Eda-activated RelB recruits an SWI/SNF (BAF) chromatin-remodeling complex and initiates gene transcription in skin appendage formation

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Ectodysplasin A (Eda) signaling activates NF-κB during skin appendage formation, but how Eda controls specific gene transcription remains unclear. Here, we find that Eda triggers the formation of an NF-κB-associated SWI/SNF (BAF) complex in which p50/RelB recruits a linker protein, Tfg, that interacts with BAF45d in the BAF complex. We further reveal that Tfg is initially induced by Eda-mediated RelB activation and then bridges RelB and BAF for subsequent gene regulation. The BAF component BAF250a is particularly up-regulated in skin appendages, and epidermal knockout of BAF250a impairs skin appendage development, resulting in phenotypes similar to those of Eda-deficient mouse models. Transcription profiling identifies several target genes regulated by Eda, RelB, and BAF. Notably, RelB and the BAF complex are indispensable for transcription of Eda target genes, and both BAF complex and Eda signaling are required to open chromatin of Eda targets. Our studies thus suggest that Eda initiates a signaling cascade and recruits a BAF complex to specific gene loci to facilitate transcription during organogenesis.

Results

Eda Signaling Triggers Linkage of RelB to a BAF Complex. We hypothesized that tissue-specific Eda signaling might require NF-κB activation to specify gene targets and a chromatin-remodeling complex to open chromatin at those sites. To test this hypothesis, we designed a workflow for the purification of BAF complexes from HaCaT keratinocytes before and after Eda stimulation (Fig. L4), using a successful purification method described previously (13, 19). We first generated a derivative of the HaCaT human keratinocyte stable cell line that expresses the Eda receptor (Edara). Immunoblotting demonstrated the expected expression of Edar, and when conditioned medium (CM) containing Eda-activated RelB recruits an SWI/SNF (BAF) chromatin-remodeling complex and initiates gene transcription in skin appendage formation

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The authors declare no conflict of interest.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE97783).

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active Eda-A1 protein (20) was added to the cultures, NF-kB luciferase activity increased 15.2-fold in Edar+ cells compared with that in untransfected (Edar−) HaCaT cells (SI Appendix, Fig. S1A).

We next fractionated nuclear extracts (NEs) from Edar− and Edar+ cells treated with Eda-A1 CM. After gel-filtration chromatography, peak fractions containing BAF components—BRG1 and other key proteins including BAF250a and BAF170 (Fig. 1B)—were pooled and immunoprecipitated with a BAF170 antibody. Polyacrylamide gel silver staining (Fig. 1C) and MS analyses (SI Appendix, Table S1) showed the presence of most BAF components including BAF and PBAF in the BAF170 immunoprecipitate. Interestingly, a fraction of BAF complex components including BAF and PBAF in the BAF170 immunoprecipitate was not affected by the presence of Edar− cells (SI Appendix, Table S1). Notably, an additional protein, Tfg, was coprecipitated, that had been suggested as possibly involved in NF-kB action (21) but had not been previously found in any BAF complex. It appeared in both BAF170 and RelB immunoprecipitates from Edar− cells (SI Appendix, Table S1), while RelB binding but not PBAF had indeed interacted with the NF-kB dimer RelB/p50 and did so only after activation of Eda signaling (Fig. 1D). In further urea denaturation experiments, either p50 or RelB was still stable in its association with the BAF complex after treatment with more than 2 M urea (SI Appendix, Fig. S1C). To rule out the possibility that NF-kB may indirectly associate with the BAF complex through DNA, IP from NE of Edar− cells was performed in the presence of ethidium bromide (EtBr), a DNA-interacting drug that dissociates proteins from DNA. The amount of RelB in the BAF170 immunoprecipitate was not affected by the presence of EtBr (SI Appendix, Fig. S1D), indicating that RelB complexes with SWI/SNF through protein interaction.

