which SUMO2 protein is fused to the N-terminal of Sfl (Sfl-SUMO). Sfl-SUMO has only 20% stimulating activity of FAdE-Luc as compared with Sfl-2KR (Fig. 4C). In order to exclude the possibility that Sfl with SUMO2 protein fused to the N-terminus engages the

transcriptional machinery differently from the natural SUMO-conjugated Sfl, we also performed FAdE activation experiments in the Y1 adrenocortical cell line in the presence or absence of overexpressed Senp1 (sentrin-specific protease 1) or SUMO-activating enzyme E1/Ubc9 (ubiquitin conjugating enzyme E2)/SUMO to manipulate the SUMOylation status of endogenous Sfl. Congruent with the inhibition of the FAdE enhancer observed with SUMO2-conjugated Sfl in comparison with wild type, SENP1-mediated inhibition of endogenous Sfl SUMOylation enhanced the Sfl-mediated activation of the FAdE enhancer (Fig. S3). Furthermore, Dax1 dramatically blunted the stimulating effect of all forms of Sfl. These data indicate that both SUMOylation modification and Dax1 decrease the stimulating effects of Sfl on the FAdE. Of note, when Sfl-SUMO and Dax1 are co-transfected in this experiment, we observe a complete inhibition (lack of activation) of FAdE activity, indicating that Sfl SUMOylation and Dax1 act synergistically to repress Sfl activity.

To explore the mechanisms related to the repressive effects of SUMOylation and Dax1 on Sfl stimulation of the FAdE, Co-IP assays were performed using Sfl antibody to pull down the protein complex of different SUMOylation forms of Sfl and Dax1 in transfected HEK293T cells. The SUMO2-conjugated Sfl significantly increases the binding of Sfl with Dax1 (2.2-fold increase), while mutation of the SUMOylation site decreases binding (Fig. 5A). The current data are in contrast to the previously published study by Campbell et al. (2008), which clearly demonstrated a modest but consistent reduction of DAX1 interaction with SUMOylated Sfl LBD (Campbell et al., 2008). However, as the previous experiments were conducted in a cell-free system using only the C-terminal region of Sfl (the ligand-binding domain of Sfl protein and a small part of the hinge region containing only one of the SUMOylation sites, K194), the two systems are not directly comparable.

Interestingly, neither SUMOylation nor the presence of Dax1 changes Sfl DNA-binding affinity to the FAdE sites. ChIP assay using different SUMOylation forms of Sfl together with Dax1 and vectors containing the DNA sequence of the FAdE Ad4 site #1 in HEK293T cell lines showed no changes in Sfl DNA-binding capacity with or without SUMOylation modification (Fig. 5B). Similar effects were confirmed in Y1 cells by examining the DNA-binding capacity of different forms of endogenous Sfl. No significant changes in all four Ad4-binding sites were observed (Fig. S4B). The data indicate that differential DNA binding of Sfl to FAdE in the context of SUMOylated Sfl and Dax1 does not occur, and therefore does not significantly contribute to changes in transcriptional activation of FAdE-mediated gene expression.

DISCUSSION

Effective Sfl dose has been shown to be a critical determinant of Sfl transcriptional activity. Effective Sfl dose is influenced by genetic dose (derived from studies in patients and in engineered mice with Sfl haplo-insufficiency manifesting with adrenal defects), mRNA/protein level, DNA binding, SF-1 ligand, post-translational modifications and co-factor recruitment – all of which ultimately influence Sfl transcriptional activity on Sfl genomic targets (Bland et al., 2004; Blind et al., 2014; Chen et al., 2005; Ozisik et al., 2002; Urs et al., 2007; Yang et al., 2009; Zubair et al., 2009).

