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REPRESSION OF SUBFAMILY V ORPHAN NUCLEAR RECEPTORS

VIA SUMO-MODIFICATION AND THE DEAD-BOX PROTEIN DP103

by

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DISSERTATION

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DEDICATION & ACKNOWLEDGMENTS

For Nadia "Noor" Barhoumeh Lee, Light of my Heart

And for Mum, Dad, and the Lee family

This work would not have borne fruit but for the creative confluence of many positive energies. I wish to express my deepest gratitude to God, the ultimate source of all creativity, intelligence and wisdom, within whose gaze my lifetime is but the blink of an eye. To dearest Mum & Dad, who gave me the gift of education when I was too young to know anything. To my beloved wife Nadia, who grounded my efforts on the home stretch and lighted my way with her undying love. To my stepsons, Daniel and Jonathan, for their terrific support. To Dr Peter Hwang, who believed in me as a scientist and facilitated my way to graduate school. To Dr Hooi Shing Chuan and the Staff of the Department of Physiology, National University of Singapore, for their faith in me and for shouldering my load these many years. To my thesis advisor and mentor, Dr Holly Ingraham, who taught me not just the craft, but the art of science and who continues to inspire me with her unique blend of scientific passion and humanity. To the exceptional crew of the Ingraham Laboratory, past and present, for scientific collaboration, invaluable help, support, companionship and laughter through the years, with special thanks to my chief collaborators Lioudmila (Lucy) and Phu. To my thesis chairperson, Synthia Mellon for feisty enthusiasm and encouragement. To my thesis committee member Dr David Pearce for insightful criticism and steadfast support. To Dr Didier Stainier and Dr John Rubenstein for help in the days of my qualifying exams and beyond. Special thanks to

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ABSTRACT

REPRESSION OF SUBFAMILY V ORPHAN NUCLEAR RECEPTORS

VIA SUMO-MODIFICATION AND THE DEAD-BOX PROTEIN DP103

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MARTIN BENG-HUAT LEE

Subfamily V nuclear receptors are transcription factors with a fascinating biology that are represented in both invertebrate and vertebrate species. Members of Subfamily V include Ftz-F1 in *Drosophila*, and Steroidogenic Factor 1 (SF-1) and Liver Receptor Homolog 1 (LRH-1) in mammals. Collectively, Subfamily V receptors play key roles in processes as diverse as larval segmentation in *Drosophila*, and sexual differentiation, neuronal maturation, stress responses and bile acid homeostasis in mammals. From this plethora of biological functions, it is clear that specific mechanisms must exist to regulate individual receptors. Although much has been done, we still lack a full understanding of how these receptors are regulated. We know that these so-called orphan receptors are active in the apparent absence of ligand, and that cofactors modulate this activity. Furthermore, post-translational modifications including phophorylation and acetylation provide an additional layer of regulation.

The main body of work in this thesis now extends our view of receptor regulation by describing post-translational SUMO-modification as a potent means of repression for Subfamily V receptors. The mechanism of SUMO-mediated repression of SF-1 was found to involve a member of the DEAD-box protein family, DP103, thus revealing an

exciting link between these ATPase/RNA helicases and transcriptional repression. Lastly, we describe dramatic subnuclear relocalization of SF-1 under conditions that strongly promote sumoylation. The challenge ahead is to analyze the relationships between receptor sumoylation, relocalization and DEAD-box proteins within the context of transcriptional repression, so as to better understand how repression subserves the biology of this subfamily of nuclear receptors.

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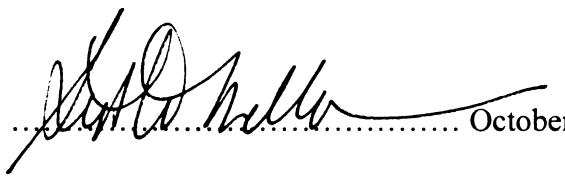

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OVERVIEW

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OVERVIEW

This overview presents the background to transcriptional regulation of Subfamily V nuclear receptors, with special emphasis on the issues that are addressed by my data. Section I introduces the biology of Subfamily V nuclear receptors. Section II describes SUMO-modification or sumoylation with emphasis on nuclear receptors and the effects of sumoylation on transcriptional activity. Section III introduces DEAD box proteins and highlights current concepts about their function that will be important in building a model of SUMO-mediated repression of Subfamily V nuclear receptors. Additional background material not directly relevant to the main thrust of the thesis may be found in the introductions of Chapter 2 and Chapter 3 and in several reviews cited therein.

ABBREVIATIONS

AF2:	Activation Function 2
AFH1:	Activation Function in Helix 1
DBD:	DNA Binding Domain
GRTH:	Gonadotropin-Regulated Testicular RNA Helicase
HDAC:	Histone Deacetylases
LBD:	Ligand Binding Domain
LRH-1:	Liver Receptor Homolog 1
NaBT:	Sodium Butyrate
NEM:	N-ethylmaleimide
NR	Nuclear Receptor
PIAS:	Protein Inhibitor of Activated Stats

PML-NBs:	PML Nuclear Bodies
RNP	Ribonucleoprotein
RTA:	Repressor of Tamoxifen Transcriptional Activity
SAE:	SUMO Activating Enzyme
SENP:	Sentrin-specific Protease
SF-1:	Steroidogenic Factor 1
SHARP:	SMRT/HDAC1 Associated Repressor Protein
SUMO:	Small Ubiquitin-like Modifier
TSA:	Trichostatin A
VMH/VMN	Ventromedial Hypothalamic Nucleus

I. Biology of Subfamily V nuclear receptors

The nuclear receptor (NR) superfamily of transcription factors consists of both ligand-dependent and so-called "orphan" receptors for which ligands have yet to be assigned (reviewed in (Mangelsdorf et al., 1995)). Members of the NR superfamily are divided into several subfamilies largely on the basis of sequence homology. Subfamily V consists of orphan receptors represented in several invertebrate and vertebrate species. *Drosophila* Ftz-F1 is the founding member of this subfamily, and interacts directly with the pair-rule gene product of *Ftz* to control parasegmention at early embryonic stages (Lavorgna et al., 1991). This thesis focuses on two mammalian orthologs of Ftz-F1, Steroidogenic Factor 1 (SF-1) and Liver Receptor Homolog 1 (LRH-1), that contain a highly conserved DNA binding domain (DBD), a large hinge domain and a ligand binding domain (LBD).

SF-1 and LRH-1 are critical in tissue development and organogenesis (Achermann et al., 2002; Ingraham et al., 1994; Luo et al., 1994; Pare et al., 2004; Tran et al., 2003). For example, during development SF-1 is essential for male differentiation, adrenogonadal morphogenesis, and development of the ventromedial hypothalamic nucleus (VMH) and pituitary gonadotropes. SF-1 null mice lack gonads and show male to female sex reversal regardless of sex chromosome makeup. SF-1 expression in the developing bipotential gonad becomes sexually dimorphic at E13.5, with SF-1 protein levels remaining elevated to drive male-specific genes in the developing testis, while in the developing ovary, SF-1 expression falls to low levels, suggesting that residual ovarian SF-1 may be in an inactive or repressed state and thus unable to activate the male developmental program.

In the postnatal adrenal, SF-1 regulates several genes required in steroid biosynthesis (Parker and Schimmer, 1997) and SF-1 is essential in both mice and humans for a normal stress response (Achermann et al., 1999). SF-1 null mice lack adrenals and die soon after birth from adrenal insufficiency, while SF-1 heterozygous mice have smaller adrenals with thinner adrenal cortices, eccentrically located adrenal medullae, and a blunted cortisol response to stress (Bland et al., 2000). This adrenal phenotype illustrates dose-dependency for SF-1 in adrenal development and function. Intriguingly, SF-1 protein levels are seemingly adequate in the heterozygous mice, but clearly the amount of active SF-1 protein is insufficient to overcome defects in adrenal morphogenesis and function, again suggesting that the expressed SF-1 protein is in an inactive state (Bland et al., 2004; Bland et al., 2000). In humans, SF-1 haploinsufficiency is associated with adrenal crisis, often presenting in infancy (Achermann et al., 2001). In the adult, SF-1 also regulates a large number of genes involved in steroid biosynthesis and endocrine signaling (Parker and Schimmer, 1997). In summary, SF-1 regulates the hormonal milieu that directs sexual differentiation, adrenal development and adrenal stress responses.

In contrast to the adrenals, loss of SF-1 function in the VMH results in defective morphogenesis of this nucleus in SF-1 null mice, but apparently normal development in heterozygous mice. This suggests that there may be differences in target organ susceptibility to SF-1 dose, or that the reduced amount of SF-1 protein in the VMH of SF-1 heterozygotes is comprised of a higher proportion of transcriptionally active SF-1. Again, one wonders what proportion of expressed SF-1 is active in any given target tissue, and how this proportion might be regulated.

On a different note, it was not clear at the outset how VMH development was derailed in SF-1 homozygous mice, and this question is addressed in Chapter 3 by work published in collaboration with the first author, Dr Phu V Tran, a postdoctoral fellow in the Ingraham Laboratory (Tran et al., 2003). This work examined the potential roles of proliferation, migration, survival, aggregation and differentiation of VMH precursors during development in wild type and SF-1 mutant mice.