Direct protein–protein binding experiments in vitro further explored the possibility that Tfg might link the BAF complex to NF-kB. We used a cell-free protein synthesis system to produce individual proteins, including 10 components of the RelB-associated BAF complex, five NF-kBs, and Tfg, detected by immunoblotting (SI Appendix, Fig. S1H). Protein–protein binding assays showed that Tfg directly bound only RelB among the five NF-kBs and bound its association with the BAF complex after treatment with more than 2 M urea (SI Appendix, Fig. S1C). Gel silver staining confirmed the production and purity of key proteins (SI Appendix, Fig. S1H). Protein–protein binding assays showed that Tfg directly bound only RelB among the five NF-kBs and bound BAF45d but no other tested BAF (SI Appendix, Fig. S2A and B). Reverse IP-Western blotting confirmed the direct binding of Tfg to both RelB and BAF45d (SI Appendix, Fig. S2 C and D).

Thus, biochemical data indicated that skin-specific Eda signaling activated a large protein complex containing BAF components, p50/RelB, and the linker protein Tfg.

Eda Signaling Is Mediated Mainly by the p50/RelB Subclass of NF-kB. NF-kB mediates Eda/Edar signaling in cell cultures and in animal models (22, 23), but the selective binding of RelB to BAF complex (Fig. 1) suggested that refined specificity of NF-kB action might be gained by the activation of a subclass of components. We therefore checked the protein levels of all five NF-kBs in cell cultures. In the cytoplasm, Eda/Edar action up-regulated the levels of p50, p105 (p50 precursor), p100 (p52 precursor), and RelB, while other NF-kBs, including p52, RelA, and c-Rel, showed no apparent change in levels (Fig. 2A). In the nucleus, upon Edar expression, p52 was unaffected, and p50, RelA, and c-Rel were somewhat up-regulated, consistent with the previous finding that Edar itself can activate NF-kB (24, 25); however, RelB accumulated to a strikingly higher level, and the application of Eda CM further augmented RelB among three Rel family members (Fig. 2B). We further performed NF-kB oligonucleotide (oligo) IP experiments and found that Eda/Edar strongly activated the binding of p50 and RelB to an oligo with the NF-kB consensus sequence but not to oligos with a mutated binding sequence. Slight oligo binding to RelA was also seen (SI Appendix, Fig. S3 A and B).

We extended studies of NF-kB subunit activation in skin appendage development using mouse Meibomian glands (MGs) as a model (25). At an early stage (E16.5), immunohistochemistry (IHC) images showed that p50, RelB, and RelA were expressed throughout the basal epidermal layer in WT mouse skin, while p52 was undetectable and c-Rel was restricted to the mucus layer. In MG germs, the RelA level was similar to that in epidermal layers, but p50 and RelB were considerably elevated (Fig.
Eda signaling specifically activates p50/RelB and recruits BAF complex during skin appendage development. (A) Immunoblotting shows levels of the NF-κB subunit as well as Tfg in the cytosol (Cyt) and NE of HaCaT cells with (+) or without (−) the indicated treatment. Actin and histone H3 (a nuclear protein) served as loading controls. (B) IHC staining (green) of p50, RelB, and Tfg in WT or Tabby (Ta) mouse eyelids at E16.5. Enlarged views of the areas within the white rectangles are shown below each image. Arrows indicate MG germs. (C) Immunoblotting shows protein levels of NF-κB subunits and Tfg in WT and Tabby eyelids at E16.5. (D) IHC of K14 (green) and indicated BAF proteins (red) in back skin (Left) and eyelids (Right) from WT mice at E16.5. Arrows indicate HFs (Left) or MGs (Right). (E) Total NE from WT (Left) or Tabby (Right) eyelids was used for RelB, Tfg, and BAF170 IP. Immunoblotting with the indicated antibodies is shown. Five percent NE from WT eyelids was used as input. IgG served as the negative control. (Scale bars, 50 μm.)

Eda-Activated p50/RelB Recruits BAF Complex in Developing Skin Appendages. To substantiate the function of the Eda-induced BAF complex in vivo, we studied two mouse skin appendage models: HFs and MGs. First, we analyzed the expression pattern of BRG1, a key component of both BAF and PBAF complexes. BRG1 IHC staining showed ubiquitous expression in whole-skin tissues for both HFs and MGs (SI Appendix, Fig. S4A). To further compare the expression of BAF and PBAF complexes, we checked for BAF250a and BAF250b as selective components of BAF (26) and for BAF180 and BAF200 as idiosyncratic PBAF subunits (14). BAF250a was widely expressed in whole skin, including the epidermal layer, labeled by the marker keratin 14 (K14), but BAF250a staining was markedly up-regulated within HFs and MGs (Fig. 2D). Unlike BAF250a, other BAF components (BAF250b, BAF170, and BAF45d) were all expressed uniformly in both dermis and epidermis (Fig. 2D). In contrast to other BAF proteins, IHC staining showed no expression of either BAF180 or BAF200 in HFs or MGs but showed their sporadic expression outside the basal epidermal layers (SI Appendix, Fig. S4B). We infer that BAF, but not PBAF, is expressed in developing skin appendages at E16.5 and that BAF250a is highly up-regulated in this stage.