Developmental studies in mice have determined that Sfl dose (Sfl mRNA/protein level) is a critical determinant of the specification/formation of the adrenal primordium (fetal zone) as it buds off of the shared adrenogonadal primordia (Bland et al., 2004). The expression of Sfl in the adrenal primordia (fetal zone) is driven by the fetal zone-restricted Sfl enhancer FAdE. The

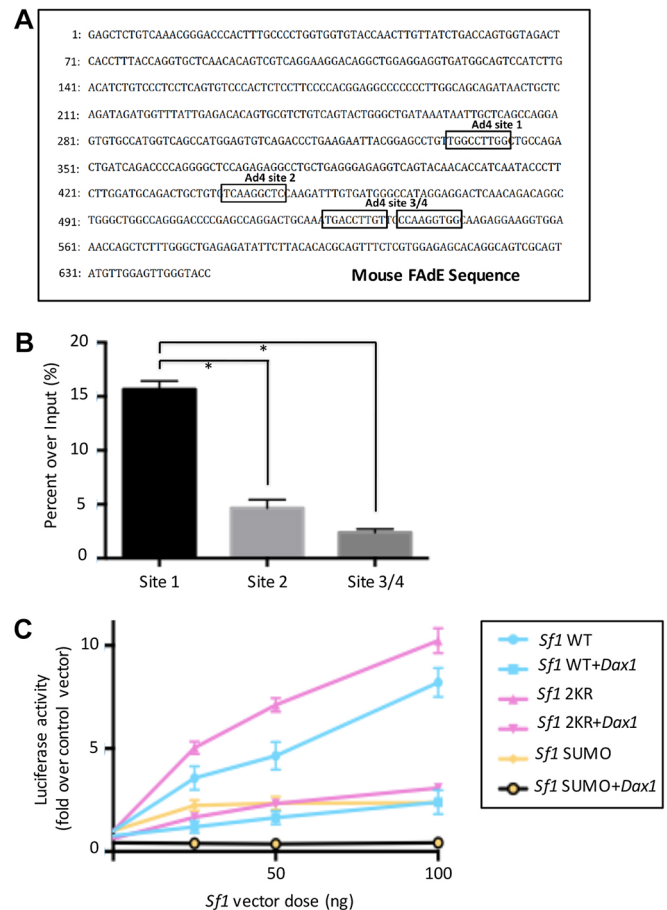


Fig. 4. Sfl SUMOylation and Dax1 modify Sfl activity on the FAdE enhancer *in vitro*. (A) Ad4 (Sfl) binding sites in the mouse FAdE region (Zubair et al., 2002). (B) Binding capacity of Sfl on the different Ad4 sites in the FAdE region. ChIP assays were performed on Y1 cell lines using anti-Sfl antibodies. Immunoprecipitates were analyzed by quantitative PCR using primers designed for each individual site. The data were normalized to values obtained for 1% input controls, and the results are presented as percentage of input. Data are mean \pm s.e.m. (C) SUMOylation and Dax regulate Sfl activity on the FAdE enhancer. HEK293T cells were plated at 10^5 cells/well in 24-well plates 24 h before transfection and were transfected in triplicate with FAdE Luc (100 ng/well), with Sfl as indicated, and with or without Dax1 (50 ng/well). Luciferase assays were carried out and the data were normalized to renilla level and shown as fold change over control vectors. $n=6$. * $P<0.05$. Error bars indicate s.e.m.

activation of FAdE in the fetal zone is maintained by Sfl itself, which binds to and activates FAdE – hence maintaining its own expression (Zubair et al., 2006). Moreover, the forced expression of Sfl under the control of a transgene driven by the fetal zone restricted Sfl enhancer FAdE results in both: (1) an expansion of ectopic adrenal tissue that persists postnatally, expresses *FAdE-LacZ* and hence is consistent with a fetal zone identity; and (2) a persistent expanded postnatal *FAdE-LacZ* population in the bona fide adrenal cortex consistent with a retained X-zone (Zubair et al., 2009). Both studies define an essential role of Sfl dose in the extent of FAdE activation and the extent of X-zone retention. Moreover, the work indicates that FAdE-mediated Sfl dose is a critical determinant of the extent of X-zone retention.