Despite very similar LBD structures, SF-1's close cousin LRH-1 has a markedly different tissue distribution and biology. LRH-1 acts far earlier in development than SF-1, as evidenced by the embryonic lethality observed in LRH-1 null embryos (Pare et al., 2004). In vitro and in vivo analyses have implicated LRH-1 in bile acid homeostasis (Goodwin et al., 2000; Lu et al., 2000), and in tissue conversion of androgens to estrogen by regulating aromatase gene expression (Clyne et al., 2004; Hinshelwood et al., 2003).

II Regulation of Subfamily V receptor activity

Clearly, Subfamily V receptors play key roles in developmental and hormonal processes in which they regulate target genes in a highly orchestrated and specific fashion. It is also clear that these receptors are active or repressed in specific contexts in the whole organism. Both SF-1 and LRH-1 are active as monomers on response elements composed of an estrogen receptor half-site that contains the core consensus sequence AGGTCA. To date, no synthetic or natural ligands have emerged for Subfamily V receptors despite the demonstration of a large hydrophobic pocket in the high-resolution crystal structure of the LBD of LRH-1 (Sablin et al., 2003). As such, the question of how

subfamily V receptors are regulated is unclear. In many cellular contexts, this subclass of receptors is active and presumably recruits coactivators in a ligand-independent manner. Cellular studies revealed that SF-1 and LRH-1-mediated gene activation depends on an activation function in helix 1 (AFH1) of the LBD and a classic AF2 in the very C-terminal region of the LBD (Desclozeaux et al., 2002; Ito et al., 2000).

For SF-1 and other NRs, several mechanisms of transcriptional activation have been described, including co-activators such as SRC1 and GRIP1, and posttranslational modification by phosphorylation (Hammer and Ingraham, 1999; Rangwala et al., 2003; Wang et al., 2003). Phosphorylation of SF-1 in vivo occurs at Ser203 and is proposed to increase receptor activity by stabilization of the LBD and enhanced cofactor recruitment (Desclozeaux et al., 2002; Fowkes et al., 2003; Hammer et al., 1999). Although much is understood about SF-1 activation, little is known about how SF-1 might be repressed. In dosage sensitive sex reversal, it is thought that duplication of the *DAX-1* gene increases the dose of the co-repressor Dax1, which represses SF-1 via AF2 (Nachtigal et al., 1998).

In both SF-1 and LRH-1 a "repression domain" was identified in the hinge region (Ou et al., 2001; Xu et al., 2003), and in SF-1 this domain (aa193-201) resides immediately proximal to the major MAP-kinase phosphorylation site at Ser203. The SF-1 domain was able to autonomously confer repression when expressed *in cis* to the LBD of estrogen receptor (ER). Interestingly, the repression domain of SF-1 was reported to interact with a known repressor of SF-1, the DEAD-box RNA helicase DP103 (Ddx20, Gemin-3) (Yan et al., 2003), although the precise mechanism of SF-1 repression by DP103 was not

elucidated. To add further complexity, mutational analysis of the repression domain showed that the amino acid substitutions K194R and S195A in SF-1, and K289R in LRH-1, resulted in significant derepression of transcriptional activity. At the time however, it was not clear how these amino acid residues facilitated repression but we now know from the work in this thesis that Lys194 in SF-1 and Lys289 in LRH-1 mediate repression by serving as attachment points for post-translational modification by the ubiquitin-like protein, SUMO.

III SUMO-modification

SUMO-modification or sumoylation is a post-translational modification where the Small Ubiquitin-like Modifier (SUMO) is reversibly attached to a protein substrate (Melchior, 2000; Muller et al., 2004). Of the four known isoforms of SUMO (SUMO1 through SUMO4), SUMO1 has been most extensively studied. The three dimensional structures of SUMO1 and ubiquitin are remarkably similar but carry markedly different surface charge, thereby offering distinct interfaces for recruitment of proteins. So far, there have been no published reports of the structure of a sumoylated substrate. Sumoylation occurs at canonical motifs of ψKXE, where ψ is a hydrophobic amino acid and K is the acceptor lysine for covalent attachment of SUMO. SF-1, LRH-1, and all other members of subfamily V receptors are predicted to be sumoylated given the presence of a conserved IKSE or I/VKQE site in the hinge region. Numerous proteins have been shown to be sumoylated, including PPARgamma and all steroid receptors except for ER. In contrast, COUP-TF-I was shown to interact with the E2 sumoylation enzyme Ubc9 (Kobayashi et al., 2004), and Nurr1 with the E3 SUMO ligase PIASy (Galleguillos et al., 2004), but

sumoylation of these receptors was not demonstrated. Unlike ubiquitination which mainly facilitates proteasomal degradation, the effects of sumoylation are diverse and substrate specific, and may include changes in transcriptional activity, protein recruitment, phosphorylation, and subnuclear relocalization. One theme which has recently emerged is that sumoylation of several transcription factors, such as Elk-1, Lef1, the liganded receptor PPARgamma, and nearly all steroid nuclear receptors, results in transcriptional repression (Chauchereau et al., 2003; Floyd and Stephens, 2004; Holmstrom et al., 2003; Ohshima et al., 2004; Poukka et al., 2000; Sachdev et al., 2001; Tallec et al., 2003; Yang et al., 2003). The mechanism of SUMO-mediated repression is not fully understood, but work by several groups has implicated histone deacetylation (Shiio and Eisenman, 2003; Yang and Sharrocks, 2004) and subnuclear relocalization (Dobreva et al., 2003; Sachdev et al., 2001).

Much of the data on the functional effect of sumoylation has been obtained from overexpression studies in cellular systems using loss-of-function approaches. A common approach is to mutate the obligatory Lys residue to Arg, which retains the charge but precludes attachment of all SUMO isoforms, or less commonly from Lys to Ala where the charge is lost. Other loss-of-function approaches include overexpressing SUMO isopeptidases to desumoylate cellular proteins, use of dominant negative sumoylation enzymes such as catalytically dead Ubc9, and finally RNAi take-down of these enzymes. All these approaches are non-specific, and may cause secondary effects by perturbing sumoylation of interacting proteins or unrelated proteins in the cell. For example, the cofactors GRIP1 and SRC1, and many of their nuclear receptor targets are sumoylated

(Chauchereau et al., 2003; Kotaja et al., 2002), thus confounding the interpretation of studies using non-specific loss-of-function approaches. Existing gain-of-function approaches have been limited to overexpressing SUMO, which increases sumoylation non-specifically, and to expressing SUMO *in cis* to the protein substrate, which results in a different composite structure than when SUMO is normally attached. Finally, studies of sumoylation in the whole organism have been hampered by the lack of antibodies specific for sumoylated substrates. Genetic approaches to investigate sumoylation of specific proteins have been published for yeast and *Drosophila* (Chan et al., 2002; Shih et al., 2002; Smith et al., 2004; Steffan et al., 2004), but so far not for mice .

The biochemistry of protein SUMO modification is analogous to ubiquitination, involving a three-step ATP-dependent reaction. Processed SUMO protein is loaded onto the heterodimeric E1 enzyme (SAE1/SAE2) and transferred from E1 to the sole E2 enzyme Ubc9, which then mediates SUMO conjugation to the protein substrate with aid from E3-SUMO ligases. Protein Inhibitor of Activated Stats (PIAS) proteins including PIASxα, PIASxβ, PIAS1, PIAS3 and PIASγ comprise the largest of three identified E3 SUMO ligase classes; the remaining two E3 classes are represented by Polycomb protein 2 and RanBP2. Sumoylation of proteins is dynamic and easily reversed by SUMO isopeptidases or Sentrin/SUMO-specific proteases (SENP/SUSP), which cleave SUMO from its substrate. It is not clear if sumoylation or desumoylation are regulated or constitutive processes. The E1 and E2 enzymes are ubiquitous, while several PIAS E3 ligases have specific tissue distributions and could theoretically regulate sumoylation in concert with substrate-recognition factors. There is evidence that a variety of cellular

stresses increase posttranslational modification of heat shock factor 1 with SUMO2 and SUMO3, but not with SUMO1, but the mechanism of such regulation is not clear (Bohren et al., 2004; Goodson et al., 2001; Hietakangas et al., 2003; Hilgarth et al., 2003; Hong et al., 2001). Likewise, SUMO isopeptidases have not been shown to regulate the level of sumoylation of specific substrates in vivo.

IV DEAD-box proteins

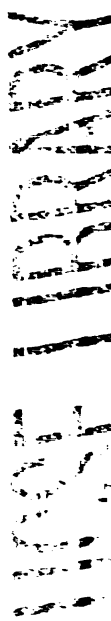
The DExD/H-box protein family is comprised of DEAD-box proteins and their relatives, the DExD, DEAH and DExH-box proteins (Linder et al., 2001). DEAD-box and DEAH-box proteins have been ascribed a numerical Ddx and Dhx nomenclature respectively (Abdelhaleem et al., 2003). Members of the DExD/H-box protein family were originally identified as components of the spliceosome and have been implicated in all aspects of RNA metabolism, including ribosome assembly and translation initiation (Tanner and Linder, 2001). It was originally thought that DEAD-box proteins function to unwind dsRNA during spliceosome rearrangements, hence these proteins were described as RNA helicases. However, recent studies suggest that these proteins also facilitate association and dissociation of nucleic acids and proteins within nucleoprotein complexes, thus acting as RNPases (Linder et al., 2001). Furthermore, recent studies suggest that DEAD-box proteins may function to repress transcription factors, including ER, Egr1-4 and Ets (Gillian and Svaren, 2003; Klappacher et al., 2002; Rajendran et al., 2003).

