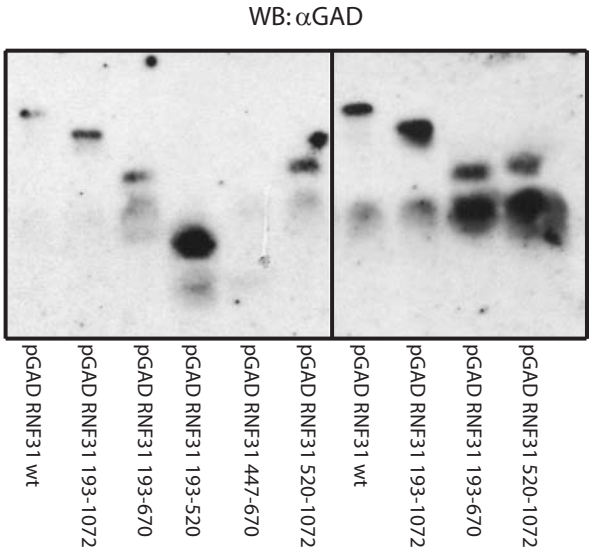
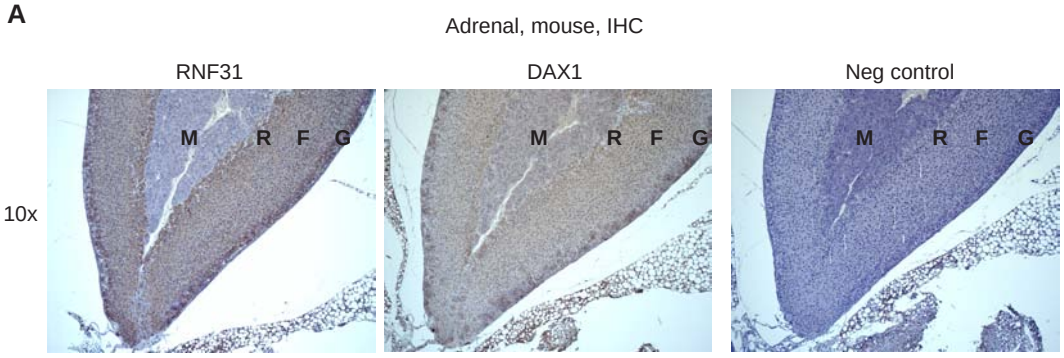
Ehrlund *et al*
Supplemental Figure 1

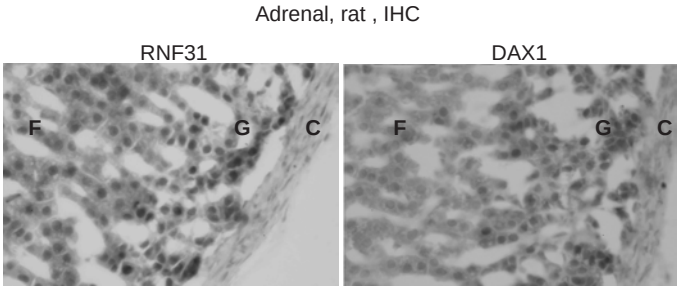


Ehrlund *et al*
Supplemental Figure 2

A



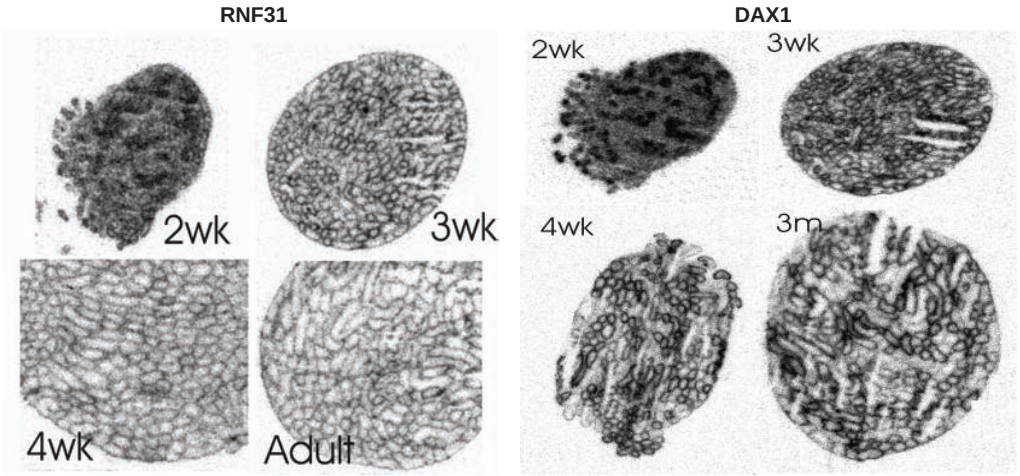
B



M:medulla; R:zona reticularis; F:zona fasciculata; G:zona glomerulosa; C:capsule

C

Testis development, mouse, ISH

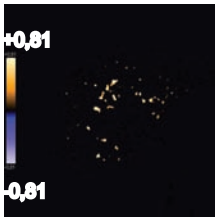


Ehrlund *et al*
Supplemental Figure 3

A

High expression

Whole Cell



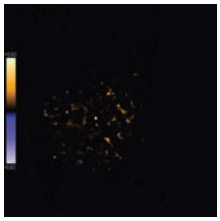
Pearsson correlation coeficient Rr=0,914 (-1<Rr<+1)