Two of the most well-studied physiological mediators of effective Sfl dose are the post-translational modification of SUMOylation of the Sfl protein and the recruitment of the transcriptional co-factor Dax-1 to the Sfl complex on DNA. Previous studies have shown that

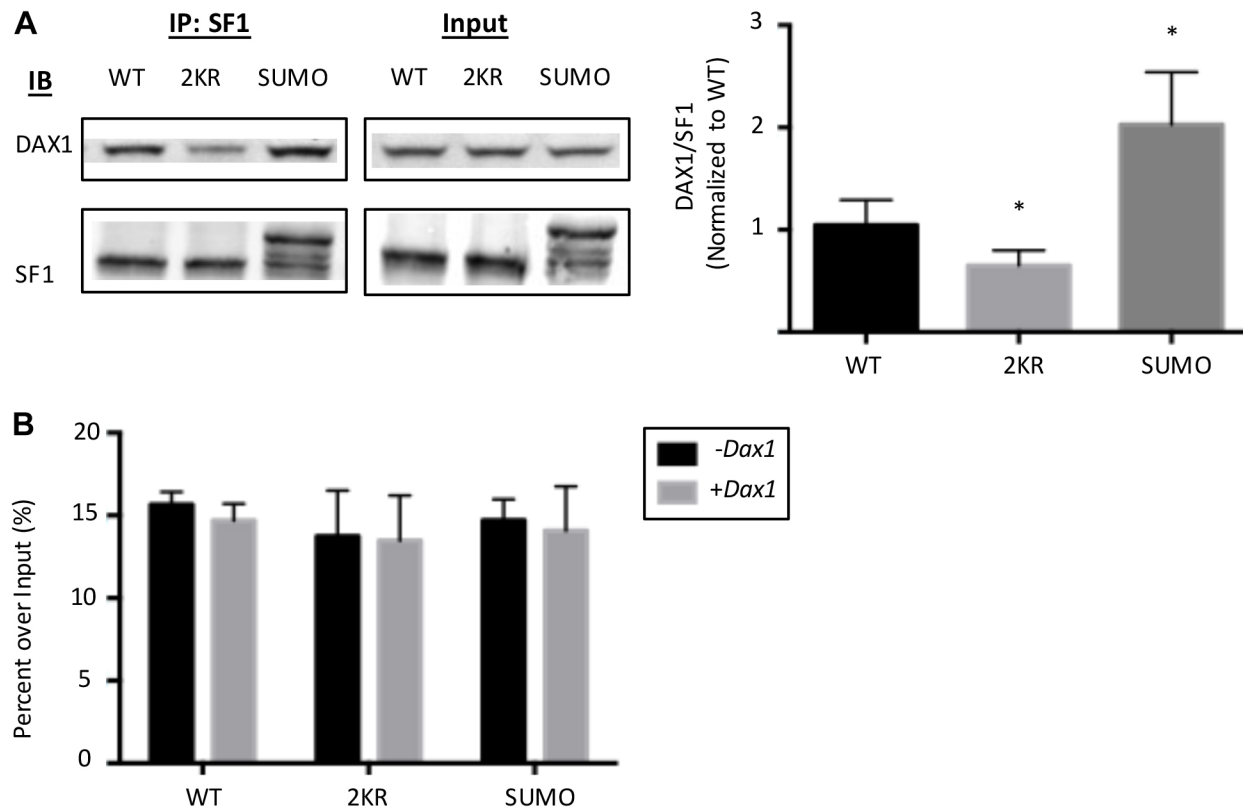


Fig. 5. SUMOylation enhances Sf1 binding to Dax1, but does not influence its binding affinity to DNA. (A) SUMOylation increases the binding of Sf1 to Dax1. HEK293T cells were plated at 10^6 cells/dish in 10 mm cell culture dishes 24 h before transfection and were transfected with 1 μ g pcDNA-Sf1 and 1 μ g pcDNA-Dax1 vectors. After 48 h, cells were lysed and co-immunoprecipitation assays performed. Lysates were precipitated using anti-Sf1 antibody and immunoblotted with anti-HA (for Dax1) or -Sf1 antibodies. The data were normalized to input and shown as fold change over wild-type Sf1. * $P < 0.05$. Error bars indicate s.e.m. (B) SUMOylation or Dax1 do not change the DNA-binding capacity of Sf1. HEK293T cells were plated at 10^6 cells/dish in 10 mm cell culture dishes 24 h before transfection and were transfected with 1 μ g pcDNA-Sf1 and 1 μ g pcDNA-Dax1 vectors with 25 ng of linearized pGL3-FAdE construct. After 48 h, cells were fixed and processed for ChIP assay using anti-Sf1 antibody. The data were normalized to values obtained for 1% input controls, and the results are presented as percentage of input ($n=5$). Error bars indicate s.e.m.