DExD/H proteins possess a conserved N-terminal helicase domain-containing region and a non-conserved C-terminal region. The N-terminal helicase domain comprises seven to

eight conserved motifs, which includes the signature DExD/H motif. Studies of motif mutants revealed that individual motifs mediate specific functions such as ATP binding, ATP hydrolysis and nucleic acid unwinding. The unique C-terminal domain is thought to confer protein specific functions and recent studies on the DEAD-box proteins DP103 (Ddx20, Gemin-3) and DP97 have shown that the C-terminal region is sufficient for interaction with and repression of several transcription factors (Klappacher et al., 2002; Rajendran et al., 2003; Yan et al., 2003). Specifically, the C-terminal region of DP103 repressed SF-1, and interacted with the repressor METS/PE-1 to repress Ets, while the C-terminal region of DP97 was shown to repress ERα. In these studies, the N-terminal helicase domain was dispensable, and its role in repression, if any, remains enigmatic. For Ets, the mechanism of DP103-mediated repression was found to involve interactions with NCoR, Sin3A, HDAC2 and HDAC5, while the mechanisms for repression of ERα and SF-1 were not investigated. In particular, no connection was established between repression by DEAD-box proteins and that observed upon sumoylation.

DEAD-box proteins have diverse tissue distributions, and several members have been implicated in biological processes and diseases. Of relevance to SF-1, expression of DP103 in the somatic cells of mouse testes parallels that of SF-1, although co-expression of DP103 and SF-1 was not demonstrated (Ou et al., 2001). DP103 is also expressed in adrenals and ovaries, both SF-1 target organs. Similarly, Gonadotropin-Regulated Testicular RNA Helicase (GRTH, Ddx25) is highly expressed in the testes and in male germ cells, where it plays a role in spermatogenesis and hormone synthesis (Dufau et al., 2001). To date, a role for DP103 and GRTH in the repression of SF-1 in these tissues has

yet to be demonstrated. DP103 also forms a complex with SMN protein, the spinal muscular atrophy (SMA) gene product (Charroux et al., 1999), in the cytoplasm and in nuclear bodies called gems (from the word Gemini or twin for their resemblance to Cajal bodies). In selected SMA patients, mutations such as Y272C or deletion of exon 7 of SMN reduced the interaction of SMN with DP103. The contribution of DP103 towards the pathogenesis of SMA is still under investigation and it remains to be seen what role DP103 might play in adrenal insufficiency in SF-1 heterozygous mice, and in SF-1 biology.



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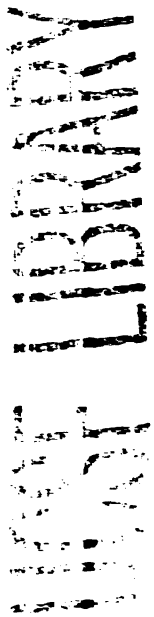
CHAPTER 2

THE DEAD-BOX PROTEIN DP103 (DDX20, GEMIN-3) REPRESSES ORPHAN NUCLEAR RECEPTORS VIA SUMO-MODIFICATION



THE DEAD-BOX PROTEIN DP103 (DDX20, GEMIN-3) REPRESSES ORPHAN NUCLEAR RECEPTORS VIA SUMO-MODIFICATION

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Short title: SUMO Repression of Orphan Receptors by a DEAD-Box Protein

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Abstract

Structural analysis of subfamily V orphan nuclear receptors suggests that ligand-independent mechanisms must regulate this subclass of receptors. Here we report that members of subfamily V, including steroidogenic factor 1 (SF-1) and liver receptor homolog (LRH-1) are repressed via posttranslational SUMO modification at conserved lysines within the hinge domain. Indeed, mutating these lysines or adding the SUMO isopeptidase SENP1 dramatically increased receptor activity. Amongst the known PIAS E3 SUMO ligases, PIASy and PIASxa strongly promoted SF-1 sumoylation. The mechanism by which SUMO conjugation attenuates SF-1 activity was found to be largely HDAC-independent and unaffected by the AF2-corepressor, Dax-1. Instead, our data suggest that SUMO-mediated repression involves direct interaction of the DEAD-box protein DP103 with sumoylated SF-1. Furthermore, DP103 promoted PIAS-dependent receptor sumoylation and facilitated PIASy-dependent receptor relocalization to discrete nuclear bodies. Collectively, these results lead us to propose that ATPases/RNA helicases are directly coupled to transcriptional repression by protein sumoylation.

Introduction

Steroidogenic factor 1 (SF-1) and liver receptor homolog 1 (LRH-1) are two closely related transcription factors belonging to the nuclear receptor subfamily V that contain a highly conserved DNA binding domain (DBD), a large hinge domain and a ligand binding domain (LBD, Fig 1A). *Drosophila* Ftz-F1 is the founding member of this subfamily, and interacts directly with the pair-rule gene product of *Ftz* to control parasegmention at early embryonic stages (Lavorgna et al., 1991). The mammalian

orthologs SF-1 and LRH-1 are also critical in tissue development and organogenesis (Achermann et al., 2002; Ingraham et al., 1994; Luo et al., 1994; Pare et al., 2004). For example, during development SF-1 is essential for male differentiation, adrenogonadal morphogenesis, and terminal differentiation of the ventromedial hypothalamus. In the adult, SF-1 also regulates a large number of genes involved in steroid biosynthesis and endocrine signaling (Parker and Schimmer, 1997; Tran et al., 2003). SF-1 null mice die at birth from adrenal insufficiency and analyses of SF-1 heterozygous mice suggest that despite seemingly adequate SF-1 protein levels, the amount of active SF-1 protein in these mice is insufficient to overcome defects in adrenal morphogenesis (Bland et al., 2004; Bland et al., 2000). In humans, SF-1 haploinsufficiency is associated with severe adrenal disease (Achermann et al., 2001). LRH-1 acts far earlier in development than SF-1, as evidenced by the embryonic lethality observed in LRH-1 null embryos (Pare et al., 2004). In vitro and in vivo analyses have implicated LRH-1 in bile acid homeostasis (Goodwin et al., 2000; Lu et al., 2000), and in tissue conversion of androgens to estrogen by regulating aromatase gene expression (Clyne et al., 2004; Hinshelwood et al., 2003).

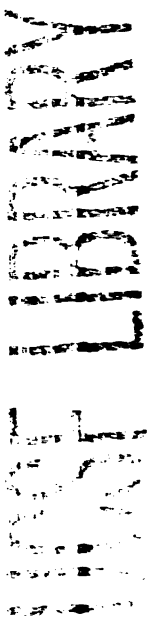
Despite the fact that the high-resolution crystal structure of LRH-1 revealed a large hydrophobic pocket within the LBD (Sablin et al., 2003), no synthetic or natural ligands have yet to emerge for this subclass of receptors. As such, the question of how subfamily V receptors are regulated is unclear. In many cellular contexts, this subclass of receptors is active and presumably recruits coactivators in a ligand-independent manner. Cellular studies revealed that SF-1 and LRH-1-mediated gene activation depends on an activation function in helix 1 (AFH1) of the LBD and a classic AF2 in the very C-terminal region of

the LBD (Desclozeaux et al., 2002; Ito et al., 2000). In both SF-1 and LRH-1 a "repression domain" has been identified in the hinge region (Ou et al., 2001; Xu et al., 2003). For SF-1, this domain is reported to interact with the DEAD-box RNA helicase DP103, (Ddx20, Gemin-3) (Yan et al., 2003b), although the precise mechanism of SF-1 repression by DP103 is unknown.

Phosphorylation and sumoylation are posttranslational modifications known to modulate both ligand-dependent and ligand-independent receptors (Hammer and Ingraham, 1999; Rangwala et al., 2003; Wang et al., 2003). Phosphorylation of SF-1 in vivo occurs at Ser203 and is proposed to increase receptor activity by stabilization of the LBD and enhanced cofactor recruitment (Desclozeaux et al., 2002; Fowkes et al., 2003; Hammer et al., 1999). On the other hand, sumoylation of several transcription factors, such as Elk-1, Lef1, and nearly all steroid nuclear receptors, results in their transcriptional repression (Chauchereau et al., 2003; Holmstrom et al., 2003; Poukka et al., 2000; Sachdev et al., 2001; Tallec et al., 2003; Yang et al., 2003). Sumoylation occurs at canonical motifs of ψKXE, where ψ is a hydrophobic amino acid and K is the acceptor lysine for covalent attachment of the Small Ubiquitin-like Modifier (SUMO) (Melchior, 2000; Muller et al., 2004). SF-1, LRH-1, and all other members of subfamily V receptors are predicted to be sumoylated given the presence of a conserved IKSE or I/VKQE site in the hinge region.