To test whether Eda activates BAF formation in developing skin appendages, we carried out RelB, Tfg, and BAF170 IP using NEs from WT or Tabby eyelids at E16.5. Indeed, p50/RelB, together with Tfg, formed a complex with BAF but not with PBAF in WT eyelids (Fig. 2E, Left). Similarly, in Eda-deficient (Tabby) eyelids, RelB failed to complex with p50, Tfg, or any BAF protein tested (Fig. 2E, Right). These data thus provide evidence that Eda mainly activates p50/RelB, which then recruits the BAF complex through Tfg in development.

Epidermal Knockout of BAF250a Inhibits Skin Appendage Formation. BAF250a showed specific up-regulation in developing appendages (Fig. 2D), which might reflect its functional importance. We thus generated skin-specific BAF250a knockout (cKO) mice by crossing BAF250a loxP/loxP mice with K14-Cre mice. In BAF250a cKO mice at E17.5, BAF250a expression was successfully ablated in K14+ cells (Fig. 3A). We noted that the cKO mice were born with open eyes and died within a few hours (SI Appendix, Fig. S5A).

We next examined eyelids from WT and cKO mice collected at birth. Results in H&E-stained tissues were consistent with a previous study (25) with an average length of MGs of 35.2 μm in WT mice. By contrast, in the cKO mice, no nascent MGs progressed to invade the dermis (Fig. 3D). To test whether BAF250a affected the pregrern initiation stage of MGs, we evaluated the length of MGs of Eda-deficient [Tabby (Ta)] mice (Fig. 3B, Left)). Immunoblotting of each NF-κB subunit as well as Tfg in the cytosol (Cyt) and NE of HaCaT cells with (+) or without (−) the indicated treatment. Actin and histone H3 (a nuclear protein) served as loading controls. (A) Immunoblotting shows levels of the NF-κB subunit as well as Tfg in the cytosol (Cyt) and NE of HaCaT cells with (+) or without (−) the indicated treatment. Actin and histone H3 (a nuclear protein) served as loading controls. (B) IHC staining (green) of p50, RelB, and Tfg in WT or Tabby (Ta) mouse eyelids at E16.5. Enlarged views of the areas within the white rectangles are shown below each image. Arrows indicate MG germs. (C) Immunoblotting shows protein levels of NF-κB subunits and Tfg in WT and Tabby eyelids at E16.5. (D) IHC of K14 (green) and indicated BAF proteins (red) in back skin (Left) and eyelids (Right) from WT mice at E16.5. Arrows indicate HFs (Left) or MGs (Right). (E) Total NE from WT (Left) or Tabby (Right) eyelids was used for RelB, Tfg, and BAF170 IP. Immunoblotting with the indicated antibodies is shown. Five percent NE from WT eyelids was used as input. IgG served as the negative control. (Scale bars, 50 μm.)

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expression of a pregerm marker, Lef1, at E15.5, when MG is
initiated by the WNT/β-catenin pathway (6). Lef1 was appreciably expressed in both WT and cKO mice, suggesting that BAF250a did not perturb the early initiation of MG germ (SI Appendix, Fig. S5B). MG morphology at E17.5 confirmed that knockout of BAF250a blocked the further growth of MG germ (Fig. 3 B and D). H&E staining during HF development in cKO mice showed a sharp reduction in HF density and length (Fig. 3 C and E), suggesting the loss of guard (tylotrich) HFs. The BAF250a cKO phenotypes thus overlap with findings in Eda-deficient Tabby or Edar-KO mice (27, 28).