SUMOylation at K194/K119 inhibits Sf1 transcriptional activity on a number of target genes active in the definitive adrenal cortex, including StAR and MC2R (Yang et al., 2009). When those two SUMOylation sites are eliminated in mice, defects in the adult differentiated cortex include decreased steroidogenesis and an enhancement in expression/signaling of progenitor cell sonic hedgehog (Shh) expression (Lee et al., 2011). In the present study, we examined the roles of Sf1 SUMOylation specifically in the fetal adrenal cortex. We demonstrate that both SUMOylation-deficient Sf1 and knockout of the Sf1 repressor Dax1 result in retention of FAdE expression (consistent with persistent FAdE-mediated Sf1 expression) and a prolonged retention (delayed regression) of the X-zone (fetal adrenal remnant) in the mouse adrenal gland (Fig. 6A). Furthermore, we determine that in cell culture, SUMOylation of Sf1 increases the interaction between Sf1 and Dax1, resulting in a near-complete inactivation of FAdE activity (Fig. 6B), providing a potential molecular mechanism for the active extinguishing of FAdE-mediated Sf1 transcription, which ultimately leads to postnatal regression of the remnant fetal cortex – the X-zone.

SUMOylation is emerging as a versatile modification and has been shown to modify protein function through regulation of protein–protein interactions, subcellular nuclear localization, protein–DNA interactions or enzymatic activity (Wilson and Rangasamy, 2001; Melchior and Hengst, 2002; Pichler and Melchior, 2002; Freiman and Tjian, 2003; Verger et al., 2003; Müller et al., 2004). Consequently,

the SUMOylation process can influence a variety of biological processes, including apoptosis, cell cycle regulation, cell growth and differentiation (Andreou and Tavernarakis, 2009; Bettermann et al., 2012; Krumova and Weishaupt, 2013). Previous studies have indicated that SUMOylation of Sf1 does not alter its nuclear localization or DNA interaction (Chen et al., 2004; Lee et al., 2005), both of which are confirmed in the current study (data not shown). *In vitro* studies using the Y1 adrenocortical cell line have shown that SUMOylation inhibits Sf1 activity by reducing phosphorylation at S203, suggesting the interplay between SUMOylation and phosphorylation (Yang et al., 2009). However, no changes in the phosphorylation level of Sf1 were detected in whole organ lysates of adrenal glands from *Sf1*^{2KR/2KR} mice (data not shown), suggesting the phenotypes we observed are unlikely to be mediated by an increase in phosphorylation of Sf1 alone. Importantly, this observation does not preclude the possibility that the deficiency in SUMOylation only enhances CDK7-mediated phosphorylation of Sf1, as shown in a previous study (Yang et al., 2009), without elevating the total phosphorylation levels. An *in vitro* CoIP assay failed to detect CDK7 protein in the Sf1/Dax1 complex (data not shown).