The biochemistry of protein SUMO modification is analogous to ubiquitination, involving a three-step ATP-dependent reaction. Processed SUMO protein is loaded onto the heterodimeric E1 enzyme (SAE1/SAE2) and transferred from E1 to the sole E2

enzyme Ubc9, which then mediates SUMO conjugation to the protein substrate with aid from E3-SUMO ligases. Protein Inhibitor of Activated Stats (PIAS) proteins including PIASxa, PIASxb, PIAS1, PIAS3 and PIASy comprise the largest of three identified E3 SUMO ligase classes; the remaining two E3 classes are represented by Polycomb protein 2 and RanBP2. Sumoylation of proteins is dynamic and easily reversed by cellular isopeptidases or Sentrin/SUMO-specific proteases (SENP/SUSP), which cleave SUMO from its substrate. However unlike ubiquitin conjugation, which primarily facilitates protein degradation, SUMO modification of transcription factors often results in transcriptional repression. Recent literature suggests that two modes of repression by protein sumoylation involve direct recruitment of histone deacetylases (HDACs) (Shiio and Eisenman, 2003; Yang and Sharrocks, 2004) or a relocalization to PML nuclear bodies (Dobreva et al., 2003; Sachdev et al., 2001); both mechanisms would result in transcriptional repression.



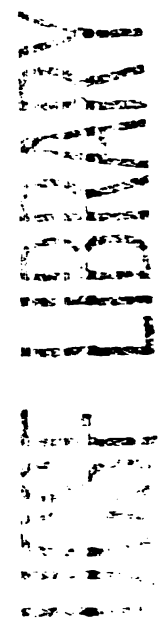
Here we identify sumoylation as an important posttranslational regulatory mechanism for dampening the activity of subfamily V nuclear receptors. Potential mechanisms that lead to receptor repression by sumoylation were investigated and found to involve a functional interaction with the DEAD-box RNA helicase DP103, as well as relocalization to distinct nuclear bodies.

Materials & Methods

Plasmids

Full length mSUMO1 (101 aa) was PCR-amplified from embryonic mouse hypothalamic-enriched cDNA using primers: 5'-CTCGAGATGT C T G A C C A G G A G G C A A A A - 3 ', 5 ' - TCTAGACTAAACCGTCGAGTGACCCCC-3', TA-cloned into pCRII (Invitrogen), and subcloned into Xho1-Xba1 pCI-neo. Processed His_6-hSUMO1 (97 aa) was subcloned from His_6-hSUMO1-pcDNA3 (F. Poulat) into pGEX4T1 at BamH1. HA-tagged mSENP1 was PCR-cloned from mouse hypothalamic-enriched cDNA using primers: 5'-CCGGAATTCATGTACCCATACGACGTACCAGATTACGCTAGCTTGGATGACA CAGCTGATGGGGTG-3', and 5'-ACCTCTAGAGTCGACTCACAAGAGCTTCCGGT GGAG-3' into EcoR1-Sal1 of pCI-neo. HA-tagged mSF1 in pCI-neo, HA SF-1 S203A and GFP-HA-SF-1 pCMV were described previously (Desclozeaux et al., 2002). K119R, K194R, and 2KR mutants of HA-SF-1-pCI-neo and GFP-HA-SF-1 were created by PCR mutagenesis (Stratagene). For all Gal4 constructs, the original pM vector (Clontech) was modified by addition of an HA-epitope tag N-terminal to the Gal4-DBD by PCR using forward and reverse primers: 5'–AGATCTATGTACCCATACGACGTACCAGATTACGCTAGCTTGTGCCTCACT A A G C T A C T G T C T T C T A T C G A A C – 3 ' and 5'–GAATTCGCGGCCGCCGGCGATACAGTCAACTGTCTTTGACC–3'. A C-terminal fragment containing the hinge-LBD (aa105-462) of SF-1 and mutants was generated by PCR from HA-SF-1-pCI-neo with primers

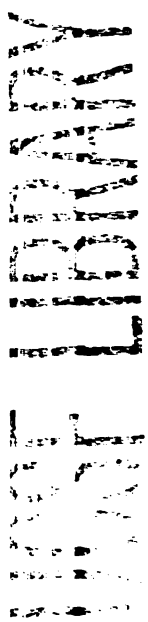
5'–ACGCGTCCTTGAAGCAGCAGAAGAAAGCA-3' and 5'–AAGCTTTCAAGTC-TGCTTGGCCTG–3', and subcloned 3' to the HA-Gal4-DBD. A similar strategy was used to create all pGAL-LRH-1 constructs with the LRH-1 (aa198-562) fused to Gal4. FLAG-mPIASxa was cloned from RIKEN clone 4921511I02 with primers [5'-CCGGAATTCATGGACTACAAAGACGACGACGACAAAGCGGATTTCGAGGAG TTG-3', 5'-CCGCTCGAGTCACTGTTGCACAGTATCAGA-3' and FLAG-mPIAS1 was cloned from mouse hypothalamic cDNA using primers: 5'-CTCGAGATGGACTACAAAGACGACGACGACAAAGCGGACAGTGCGGAACTA AAG-3', 5'-CCGCTCGAGTCAG-TCCAATGAGATAATGTC-3'. PCR products were subcloned into pCI-neo, pBH4 and pGADT7. pVP16-PIAS1 and pVP16-PIASxα were generated by inserting FLAG-mPIAS1 and FLAG-mPIASxα PCR fragments downstream of the VP16 activation domain in a pVP16 vector (Clontech). The following constructs were generous gifts: pCMV GFP-SUMO1 (M. Pederlin, M. Adams, D. Pearce); T7 tagged-mPIASy pCMV (R. Grosschedl); FLAG-mPIAS3 pCMV (K. Shuai); full length mDP103 pcDNA3 (Y. Sadovsky); C-terminal hDP103 pGEX (aa414-824) and full length 2FLAG-hDP103 pcDNA3 (from C. Glass).



Cell transfections, luciferase assays, and metabolic labeling

COS-7 cells were plated at a density of 50,000 cells/mL/12-well plates or 1.5 x 10^6 cells/10cm plate in media (DME H21 4.5g/L glucose with 10% calf serum and antibiotics) 18 hrs prior to transfection. Transfections were carried out using FuGene 6 (Roche). For luciferase assays, cells were transfected with no more than 500ng total DNA per well, and harvested 48 hrs after transfection (BD Pharmingen). All

transfections were performed in triplicate and repeated at least twice. Results were normalized to β-galactosidase activity and are expressed as relative luciferase units (RLU) or fold activation as indicated. For metabolic labeling, COS-7 cells were plated in full media and transfected 18 hrs after plating. Cys/Met deficient media (DME H21 4.5 g/L glucose, 10% dialyzed fetal bovine serum, 2mM glutamine and antibiotics) was added to washed cells 48 hrs post-transfection, followed by 1 hr pulse-labeling with 350 μCi of ^{35}S-Cys and ^{35}S-Met (Redivue, AGQ0080, Amersham), washing, and incubation in full media for relevant chase periods. Cells lysates were subjected to immunoprecipitation, SDS-PAGE electrophoresis, and autoradiography, and signal was quantified by phosphor-imaging.



Yeast Interaction System

An expression cassette containing full-length mouse SF-1 (no heterologous activation domain) was integrated in yeast strain YM4271 containing two integrated reporters, *HIS* and LacZ, driven by four tandem copies of the SF-1 response elements, using the manufacturer's protocols (Clontech). Full length FLAG-tagged mPIAS1 and mPIASxα were subcloned into pGADT7 for transformation into yeast reporter strains. Transformants were plated on selective media, and an X-gal overlay assay was used to detect SF-1/PIAS interactions using 5% X-gal, 1M $NaPO_4$ pH 7.0, 1% agarose, 20% SDS, and color developed at 37°C for 20 min.

Western analysis, immunoprecipitation and co-immunoprecipitation

Cells were washed twice in cold PBS (calcium and magnesium free), lysed in 50mM Tris-HCl pH 7.6, 150mM NaCl, 1mM EDTA, 0.1% NP40, 0.5mM PMSF, 0.5mM DTT, protease inhibitors (Roche), and pre-cleared by centrifugation at 14000 rpm for 30 min. When appropriate, all solutions contained 20 mM N-ethylmaleimide (NEM, Sigma) to inhibit SUMO isopeptidases. Protein concentrations were determined by the Bradford method (Pierce). Equal amounts of total protein were loaded for Western blot analysis. Wild type and mutant receptors were affinity purified using anti-HA affinity matrix (Covance/Babco) in lysis buffer (as described above), washed in a modified lysis buffer containing 300mM KCl and 0.05% NP40, subjected to 8.5% SDS-PAGE and Western blotting following incubation with primary antibodies (anti-HA, 1:2000, Covance/Babco; anti-FLAGM2 1:2000 Sigma; anti-SUMO1, 1:500 Zymed) and a HRP goat anti-mouse 1:10,000 secondary antibody (BioRad). Signal was developed by chemiluminescence (ECL, Amersham). For coimmunoprecipitation of FLAG-hDP103 and sumoylated SF1, cells were transfected and lysed as for in vivo sumoylation in 10 mM NEM. Lysates were incubated with anti-FLAG M2 agarose beads (Sigma) in pull down buffer (50 mM Tris HCl pH 7.6, 150 mM NaCl, 0.5 mM EDTA, 0.01%NP40, 2 mM NEM, protease inhibitors) and precipitates analyzed by Western blotting (anti-HA 1:2000, Covance/Babco; anti-hDP103 1:2000, BD Biosciences).