**Tfg Up-Regulation Is Mediated by Eda-Induced RelB Activation.** To clarify how Eda signaling induces the formation of the RelB–BAF complex, we first checked the expression of the linker protein Tfg in MGs at the initiation stage. Consistent with data in Fig. 2 A–C, IHC staining showed a specific increase of Tfg in WT MG pregerms, with some accumulation in nucleus, but the increase was blocked in Tabby mice (Fig. 4 A). To further characterize the sequential molecular cascade, we used lentivirus encoding shRNA against NF-kB, Tfg, and BAF45d, respectively. Knockdown (KD) efficacy was validated as more than 80% of each target protein down-regulated (SI Appendix, Fig. S6A). Promoter luciferase activity assays in Kera308 cells showed that Eda indeed activated Tfg transcription through p50 and RelB but independently of RelA and BAF45d (Fig. 4B). Thus, Tfg, induced by Eda-mediated RelB activation, then bridges RelB and BAF45d to form the Eda-induced BAF complex.

**RelB, Tfg, and BAF45d All Promote MG Growth During Development.** To test for the indispensability of the three linker proteins in skin appendage development, we used the MG growth model previously described (25). A schematic shows the MG development from E15.5 to E17.5 (Fig. 4C). We then used lentivirus and transfected the shRNAs into cultured eyelids and examined MG morphology. Compared with control shRNA (LentiCon), RelB KD arrested MG growth, leaving pregerm morphology (Fig. 4D), the same phenotype seen in Tabby mice (25). Furthermore, the same repression of MG germ was observed with KD of Tfg or BAF45d (Fig. 4D). The defect was highly significant: MGs transfected with LentiCon showed an average length of 5.6 μm, whereas the length was reduced to 1.5 μm by RelB KD and to 0.6–0.8 μm by Tfg or BAF45d KD (Fig. 4E). As negative controls, neither RelA KD nor p52 KD inhibited MG growth (Fig. 4 D and E). As confirmation, in 4-d cultures, Lef1+ MG germs invaded deeply into dermis, but RelB KD inhibited further MG progression (Fig. 4F). We thus conclude that each of the trio of linked proteins is required for MG germ growth during early development.

**Genome-Wide Screen Identifies Gene Targets Regulated by Both Eda Signaling and BAF Complex.** To identify Eda targets regulated by both RelB and BAF, we profiled gene expression in HaCaT cells exposed to Eda-A1 CM. Three comparisons were carried out: (i) Edar−/− cells vs. Edar+; (ii) Edar+ cells with control siRNA vs. Edar+ cells with RelB siRNA; and (iii) Edar+ cells with control siRNA vs. Edar−/− cells with BRG1 siRNA. The efficacy of siRNAs was verified in HaCat cells (SI Appendix, Fig. S6B). A large group of 1,681 genes was significantly up-regulated by Eda/Edar signaling. Among these genes, 314 were also notably down-regulated by both RelB KD and BRG1 KD, making them stronger candidates for up-regulation by Eda-induced BAF (Fig. 5A). The 314 genes included the known Eda targets Ltb, Kremen2, and Mmp9 (29, 30). Consistent with recent findings that Eda may regulate the chemokine pathway and cell migration (31, 32), we also observed some gene targets implicated in chemokine/cell motility pathways (SI Appendix, Table S2). Interestingly, among some TFs, and genes in the TNF pathway were also observed (SI Appendix, Table S2), hinting at more probable components regulated by Eda-induced BAF complex. qPCR confirmed the expression levels of most genes selected as markedly up-regulated by Eda/Edar signaling. Among them, Ltb and Tnfrsf9 were the most strikingly augmented, by 4.1-fold and 11.1-fold, respectively (Fig. 5B). Again, these Eda targets identified in cell culture were also verified in eyelids from WT and Tabby mice (SI Appendix, Fig. S7G).

To further determine the involvement of BAF complex in the regulation of these Eda targets, we used T47D cells (BAF250a-deficient mammary gland cells) and SW13 cells (BRG1-deficient adrenal gland cells). qPCR showed that the mRNA levels of Ltb and Tnfrsf9 did not change in T47D or SW13 cells with Eda/Edar stimulation but were sharply stimulated in T47D cells transfected with BAF250a vector or in SW13 cells transfected with BRG1 WT vector but not BRG1 Mu (an ATPasive-mutant version) (Fig. 5C and SI Appendix, Fig. S6C). Consistent with the action of BAF250a and BRG1, KD of RelB, Tfg, or BAF45d all blocked Eda-induced up-regulation of Ltb and Tnfrsf9 (Fig. 5D).