Intensity correlation quotient ICQ=0,222 -0,5<ICQ<+0,5

B

Low expression

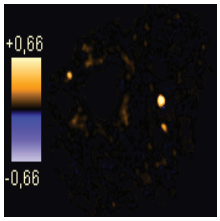
Whole cell



Pearson's correlation coeficient Rr=0,835 (-1<Rr<1)

Intensity correlation quotient ICQ=0,309 (-0,5<ICQ<0,5)

Nucleus

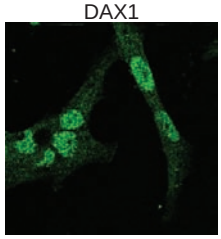


Pearson's correlation coeficient Rr=0,768 (-1<Rr<1)

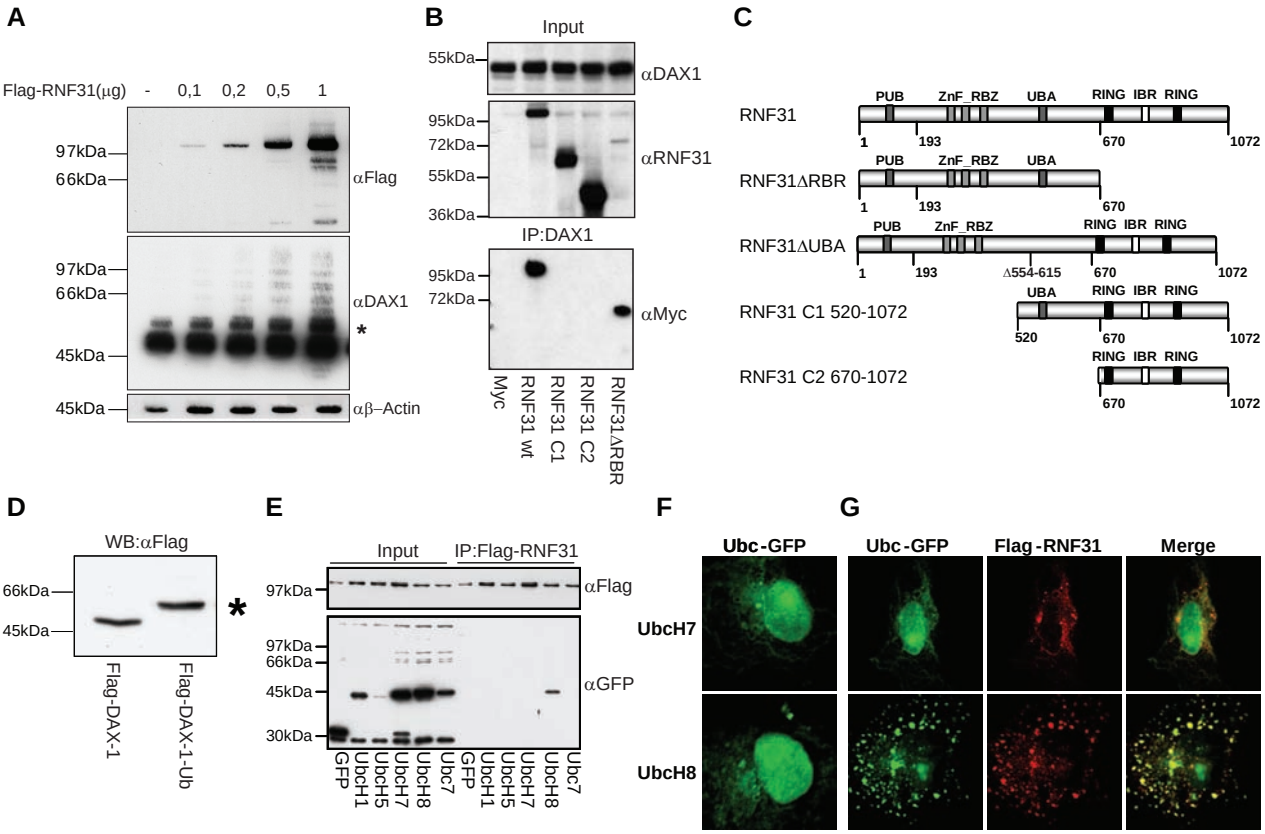
Intensity correlation quotient ICQ=0,280 (-0,5<ICQ<0,5)

C

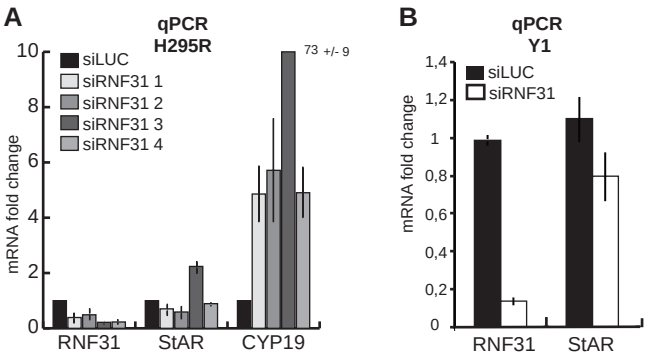
H295R
DAX1



Ehrlund *et al*
Supplemental Figure 4



Ehrlund *et al*
Supplemental Figure 5



SUPPLEMENTAL DATA

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Expression of GAD-RNF31 variants in yeast. Control western blot showing the expression of the different pGAD-RNF31 constructs used in yeast two-hybrid assay (Figure 1). Whole cell extracts from exponentially grown yeast liquid cultures were analyzed using an antibody recognizing the GAL4 activation domain (GAD). Results from two independent experiments are shown.