Recombinant protein expression, in-vitro sumoylation assay and GST pulldowns

Recombinant His_6-hSUMO1 (aa1-97) was expressed and purified by TALON chromatography (Clontech). Recombinant His_6-hE1 (SAE1/SAE2) and His_6-hUbc9 were

obtained commercially (LAE Biotech). In vitro transcribed-translated ^{35}S-SF-1 and variants thereof were produced (Promega) and incubated with 150 ng of E1, 750 ng His_6-Ubc9, 900 ng His_6-SUMO1 in 50 mM Tris pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 2.5 mM ATP at 37°C for 1.5 hrs and the reaction stopped by boiling in protein loading buffer. Samples were subjected to 8% SDS-PAGE followed by autoradiography. GST pulldown assays were carried out with ^{35}S-SF-1 or variants thereof, and purified GST-C-terminal hDP103 as described (Hammer et al., 1999; Klappacher et al., 2002).

Chromatin Immunoprecipitation Assay

HeLa Luciferase Reporter HLR (Stratagene) cells containing an integrated promoter-reporter of five Gal4 binding sites fused to the luciferase gene were electroporated with pCI-Neo and HA-tagged pGal-SF-1 constructs (4μg). The method used follows that described in (Wu et al., 2001) with PCR conditions of 25 cycles at 95°C for 30 s, 53°C for 1 min, 72°C for 1 min, using primers as described (Shiio and Eisenman, 2003) to amplify a 5' 330 bp region of luciferase cDNA.

Nuclear Localization And Immunohistochemistry

COS-7 cells were plated at 6000 cells/well in 4-well chamber slides (Lab-Tek) and transfected in duplicate 24 hrs later (total DNA: 0.5 mg/well). 48 hrs post-transfection, cells were fixed in 4% paraformaldehyde, permeabilized in PBS containing 0.3% Triton-X100 followed by incubation with primary antibodies (rabbit anti-T7 1:300, ICL; mouse anti-FLAGM2 1:5000, Sigma; mouse anti-SF2/ASF 1:1000, Zymed; goat anti-Sp100 1:50 Zymed; and mouse anti-PML (PG-M3), 1:75, Santa Cruz), followed by secondary

antibodies (Cy-3 goat anti-rabbit 1:1000; Cy3-donkey anti-mouse 1:1000, Molecular Probes; Texas Red rabbit anti-goat 1:500, Vector) and imaged on a Zeiss LSM510 confocal microscope.

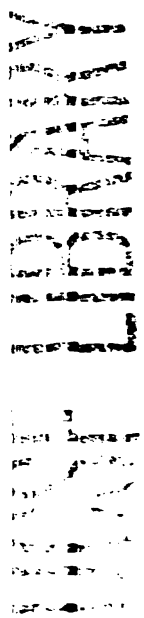
Results

Subfamily V receptors are sumoylated in the hinge region

Although sumoylation is known to repress steroid receptor activity, this modification has not been investigated for so-called orphan nuclear receptors, which can function in a ligand-independent manner. In a modified one-hybrid yeast screen for SF-1 protein partners, we identified Ubc9 or the E2 SUMO conjugating enzyme, as a strong interacting protein (data not shown, see supplementary data, P1). We next asked if SF-1 and LRH-1 could be sumoylated. Indeed, sequence analysis of all vertebrate species of SF-1 and LRH-1 revealed two highly conserved canonical sumoylation motifs at the N- and C-terminal hinge region, while insect Ftz-F1 variants contained one site in the N-terminal hinge region (Fig 1A).

Sumoylation of both SF-1 and LRH-1 was demonstrated in a cellular system as evidenced by slower migrating bands after coexpression of receptor with either SUMO1 or GFP-SUMO1 (Fig 1B). Further analysis revealed that Lys194 served as the major acceptor lysine for SF-1 sumoylation as evidenced by the loss of the slower migrating band with the single mutation K194R and double mutation (K119R and K194R, referred to as 2KR), but not with K119R (Fig 1C). Our results for SF-1 are similar to other recent reports (Chen et al., 2004; Komatsu et al., 2004). The identity of these slower migrating

SF-1 species as sumoylated receptor was confirmed by immunoprecipitation of HA-epitope tagged SF-1, followed by Western blotting with an anti- SUMO1 antibody (Fig 1D), and as predicted no sumoylated species were observed with K194R or 2KR mutant proteins. An in vitro sumoylation assay confirmed that SF-1 is sumoylated at Lys194 with a minor sumoylation site presumed to reside at Lys119 (Fig 1E). The amounts of sumoylated SF-1 diminish in both the K194R and 2KR mutants; the faint residual upshifted band observed in the 2KR variant imply that a minor third site can be sumoylated in vitro. In addition, a similar slower-migrating SF-1 species was detected in NEM-treated lysates made from both Y1 and aT3 cells (data not shown), suggesting that endogenous SF-1 is sumoylated. Taken together, we conclude that subfamily V receptors are sumoylated in vivo and in vitro.



Sumoylation of SF-1 attenuates transcriptional activity

Previous studies identified a regulatory domain which when mutated led to increased receptor activity; this domain contained the major sumoylation site for SF-1 and LRH-1 (Fig 1A and (Ou et al., 2001; Xu et al., 2003). As predicted from these previous findings, SF-1/LRH-1 sumoylation mutants showed increased transactivation of SF-1/LRH-1 promoter reporters compared to wild type receptor in either COS-7 or in HepG2 cells (Fig 2A). SF-1 sumoylation mutants showed little difference in protein stability as determined in pulse chase metabolic labeling, excluding the possibility that increased receptor activity observed in the lysine mutants results from increased SF-1 protein levels (Fig 2B); although we noted increased stability of wild type receptor at an intermediate time point.

The functional effects of loss of sumoylation were also apparent with a Gal4-SF-1 fusion containing the full hinge-LBD of SF-1. As with native receptor, Gal4-SF-1 and Gal4-K119R are efficiently sumoylated, whereas Gal4-K194R and Gal4-2KR exhibit no detectable sumoylation (Fig 2C, left lower panel). Strikingly, the single mutant K194R was at least 70-fold more active than wild type and mutation of both sumoylation sites (2KR) resulted in greater than 300-fold activation (Fig 2C, left panel). While K119R exhibited comparable activation to that of wild type consistent with our earlier observations that sumoylation at Lys119 is minor, the double mutant at both Lys119 and Lys194 showed remarkable synergism. Nearly identical results were observed for Gal4-LRH-1 constructs, where mutating Lys213 and Lys289 in the hinge region led to strong receptor activation (Fig 2C, right panel and refer back to Fig 1A).

To confirm that receptor sumoylation served to repress SF-1 activity, we asked if removing the SUMO conjugate from SF-1 with the SUMO isopeptidase, SENP1 would yield similar results as observed with the SF-1 lysine mutants. Indeed, coexpression of SENP1 with SF-1 and SUMO1 resulted in a marked attenuation of sumoylated SF-1 (Fig 3A). Furthermore, activities of both wild type and the K119R mutant were markedly enhanced after addition of small amounts of SENP1 expression vector (25ng); reaching levels observed with the K194R mutant (Fig 3B, left panel). Addition of SENP1 failed to activate the 2KR variant providing further evidence that Lys119 and Lys194 are the major sites of sumoylation (Fig 3B, right panel). Collectively our data suggest that

Lys194 plays a dominant role in mediating repression of SF-1 via sumoylation and that receptor sumoylation represents a major silencing mechanism.

PIASxa and PIASy act as E3 SUMO ligases for SF-1

One of the defining characteristics of an E3 SUMO ligase is its ability to promote further sumoylation of its substrate. In a survey of four PIAS members, we found that only PIASxa and PIASy promoted SF-1 sumoylation in a dose-dependent manner; this effect was not observed for PIAS1 or PIAS3 (Fig 4A, left panel and see supplementary data, P2). Additionally, the appearance of sumoylation at the second minor site was observed faintly only after addition of PIASy (see PIASy lane and Fig 7A). In contrast to the in vitro sumoylation assay, overexpression of PIAS proteins in vivo does not reveal detectable sumoylation at non-canonical sites, as evidenced by the 2KR mutant (Fig 4A, right panel). Interestingly, mutating the major phosphorylation site of SF-1 adjacent to Lys194 (S203A), had no effect on receptor sumoylation (Fig 4A, right panel). Despite the fact that PIAS1 interacted strongly with SF-1 in yeast (Fig 4B) as well as in a mammalian two-hybrid system (Fig 4C), PIAS1 does not serve as an efficient E3 SUMO ligase for SF-1.

A DEAD-box protein mediates repression via SF-1 sumoylation.

The mechanisms by which protein sumoylation leads to transcriptional repression are diverse. Recent literature suggests that repression by sumoylation involves 1) nuclear relocalization with a concomitant decrease of promoter occupancy, or 2) direct

recruitment of histone deacetylases (HDACs). Therefore, we asked whether sumoylation mutants differ in their subnuclear localization. Both GFP-wild type and GFP-SUMO mutants yielded nearly identical patterns of nuclear localization (Fig 5A). Consistent with these results, no apparent differences were noted in the promoter occupancy of Gal4-wild type compared to the K194R mutant as judged by chromatin immunoprecipitation (ChIP) results using a HeLa cell line containing a stably integrated Gal4 reporter (Fig 5B). Thus, no apparent differences in subnuclear relocalization are observed between wild type and SF-1 sumoylation mutants under basal conditions with added SUMO1, but without PIASxa or PIASy. We next asked if SF-1 sumoylation promotes recruitment of HDACs by using the Class I and II HDAC inhibitors, trichostatin A (TSA) and sodium butyrate (NaBT). If HDAC recruitment is essential for SUMO-mediated repression, then mutating the major sumoylation sites within SF-1 should prevent derepression by TSA or NaBT. Instead, addition of TSA or NaBT leads to a dramatic increase in the activity of all receptor variants (Fig 5C, D). These results differ from those recently shown for Elk-1 where loss of sumoylation eliminates TSA sensitivity (Yang and Sharrocks, 2004), and suggest that repression of SF-1 via sumoylation is largely HDAC-independent.