**Eda-Induced BAF Components Bind to the Promoter Regions of Eda Targets.** To test whether RelB, Tfg, and BAF proteins bind to the promoter region of Eda targets, we analyzed the core promoter regions of Ltb and Tnfrsf9 across species using ConTra v2 software (SI Appendix, Fig. S7 A and D). Using CHIP-qPCR, we confirmed that RelB bound to the promoter region of Ltb and

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**Fig. 4.** Eda-induced Tfg recruits the BAF complex and promotes MG growth. (A) IHC shows the staining of Tfg (red) and K14 (green) in WT eyelids at E15.5. (B) Luciferase activity of the Tfg promoter in Kera308 cells with indicated treatment. Data are mean ± SEM for triplicate samples. **P < 0.01, Student’s t test.** (C) A schematic showing the development of an MG germ (red, Lef1) from E15.5 (eyelid open) to E17.5 (eyelid fused). (D) IHC staining of GFP (green) and K14 (red) in cultured WT eyelids transduced by lentivirus coding a GFP marker and scrambled shRNA (LentiCon) or shRNA against each indicated protein. Enlarged views of the areas marked by white rectangles are shown below each image. (E) Average length of MG germs in D. Error bars indicate mean ± SEM from at least 15 MGs of total three cultures. **P < 0.001, Student’s t test.** (F) IHC of K14 (green) and Lef1 (red) in LentiCon or RelB KD-treated eyelids cultured for 4 d. Arrows, MG germs; dotted lines separate MG germs and dermal cells. (Scale bars, 50 μm.)
cells and that Eda augmented this binding activity. As expected, RelB bound to the Ltb promoter only in WT skin tissue but not to the Tnfrsf9 promoter in mouse skin tissues, due to the lack of the NF-κB binding site in mouse (Fig. 5F).

Similar to RelB, the other Eda-induced BAF components BAF250α, Tfg, and BAF45d all bound to both Ltb and Tnfrsf9 promoters stimulated by Eda/Edar treatment in human HaCaT cells (Fig. 5E). Again, all three bound to the Ltb promoter only in WT but not in Tabby mice, and none bound to the Tnfrsf9 promoter in mouse tissue (Fig. 5F). The data thus suggest that, for gene regulation, Eda-activated RelB can recruit the BAF complex to gene loci containing NF-κB binding sites.

Eda-Induced BAF Complex Increases Chromatin Accessibility of Eda Targets. To test whether the Eda-triggered RelB–Tfg–BAF protein complex indeed modulates the chromatin structure of target genes, we evaluated the chromatin status of the distal control (Ctl) loci and proximal promoter loci of Ltb and Tnfrsf9 (SI Appendix, Fig. S7 B and E). In HaCaT cells treated with Eda/Edar, the DNase I sensitivity of both the Ltb and Tnfrsf9 loci, but not of each Ctl locus, was notably increased in Edar+ compared with Edar− cells, suggesting that a more "open" chromatin state is induced by Eda signaling (Fig. 6A and B). Next, we chose Ltb loci for the following study in several conditions. RelB KD, as expected, blocked the increase of DNase I sensitivity in Edar− cells (SI Appendix, Fig. S8A). We also checked the Ltb chromatin status in T47D and SW13 cells. Consistent with data in Fig. 5C, the Ltb region of chromatin remained “closed,” as indicated by low DNase I sensitivity, in the both T47D and SW13 cells, irrespective of Eda/Edar stimulus (SI Appendix, Fig. S8 B and D). Reexpression of BAF250α in T47D cells or of WT BRG1 (but not mutated BRG1) in T47D cells restored DNase I sensitivity of the Ltb promoter in response to Eda (SI Appendix, Fig. S8 C, E, and F). Thus, Eda-induced expression of Ltb requires both BAF250α and BRG1 for chromatin remodeling.