In the current system, the auto-activation of FAdE by Sf1 itself is almost completely inhibited by SUMOylation of Sf1 and an enhanced recruitment of the co-repressor Dax1, resulting in a complete repression of FAdE-mediated transcription of the Sf1 gene. It is expected that SUMOylation can alter the surface of the target protein and cause either general conformational changes or

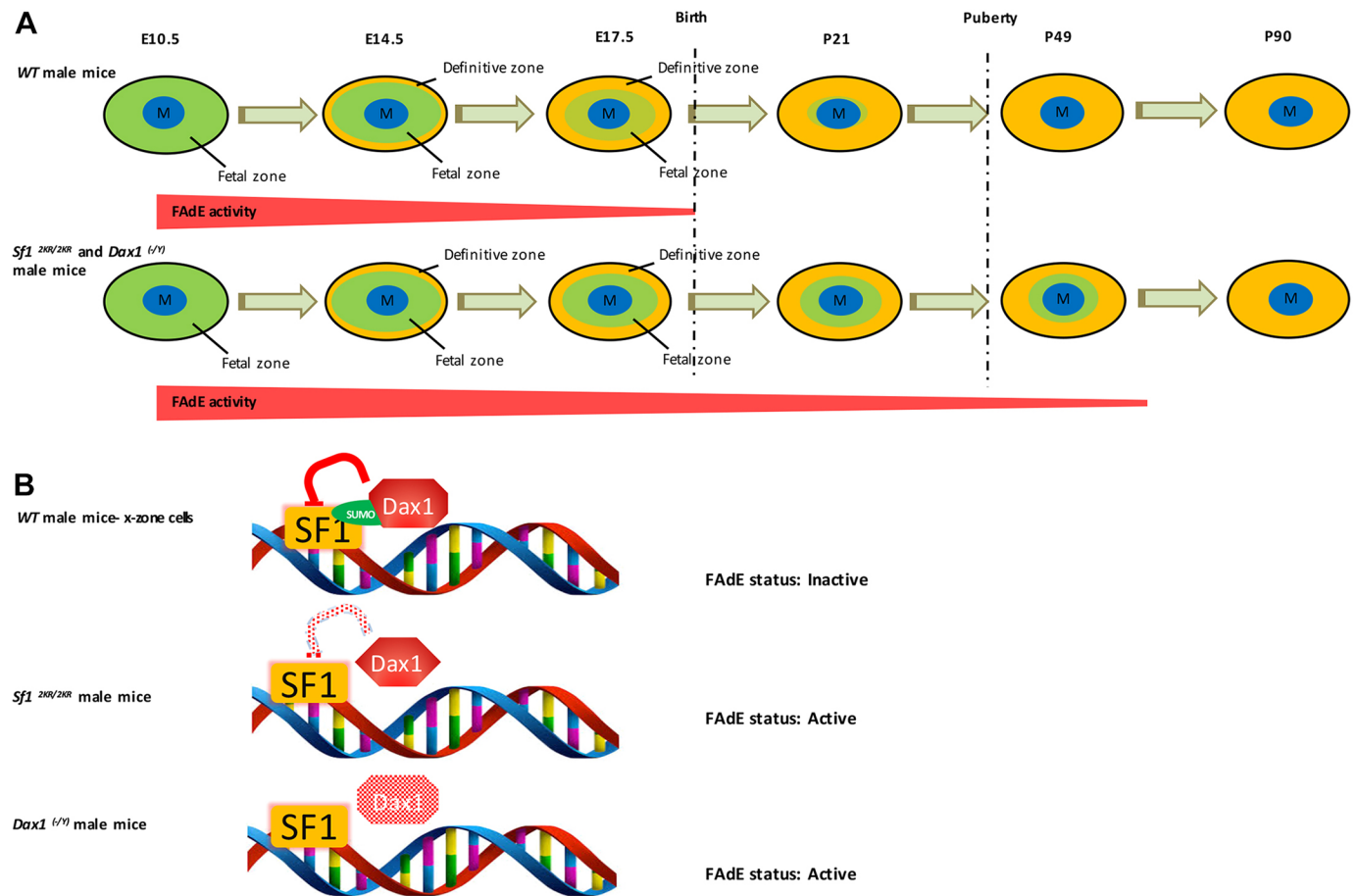


Fig. 6. The synergistic interaction between Sf1 SUMOylation and Dax1 controls the timing of X-zone regression. (A) Genetic depletion of Sf1 SUMOylation or Dax1 expression in mouse adrenal cortex delays the timing of X-zone regression by activating FAdE in postnatal adrenal glands. (B) SUMOylation of Sf1 enhances Dax1 binding to Sf1 protein and subsequent inhibition of FAdE activity in adrenocortical cells.