For subfamily V, two types of repressors have been identified. The first includes the orphan nuclear receptors Dax-1 or SHP, which interfere with the AF2 in the LBD. The second is the RNA helicase DEAD-box protein DP103 (Ou et al., 2001). Indeed, while Dax-1 was able to repress the Gal4-K194R mutant as effectively as Gal4-WT (Fig 6A, left panel), DP103 was ineffective at repressing the Gal4-K194R and 2KR mutants (Fig

6A, right panel and data not shown). Moreover, addition of SENP1 failed to abolish Dax-1-mediated repression of SF-1 (Fig 6B, left panel). In contrast, addition of SENP1 completely abrogated DP103-mediated repression of Gal4-SF-1 (Fig 6B, right panel). Our work contrasts a recent report showing no difference between DP103-mediated repression in wild-type and K194R (Komatsu et al., 2004). This discrepancy may reflect a difference in cell-types or the significantly greater amounts of DP103 used compared to experiments shown here. Nonetheless, our data agree with those reported by Sadovsky and colleagues that Lys194 is essential for DP103 repression of SF-1 in two separate cell types (Ou et al., 2001), suggesting that sumoylation at Lys194 allows DP103 to function as a repressor

The role of DP103 in repressing SF-1 via sumoylation was explored by direct binding assays. As shown previously, only the C-terminal half of DP103 interacts with SF-1 (Ou et al., 2001). Mutation of the Lys194 and/or Lys119 did not result in an appreciable loss of binding, suggesting that Lys194 is not the sole determinant for DP103 interaction with SF-1 (Fig 6C). Furthermore, DP103 is able to interact efficiently with in vitro sumoylated forms of SF-1 (Fig 6D). These results provide evidence that the DEAD-box protein, DP103 interacts with sumoylated SF-1 and directly participates in receptor repression.

DP103 promotes PIAS-dependent sumoylation and subnuclear relocalization of SF-1

The direct binding of DP103 to sumoylated SF-1 was confirmed by coimmunoprecipitation after promoting high levels of receptor sumoylation with PIASy;

no signal was observed with basal levels of sumoylation or after addition of SENP1 (Fig 7A). Surprisingly, DP103 enhanced PIAS-mediated sumoylation, potentially by enhancing PIAS E3 ligase activity or by protecting sumoylated SF-1 from desumoylation. Indeed, all PIAS proteins with the exception of PIAS3, exhibited a 2-3 fold enhancement of sumoylated receptor after addition of DP103 (Fig 7B and supplementary data, P3). No significant increase in sumoylation was observed with DP103 alone (Cont.). Finally, we asked if DP103 would alter the subnuclear localization of SF-1. Although our previous results suggested that the nuclear pattern of SF-1 does not change under basal levels of sumoylation, a dramatic relocalization of GFP-SF-1 was revealed when either mouse or human DP103 was coexpressed with PIASy and SUMO1; two representative cells with prominent nuclear bodies are shown (Fig 7C, +DP103+PIASy). Relocalization of SF-1 was not observed with SUMO1 alone, PIASy alone, DP103 alone, or with a combination of DP103 and PIAS1, PIASxa, or PIAS3 (Fig 7C and data not shown). However, we noted the presence of fine GFP-SF-1 foci in some cells with PIASy alone (Fig 7C, +PIASy). The ability of DP103 and PIASy to shuttle SF-1 to discrete nuclear bodies does not apparently require SF-1 sumoylation, as evidenced by a speckled pattern after addition of SENP1 or with the K119R, K194R and 2KR GFP-SF-1 mutants (Fig 7C, right panels and data not shown). Further analysis revealed colocalization of GFP-SF-1 with PIASy, but not with DP103, which localizes to Cajal bodies or gems (Fig 7D). These GFP-SF-1 nuclear bodies appear distinct from endogenous splicing speckles as shown by the non-overlapping patterns between GFP-SF-1 and SF2/ASF. Moreover these foci do not resemble PML nuclear bodies (PML-NBs) given that the size and number of nuclear foci observed here are much greater. More important, we failed to

detect obvious PML-NBs in COS-7 cells under our culture conditions using two markers, Sp100 and PML (Fig 7D and data not shown). Collectively, our data suggest that DP103 promotes PIAS-mediated sumoylation, and together with PIASy, relocalizes SF-1 to discrete nuclear foci.

Discussion

In this study we report that subfamily V nuclear receptors are sumoylated at evolutionarily conserved sites. As established for other transcription factors, SUMO-modification of SF-1 and LRH-1 attenuates significantly transcriptional activity. Mutating the acceptor lysines in both SF-1 and LRH-1 resulted in a more active receptor, and at least in the Gal4 context, the fold-increase is reminiscent of ligand-dependent receptor activation. Thus, for ligand-independent subfamily V receptors the extent of sumoylation represents one mechanism to both regulate and restrain receptor activity. Our data also suggest that sumoylation of the so-called "repression domain" in SF-1/LRH-1 marks the receptor for repression by the DEAD-box protein DP103. Moreover, this ATPase/RNA helicase was found to enhance PIAS-dependent receptor sumoylation and to promote PIASy-dependent shuttling of SF-1 to discrete nuclear bodies or foci. Subnuclear relocalization of SF-1 correlated strongly with conditions that promote extensive receptor sumoylation, suggesting a potential link between transcriptional repression and accumulation of SF-1 in distinct nuclear bodies.

Repression of SF-1 via sumoylation

In contrast to the ubiquitously expressed E1 and E2 sumoylation enzymes, most of the known E3 SUMO ligases exhibit restricted expression patterns, and therefore might direct tissue specific sumoylation of protein substrates (Yan et al., 2003a). In considering SF-1 sumoylation, three E3 SUMO ligases (PIASxa, PIASy and PIAS1) are all highly expressed in the adult testes (Gross et al., 2001; Yan et al., 2003a), where SF-1 regulates multiple genes. SF-1 is also needed for male sexual differentiation (Roberts et al., 1999; Vilain, 2000), and it is possible that sumoylation of SF-1 is sexually dimorphic during development. Thus, silencing of male-specific genes in the ovary can be partially explained by lowered levels of SF-1 or by the actions of Dax1 (Nachtigal et al., 1998; Swain et al., 1998), but may also involve sumoylation. Interestingly, other factors that function in sexual differentiation, namely Sox9 and WT-1, contain sumoylation sites and the combinatorial effects of sumoylation may ensure gene silencing in the female. Finally, it is worth considering the in vivo ratio of non-sumoylated to sumoylated receptor. In this regard, SF-1 haploinsufficiency (Bland et al., 2004) may stem from inadequate active SF-1 due to a reduction of protein levels, which is compounded by extensive receptor sumoylation.

Currently, our studies are limited to a loss-of-function analysis. Attempts to provide SUMO1 in *cis* to SF-1, as shown for other proteins (Holmstrom et al., 2003; Yang et al., 2003), have failed due to the precise excision of SUMO1 in COS-7 cells (L.A.L. and H.A.I., unpublished data). Whether SF-1 or LRH-1 sumoylation confers any structural changes to the DBD, hinge, or LBD remains unclear, however results from our ChIP

analysis suggest that sumoylation does not alter the apparent affinity of a heterologous DBD. Moreover, given that Dax-1-mediated repression of K194R SF-1 mutant is intact, we suggest that no gross conformational changes occur in the LBD of a sumoylation defective receptor. Further structural analyses are needed and will require an appropriate SUMO-SF-1 chimera or SUMO stably conjugated to SF-1/LRH-1. Although our findings point to a functional role for Lys194 and Lys289 in SF-1 and LRH-1, respectively, the role of the minor sumoylation sites at Lys119 or Lys213 (Fig 1A) is less apparent. Despite the fact that disumoylated SF-1 is only observed in vivo under conditions that promote efficient sumoylation, results from transfection assays suggest that both the minor and major sumoylation sites act in concert to dampen receptor activity. It remains to be established if an ordered sumoylation of SF-1/LRH-1 occurs.

Recent studies report interdependency between sumoylation and phosphorylation. MAP-kinase-mediated phosphorylation of Elk-1 greatly reduced sumoylation at adjacent lysines and led to increased transcriptional activity (Yang et al., 2003) and phosphorylation of heat shock factor 1 is a prerequisite for stress-induced sumoylation (Hietakangas et al., 2003). Currently, we find no apparent relationship between phosphorylation of Ser203 and sumoylation of SF-1. Indeed, the phospho-deficient S203A mutant was efficiently sumoylated, and all SF-1 SUMO mutants showed equivalent levels of phosphorylated Ser203 in SF-1 (unpublished data). However, it remains possible that the rate and extent of either phosphorylation or sumoylation are altered following modification of the Ser203 or Lys194, respectively.