Supplemental Figure 2. Coexpression of RNF31 and DAX-1 in rodent adrenal and testis. (A) Immunohistochemistry of paraffin embedded mouse adrenal gland at 10x magnification showing stain with RNF31 and DAX-1 antibodies and a negative control stained without primary antibody. (B) Immunohistochemistry using DAX-1 and RNF31 antibodies on frozen rat adrenal cortex showing the capsule, zona glomerulosa and zona fasciculata. (C) *In situ* hybridisation of developing mouse testis using DAX-1 and RNF31 oligonucleotide probes.

Supplemental Figure 3. Quantitative analysis of colocalization of RNF31 and DAX-1 in COS-7 cells. Shown are representative images of (A) cells with relatively high expression levels or (B) cells with relatively low expression levels. To quantify the level of co-localization displayed by RNF31 and DAX-1 in single cells, we used Image Correlation Analysis Tool for Image J, where Rr = 1 indicates perfect colocalization (Qi Li, A.L. et al. J Neuroscience 24, 4070-4081, 2004). Additionally, the Intensity Correlation Quotient (ICQ), which is a measure of whether the fluorescent intensities of two images vary in synchrony with ICQ = 0.5 indicating perfect synchrony. (C) H295R cells stained with the mouse monoclonal DAX-1 2F4 antibody to reveal localization of endogenous DAX-1.

Supplemental Figure 4. Characterization of RNF31 interactions and ubiquination. (A) Western blots from whole cell extracts from cells overexpressing DAX-1 and increasing levels of Flag-RNF31. (*) indicates the position of presumably monoubiquitinated DAX-1. (B) Extracts from COS-7 cells transfected with Flag-DAX1 and indicated Myc-RNF31 derivatives were subjected to immunoprecipitation (IP) using Flag rabbit polyclonal antibody. IP:ed proteins were analysed by western blot with Myc mouse monoclonal antibody for co-precipitated Myc-RNF31 derivatives. (C) Schematic representation of RNF31 constructs. (D) Western blot showing the mobility difference between DAX-1 and a DAX-1-ubiquitin fusion protein. (E) Cell extracts with Flag-RNF31 and indicated GFP-tagged E2 ubiquitin conjugating enzymes were subjected to Flag IP. Input and precipitates were analysed with GFP antibody for the presence of E2s. Note that all E2s except UbcH5 express properly. (F) Localization of GFP-UbcH7 and GFP-UbcH8 expressed in the absence of Flag-RNF31 in COS-7 cells. (G) Colocalization (yellow) analysis of Flag-RNF31 (red) and GFP-UbcH7 or GFP-UbcH8 (green) in COS-7 cells.

Supplemental Figure 5. Specificity of the siRNF31 knockdown effects. (A) qPCR analysis of mRNA from H295R cells treated with the four individual siRNF31 oligos included in the pool of oligos used in Figures 5-7. Data are presented as fold difference from control siRNA (siLUC). Representative data with standard deviations are shown. (B) qPCR analysis of mRNA from mouse adrenal Y1 cells treated with siRNA targeting mouse RNF31. Data are presented as fold difference from control siRNA (siLUC). Representative data with standard deviations are shown.

SUPPLEMENTAL MATERIAL AND METHODS

Rat adrenal gland immunohistochemistry

Tissues were fixed in 4 % paraformaldehyde, cryoprotected with 20% sucrose in PBS, frozen with carbon dioxide, and 6 µm sections were cut with a Microm HM 560 cryostat and thaw-mounted onto Polysine glass slides (Menzel, Braunschweig, Germany). The sections were incubated for 12-24 h at 4ºC with rabbit antiserum to RNF31 rabbit polyclonal(1:200 in PBS containing 1% BSA; Abcam) or mouse monoclonal antiserum to Dax-1 mouse monoclonal (1:200) antibodies followed by biotinylated goat anti-rabbit (Vector Labs. Burlingame, CA, USA) or sheep anti-mouse (Amersham International, Buckingshamshire, UK) diluted 1:300 for 30 min. Subsequently, the sections were incubated with ABC-complex for 30 min and diaminobenzidine was used as a chromogen chromogen to visualize immunoreactivities. The sections were dehydrated, mounted in Entellan (Merck, Darmstadt, Germany), and examined under the Nikon FXA light microscope (Nikon,Tokyo, Japan). Images were processed using Corel Draw software (Corel Corporation Ltd., Ontario, Canada).