specific changes at crucial interfaces, which consequently may modify the interaction of the protein with co-activators or co-repressors. This change in transcriptional complexes leads to regulation of target gene expression. Based on the high sequence and 3D-structure homology with mLRH-1, Sf1 is proposed to have two Dax1-binding sites: one at AF2 region and one at the hormone pocket entrance region. Owing to the proximity of SUMOylation sites K119 and K194 with LBD (ligand-binding domain), it is possible that SUMOylation at the hinge region changes the conformation/ligand-binding property of the ligand-binding pocket, which in turn enhances Dax1 binding with Sf1. No traditional co-partners (i.e. SRC or P300) were detected in the Sf1/Dax1 complex (data not shown), but it is reasonable to speculate SUMOylation/binding with Dax1 may lead to exchange of other co-activators or co-repressors.

MATERIALS AND METHODS

Experimental animals

All animal experiments were carried out in accordance with protocols approved by the University Committee on Use and Care of Animals at the University of Michigan. The *Sf1*^{2KR/2KR} mouse line were generated as described and maintained on a C57BL/6J background (Lee et al., 2011). Dax1-deficient mice were obtained previously (Scheyset al., 2011) and maintained on a 129S1/SvImJ background. To obtain *Dax1*^{+/-} mice, wild-type males were mated with heterozygous females (Babu et al., 2002; Yu et al., 1998). Both lines were crossed with *FAdE-LacZ* reporter mice (Zubair et al., 2006) for experimental purposes.

LacZ (β -galactosidase) staining, histology and immunohistochemistry

LacZ activity in fetal tissues was examined as described previously (Zubair et al., 2009). After being stained, the tissue samples were fixed with 4% paraformaldehyde in phosphate-buffered saline (PFA-PBS) for 6 h at 4°C, embedded in optimal-cutting-temperature compound, and sectioned at 16–18 μ m. Hematoxylin and Eosin and immunofluorescence staining was performed following a previously published protocol (Kim et al., 2008). Images were captured using a Nikon Optiphot microscope and color Nikon digital camera. Images were processed using Photoshop software (Adobe System). Primary antibodies used for immunohistochemistry were anti- β -galactosidase (1:1000; Abcam, ab9361), anti-tyrosine hydroxylase (TH) (1:500; Cell Signaling Technology, 2792), anti-Sf1 (1:2000, Lab custom antibody) (Walczak et al., 2014), anti-Dax1 [1:1000; generously provided by Enzo Lalli (Centre National de la Recherche Scientifique, France) (Tamai et al., 1996)] and anti-20 α hydroxysteroid dehydrogenase (20 α HSD) (1:2000; Dr Yacob Weinstein, Ben Gurion University, Israel; Hershkovitz et al., 2007). Secondary antibodies used were goat anti-mouse Alexa 488 and goat anti-rabbit 549 (1:1000; Molecular Probes, A-10680 and A-27039).

RNA isolation and real-time qPCR

RNA was isolated from paraffin wax-embedded adrenal tissue section of wild-type, *Dax1*^{+/-} and *Sf1*^{2KR/2KR} male mice using the RNeasy FFPE kit from Qiagen following the manufacturer's manual. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Sequences of primers used for qPCR amplification of x-zone markers were: 20 α HSD F, 5'-TGGTCACTCCATTCCTGTGG-3'; R, 5'-TGAGATGCTCTTCAGTTGCA-3'; Thr β F, 5'-GCTGGTAGGAATGTC-

TGAAGC-3'; R, 5'-AGTCTGGAAAGTCTGGGCAC-3'; Pik3c2g-F, 5'-GTGGACCCAGGTGAGAACT-3'; R, 5'-GGAACACACTTTGTTTCTTCTC-3'.