DEAD-Box Proteins and Transcriptional Repression

Historically, DEAD-box (Ddx) RNA helicases are associated with splicing, in part, because they were initially identified as protein components of the spliceosome (Tanner and Linder, 2001). However, other functions for Ddx family members have been noted and there is mounting evidence that they function to silence transcription factors, including nuclear receptors, Egr1-4, and the Ets-like repressor, METS (Gillian and Svaren, 2003; Klappacher et al., 2002; Rajendran et al., 2003; Yan et al., 2003b). Additionally, GRTH (Ddx25), which is expressed in testes, is reported to attenuate expression of SF-1 target genes, including steroidogenic enzymes (Dufau et al., 2001). Intriguingly, while an interaction was noted between Gu-binding protein (PIAS-like) and Gu/RHII (Ddx21A) (Valdez et al., 1997), the connection between sumoylation and DEAD-box proteins was not explored. For DP103 and another DEAD-box protein DP97, the repression domain has been mapped to the C-terminal region and does not require the N-terminal ATPase/helicase domain characteristic of this gene family (Klappacher et al., 2002; Rajendran et al., 2003). . Our study thus provides a direct link between DEAD-box protein repression and SUMO-modification.

Attenuation and silencing of transcription are known to be multi-layered and multi-dimensional. So how might Ddx proteins and sumoylation lead to transcriptional repression? Recruitment of HDACs upon protein sumoylation, or by Ddx proteins, offers the most plausible explanation and is consistent with prior literature. Indeed, DP103 interacts with the N-terminal repression domain of METS and promotes HDAC recruitment (Klappacher et al., 2002). However, our data imply that repression through

DP103 is TSA- and NaBT-insensitive; and suggest that repression by Ddx proteins must involve additional mechanisms other than recruitment of Class I or II HDACs. In considering other mechanisms, it is possible that DP103 protects SF-1 from desumoylation, consistent with our observations that addition of DP103 increased PIAS-dependent SF-1 sumoylation. This hypothesis is consistent with our findings that additional SENP1 eliminates repression by DP103 and that sumoylated SF-1 binds directly to DP103. The interaction between DP103 and SF-1 remains to be mapped and is likely to involve multiple interfaces based on our finding that Lys194 and/or sumoylation at Lys119/Lys194 are not sole determinants of this interaction. Another possible scenario is that DP103 represses SF-1 by facilitating PIASy-mediated relocalization of SF-1. However, we noted that sumoylation is dispensable for movement of SF-1 to nuclear bodies, as evidenced by persistence of these bodies with both SENP1 and with the K119R, K194R and 2KR mutants. Our observations are reminiscent of PIASy-dependent relocalization of both wild type and sumoylation defective Lef1 into nuclear bodies that partially overlap with overexpressed Sp100, a marker of PML-NBs (Sachdev et al., 2001). However, we failed to detect endogenous PML-NBs in culture conditions where relocalization of GFP-SF-1 was observed, consistent with the fact that GFP-SF-1 nuclear bodies appear distinct from PML-NBs. Thus, while sumoylation is not required for subnuclear relocalization of SF-1 (or Lef1), conditions that promote optimal sumoylation do correlate with relocalization. As such, one might speculate that this modification occurs in transit to, or within these nuclear bodies, and results in different functional outcomes for sumoylated versus non-sumoylated receptor.

Given that DEAD-box proteins are present in both splicing and translational complexes (Nelson et al., 2004), repression might be coupled to transcript processing or translational control. However, studies to date, including ours, have yet to identify a function for the RNA helicase (unwindase) and RNA binding motifs in repression. Indeed, the N-terminal portion of DP103 is dispensable for interaction and repression of SF-1 and METS (Klappacher et al., 2002; Yan et al., 2003b), and for relocalization of SF-1 to nuclear bodies (our unpublished data). It is intriguing that other RNA binding proteins such as SHARP and RTA (Fox-1 Ataxin-2 BP1) have been identified as negative coregulators of nuclear receptors (Norris et al., 2002; Shi et al., 2001). Further in vitro and in vivo experiments aimed at delineating the precise role of sumoylation in DEAD-box-mediated transcriptional repression will be of interest.

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Figure Legends

Figure 1. Subfamily V receptors are sumoylated in the hinge region. **(A)** A schematic of the domain structures and percentage protein identity for *Drosophila* Ftz-F1 and mouse SF-1 and LRH-1 are shown with SUMO sites (S), and phosphorylation sites (P) indicated. The "repression domain" is also shown (R, black square). **(B)** Anti-HA Western blot of COS-7 lysates is shown after transfection with HA-epitope tagged SF-1 or LRH-1 and SUMO1 or GFP-SUMO1. The slower-migrating forms of each receptor are indicated (*arrowheads*) and all lysates were prepared in the presence of N-ethylmaleimide (NEM), an inhibitor of SUMO isopeptidases. **(C)** Anti-HA Western blot of COS-7 cells is shown for empty vector control (pCI), HA-SF-1 wild type or lysine mutants with sumoylated SF-1 (*SF-1) and non-sumoylated SF-1 (SF-1) indicated; SUMO1 was coexpressed in all conditions. A control immunoblot for SUMO1 is shown below. **(D)** Anti-SUMO1 Western blot of HA-immunoprecipitated lysates from COS-7 cells transfected with wild type or lysine mutants of SF-1 is shown with sumoylated SF-1 (*arrowhead*) and non-specific bands (NS) indicated. A control immunoblot for HA-SF-1 expression is shown below. One μg of each plasmid was added for all transfections. **(E)** In vitro sumoylation of in vitro transcribed-translated ^{35}S-labeled wild type and lysine mutants of SF-1 (1μl) was carried out as described in Materials and Methods. Unmodified SF-1 (SF-1) and sumoylated SF-1 (*arrowheads*) are indicated.

Figure 2. Sumoylation represses SF-1 transcriptional activity. **(A)** Transcriptional activity of wild type and lysine mutants of SF-1 (50ng) on the aromatase-luciferase

reporter (Aro-Luc, 500ng) is shown for both COS-7 (no SUMO1 added), and HepG2 cells (50ng SUMO1 added). Other promoter-luciferase reporters used in HepG2 cells were the 3b-hydroxysteroid dehydrogenase promoter (3bHSD Luc, –153/+2 bp); a synthetic promoter containing tandem SF-1 response elements from the mouse Müllerian Inhibiting Substance promoter (2XRE MIS); and the StAR promoter (StAR Luc, -966/+1), 250 ng of each promoter used. **(B)** Stability of wild type (WT) and lysine mutant (K194R or 2KR) SF-1 proteins in COS-7 cells was determined after metabolic labeling, followed by chase for 0, 2, 5 and 12 hours, as described in Materials and Methods. An autoradiogram of immunoprecipitated HA-proteins from whole cell lysates is shown with phosphor-image data graphed as the percentage of labeled protein remaining after each chase period; levels of protein at time 0 were taken to be 100%. **(C)** Transcriptional activity is shown for Gal4-SF-1 wild type (pGalWT, aa105-462, 25ng) or Gal4-SF-1-Lys mutants (pGalK119R, pGalK194R, pGal2KR, 25ng) on the Gal4-luciferase reporter (pFR-Luc, Stratagene, 200ng) in COS-7 cells (left panel). Anti-HA Western blotting shows expression levels of the Gal4-SF-1 WT or KR mutants with slower-migrating forms of sumoylated Gal4-SF-1 protein indicated (arrowhead). Transcriptional activity of Gal4-LRH-1 wild type (pGalWT, aa198-561, 25ng) and lysine mutants (pGalK213R, pGalK289R, pGal2KR) are shown (right panel). All luciferase activity is expressed as fold-activation over parent vectors: pCI-neo (C) for panels in A and pM (pGal) for panels in C.

Figure 3. Adding SENP1 increases activity of wild type SF-1, but not the 2KR sumoylation mutant. **(A)** A western blot is shown for COS-7 cells transfected with

empty vector (pCI) or wild type SF-1 (1μg each) in the presence or absence of SUMO1 and SENP1 (1μg each); with sumoylated SF-1 (*SF-1), and non-sumoylated SF-1 (SF-1) indicated. **(B)** Effects of increasing amounts of SENP1 (0, 25 and 50ng) are shown for transcriptional activity of Gal4-SF-1 wild type (pGalWT) and Gal4-SF-1-lysine mutants (pGalK119R, pGalK194R, pGal2KR) on the pFR-Luc Gal4 reporter in COS-7 cells. Luciferase activity is expressed as relative light units. Amounts of transfected plasmids are identical to those used in Fig 2C.

Figure 4. PIASxa and PIASy are E3 SUMO ligases for SF-1. (A) Western blots for COS-7 cells cotransfected with wild type HA-SF-1, SUMO1, and with individual PIAS proteins or SENP1 are shown (left panel). The right panel shows a Western blot for HA-SF-1 (WT), lysine or S203A SF-1 mutants after addition of PIASxa and SUMO1; 1μg of each plasmid was added. Sumoylated SF-1 (*SF-1) and non-sumoylated SF-1 (SF-1) are indicated. **(B)** Interactions between full-length SF-1 and Gal4AD-PIASxa or Gal4AD-PIAS1 fusion proteins were tested in yeast expressing SF-1, driving SF-1 response elements fused to LacZ. The middle sector (Control) shows b-galactosidase activity in a control strain with the LacZ reporter but no integrated SF-1 or transformed PIAS fusion proteins. The upper sector shows basal activity resulting from yeast expressing SF-1 (SF-1), and the empty vector pGADT7. The lower sector shows activity for yeast expressing SF-1 and Gal4AD-PIAS fusion proteins (SF-1+PIASxa, SF-1+PIAS1). **(C)** A mammalian two-hybrid system showing transcriptional activity of wild type pGal4-SF-1 with increasing concentrations of VP16-PIAS fusion proteins (25, 50, 150ng). Empty vector control is shown (pGal).