To further confirm the function of the Eda-induced BAF complex in gene regulation of Ltb, we performed CHIP-qPCR assays using antibodies against H3K27me3 (a marker of inactive genes) and H3K27ac (a marker of active genes). Indeed, anti-H3K27me3 bound to the Ltb promoter in HaCaT cells without Eda/Edar stimulation; by contrast, anti-H3K27ac bound to the Ltb promoter after Eda/Edar stimulus (SI Appendix, Fig. S8G). Using anti-H3K27ac CHIP-qPCR assays, we confirmed the requirement for BAF250α and BRG1 as well as for RelB, Tfg, and BAF45d for Eda-mediated Ltb transcription (Fig. 6C and SI Appendix, Fig. S8 H and I). Consistent with recent findings of the BAF-mediated polycomb repressive complex (PRC) eviction mechanism (33, 34), Ring1b and Ezh2 CHIP assays showed that the activation of two Eda targets, Ltb and Tnfrsf9, involved the removal of both the PRC1 and PRC2 complexes (Fig. 6 C and D).

Discussion

How cells and developing tissues select genes for expression in a precise spatiotemporal manner remains largely unknown. In the example of Eda signaling, Eda activates NF-κB for downstream gene expression (7). However, the powerful NF-κB can be
activated by many stimuli and may in turn regulate many hundreds of genes (8). One source of specificity in the Eda paradigm was determined early on. The action of Eda in initiating skin appendage growth is restricted to cells that express its receptor Edar and its adaptor protein Edaradd (6). This study focuses on the steps at which specific gene activation is implemented in the regulatory cascade. The steps delineate three features: selective activation of NF-κB subunits, transcriptional induction of a linker protein, and recruitment of a chromatin-remodeling complex to NF-κB-binding loci to facilitate gene transcription (SI Appendix, Fig. S9).

The expression pattern of NF-κB subunits in normal epidermis has been reported (35), but which subunits are involved in Eda signaling was unclear. Here, we find that Eda signaling slightly activated RelA and c-Rel, which may have specific transcription targets, but predominant activation of RelB was seen (Fig. 2). Consistently, downregulation of RelB arrested MG growth (Fig. 4D), producing a phenotype similar to that of the Eda-ablated Tabby mouse.

We started from unbiased protein purification and found that Eda induced an NF-κB-associated BAF complex, which comprises most, if not all, BAF components and in addition contains the p50/RelB dimer and a linker protein, Tgf (Fig. 1). Thus, tissue-specific Eda signaling may co-opt the chromatin remodeler to regulate organ development. In support of that possibility, the BAF component BAF250a is co-opted in developing skin appendages, and skin-specific KO of BAF250α led to the malformation of HF and arrested MG growth (Fig. 3); again, the BAF-mediated process downstream of Eda is consistent with the striking similarity of these phenotypes to those seen in Eda-deficient mice.

Using unbiased expression profiling, we identified several genes activated by Eda, RelB, and the BAF core ATPase BRG1 (Fig. 5A and B). Some known Eda targets, including Ltb, Kremen2, and Mmp9, as well as previously unidentified candidate targets, were activated. Interestingly, the most discriminated gene identified from our human cell model was Tnf receptor 3, which had never appeared as an Eda target in several mouse models. Tnf receptor 3 had been previously implicated in NF-κB signaling and function in T cell development (36), and its possible specific function in human skin appendage development remains to be investigated.

In the context of Eda- and BAF complex-coordinated gene regulation (SI Appendix, Fig. S9), there appear to be two successive phases of gene transcription: a first phase in which RelB and Tgf are up-regulated and a second phase in which the RelB recruits BAF complex through Tgf and activates subsequent NFκB targets. Our study has identified a critical protein, Tgf, which links the two phases, and we have further focused on BAF complex-mediated NFκB target activation. It remains unknown how other epigenetic mechanisms are dynamically controlled. However, in a larger context, our results are in line with converging thinking that reciprocal modulation between NFκB and chromatin organization may regulate gene expression at the right times and places (37). Given the critical roles of both NFκB and BAF complexes in cancer development (38, 39), our study may also provide a model for the mechanism of gene expression coordination by NFκB and BAF complexes in tumorigenesis.

Materials and Methods

All animal study protocols were approved by the National Institute on Aging Animal Care and Use Committee. The materials and methods used in this study, including cell culture, molecular and biochemical assays, histology, mice, and organotypic culture are described in detail in SI Appendix, SI Materials and Methods.

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