Construct engineering

Mouse Sfl cDNA was amplified by PCR using the forward primer 5'-TCGTGGATCCATGGACTACTCGTACGACGAG-3' and the reverse primer 5'-ACGAAAGCTTCAAGTCTGCTTGGCCTGCAG-3', and integrated into pcDNA3 construct. The pcDNA3-HIS-FLAG-SF1 2KR, pcDNA3-HA-SUMO2-SF1 plasmids were derived from the wild-type pcDNA3-HIS-FLAG-SF1 vector using the QuikChange site-directed mutagenesis approach (Stratagene). The reporter plasmid was constructed by inserting the FAdE fragment (Zubair et al., 2006) together with the minimal promoter of thymidine kinase of herpes simplex virus into the *KpnI-HindIII* sites of pGL3-Basic (Promega).

Cell culture and transient transfection

Mouse adrenal Y1 cells and human embryonic kidney HEK293T cells were cultured in DME/F-12 medium (Invitrogen Life Technologies) supplemented with 10% Cosmic Calf Serum (ThermoFisher Scientific) and antibiotics in humidified air containing 5% CO₂ at 37°C. Twenty-four hours before transfection, HEK293T cells were plated at 10⁵ cells/well in 24-well plates. Transfections were carried out in triplicate with a combination of plasmids and using FuGENE 6 (Roche, Basel, Switzerland) according to the manufacturer's protocol. Plasmids used include pGL3-FAdE luciferase reporter plasmid at 100 ng/well; pcDNA-Sfl (wild type, SUMO2 or 2KR) in varying amounts, pcDNA-Dax1, Senp1, Ubc9 and pSA2 (Addgene); cytomegalovirus- β -galactosidase (40 ng/well) as a control for transfection efficiency; pcDNA to bring the total to 265 ng/well. The cells were harvested 48 h after transfection, and activities in cell lysates were determined using the dual luciferase reporter assay system (Promega). The luciferase values were normalized to the β -galactosidase activity.

CoIP and western analysis

Protein extracts were prepared by homogenization of cells and tissues in RIPA buffer [50 mM Tris HCl (pH 8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS] containing protease inhibitor cocktail (Sigma). Soluble protein was collected and immunoprecipitated with the Sfl antibody overnight. Protein G-dynabeads were added to the protein lysates and incubated for 2 h at 4°C. The beads were precipitated and washed with buffers of increasing stringency. The proteins were eluted by boiling in 30 μ l of sample buffer, resolved by 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane. Immunoblot was performed using the Odyssey system for protein detection. Primary antibodies used were rabbit polyclonal anti-Ad4BP/SF1 [1:1500; custom antibody (Walczak et al., 2014)] and mouse anti-HA antibody (1:300, Covance).

Chromatin immunoprecipitation (ChIP) assay

ChIP was performed using the ChIP-Easy kit (Upstate) following the manufacturer's protocol. Antibodies used for ChIP were non-immune rabbit IgG (Santa Cruz Biotechnology) and anti-Ad4BP/Sfl (Custom antibody). Sequences of primers used for qPCR amplification of immunoprecipitated chromatin were as follows: FAdE site 1 F, CATGGTCAGCCATGGAGTG; FAdE site 1 R, CTGGGGTCTGATCAGTCTG; FAdE site 2 F, CTGCT-GAGGGAGAGGTCAGT, FAdE site 2 R, TCCTCTATGGCCCATCA-CA; FAdE site 3/4 F, TGTGATGGCCCATAGGAGGA; FAdE site 3/4 R, ACCTTCTCTTGCCACCTTG.

Statistical analyses

Data are expressed as means \pm s.e.m. A one-way ANOVA was applied to compare means among the groups. Appropriate post-hoc pair-wise multiple comparisons were performed. Prism was used for the statistical analysis. $P < 0.05$ was considered significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: Y.X., G.D.H.; Methodology: Y.X., K.M.; Formal analysis: Y.X.; Investigation: Y.X.; Resources: H.A.I., K.M.; Data curation: Y.X.; Writing - original draft: Y.X.; Writing - review & editing: H.A.I., K.M., G.D.H.; Supervision: G.D.H.; Funding acquisition: H.I., G.D.H.

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Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.150516.supplemental>

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