Figure 5. SF-1 sumoylation mutants exhibit wild-type localization, promoter occupancy and sensitivity to HDAC inhibitors. **(A)** Nuclear localization is shown for transfected GFP-SF-1 wild-type (WT) and lysine mutants (K119R, K194R, 2KR) in COS-7 cells; 100ng of each plasmid was used and resulted in expression of GFP-SF-1 in 15% of all cells. **(B)** ChIP assays are shown for control vector, N-terminal HA tagged Gal4-SF-1 (WT) or Gal4-lysine mutants in HeLa cells containing integrated Gal4 response elements fused to luciferase using anti-HA or control IgG. **(C)** Trichostatin A (TSA) and **(D)** sodium butyrate (NaBT) effect on transcriptional activity of Gal4-SF-1 wild-type (pGalWT) and lysine mutants (pGalK119R, pGalK194R, pGal2KR) in COS-7. TSA (0, 333 nM) or NaBT (0, 0.1, 1 or 10 mM) was added to cells 12 hrs post transfection and incubated for 24 hrs.

Figure 6. The DEAD-box protein DP103 mediates SF-1 repression by sumoylation at Lys194 and binds to sumoylated SF-1. **(A)** Repression of SF-1 by Dax1 and DP103. Increasing amounts of mDax1 or mDP103 (0, 25, 50, 150ng) were cotransfected in COS-7 cells with Gal4-SF-1 (pGalWT) or the pGalK194R (25ng each) on Gal4-luciferase reporter (pFR-Luc, 250ng). **(B)** Effect of SENP1 on repression by Dax1 and mDP103. Increasing amounts of Dax1 or DP103 (as in A) cotransfected with control vector (pGal) or Gal4-SF1 wild type (pGal-SF-1), with or without SENP1 (25ng), on Gal4-luciferase reporter (as in A). **(C)** GST pulldown assays show binding of [^{35}S]-SF-1 wild-type and lysine mutants (WT, K194R, 2KR) to increasing amounts of GST-hDP103 C-terminal aa414-824 (GST-DPC; 1X, 2X indicate relative amounts used). 10% input and GST

controls are indicated. A schematic of human DP103 shows the unique C-terminal region and conserved helicase domain motifs (gray rectangles), including the signature DEAD-box motif (black rectangle). **(D)** GST pulldown assays show binding of in vitro sumoylated [^{35}S]-SF-1 (+E1, *SF-1, upper panel) to increasing amounts of GST-hDP103 C-terminal (1X, 3X, 9X) and to a nonsumoylated SF-1 control made in reactions lacking E1 enzyme (-E1, SF-1, lower panel). Amounts of GST proteins used in panels C, D are shown in Supplemental Data P3.

Figure 7. DP103 interacts with sumoylated SF-1 in vivo and promotes PIASy-mediated SF-1 relocalization into nuclear bodies(A) Coimmunoprecipitation of sumoylated SF-1 from COS-7 cells transfected with wild-type HA-SF-1, SUMO1 and indicated combinations of FLAG-DP103, T7-PIASy and SENP1 (1μg each) using anti-FLAG-M2 agarose beads. Western blots for HA-SF-1 (3% input lysate, upper panel) and immunoprecipitated (IPed) DP103 (10% IPed protein, middle panel) are shown with sumoylated SF-1 indicated (*SF-1). An anti-HA Western blot of IPed DP103 protein is shown with coimmunoprecipitated sumoylated SF-1 (black arrowhead) and non-specific bands (NS) indicated (lower panel). **(B)** Western blots are shown of COS-7 cells cotransfected with wild type HA-SF-1, SUMO1 and individual PIAS proteins or empty vector (Cont.) with (+) or without (-) DP103 (1 mg of each plasmid was used). Sumoylated SF-1 (*SF-1) and non-sumoylated SF-1 (SF-1) are indicated. **(C)** Nuclear localization of GFP-SF-1 transfected into COS-7 cells is shown with different combinations of FLAG-DP103, T7-PIASy, FLAG-PIAS1 and SENP1, as indicated. All cells were transfected with SUMO1 and 100ng of each plasmid was used in all

experiments. **(D)** Subnuclear signals are shown for wild-type GFP-SF-1 (green) and indirect immunofluorescence is shown for T7-PIASy (red), or FLAG-hDP103 (red). Colocalization of GFP-SF-1 and T7-PIASy signals are shown in the merged figure (upper panels), or the endogenous DP103 signal (lower panels) are indicated (arrowheads). Staining for endogenous SF2/ASF (marker for splicing speckles) or Sp100 (marker for PML-NBs) is shown in similarly transfected COS-7 cells (red), as indicated. Note that no positive staining is observed for endogenous Sp100. In all conditions, cells were transfected with 100 ng each of GFP-SF-1, PIASy, hDP103, and SUMO1.

Supplemental Data P1

Interaction of SF-1 and LRH-1 with Ubc9. **(A)** Ubc9 interacts strongly with SF-1 and LRH-1 in vivo. ß-galactosidase activity was measured in yeast expressing stably integrated mouse SF-1 and driven by four tandem copies of the SF-1 response element from the Müllerian Inhibiting Substance promoter. Values are shown for SF-1/LRH-1 binding alone (-), with the control pGADT7 vector (pGADT7) or with Ubc9 fused to the Gal4AD (AD-UBC9) (left and middle panels). Values for yeast with no integrated SF-1 or LRH-1 are also shown (right panel). **(B)** Mouse Ubc9 was cloned into GST expression vector pGEX4T1 and expressed in BL21 after induction with IPTG. Equivalent amounts of GST-Ubc9 and GST proteins were bound to radiolabeled SF-1 in 20mM Tris pH 8.0, 0.1M NaCl, 1mM EDTA, 1mM DTT, 0.01% NP40, 10% glycerol, 0.1mM PMSF at 4°C for 3 hrs. Beads were washed, and analyzed by SDS-PAGE gel, followed by autoradiography.

Supplemental Data P2

Supplemental Data for Figure 4. Anti-HA immunoblot of COS-7 cells co-transfected with wild-type HA-SF1 and SUMO1 (1 μg each) with increasing amounts of PIASxa or PIASg (0, 0.15, 0.5, 1.5 mg), with sumoylated SF-1 (*SF-1) and non-sumoylated SF-1 (SF-1) indicated.

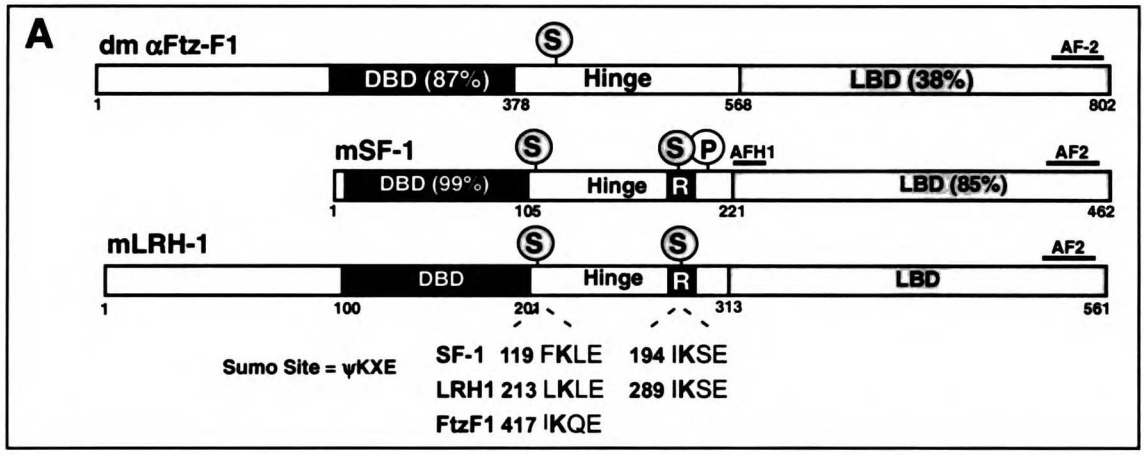
Supplemental Data for Figure 5. Effect of trichostatin A (TSA: 0, 10, 100, 333 nM; *left* panel) and sodium butyrate (NaBT: 0, 0.1, 1, 10 mM; *right* panel) on transcriptional activity of Gal4SF-1 wild-type (pGalSF-1), lysine mutants (pGalK119R, pGalK194R, pGal2KR) or empty vector (pGal) in HeLa cells containing integrated Gal4 response elements driving luciferase, with data expressed as raw light units uncorrected for beta-galactosidaase activity.

Supplemental Data P3

Supplemental Data for Figure 6. Coomassie-stained 10% SDS-PAGE gel of GST and increasing amounts of GST-DP103 C terminal (1X, 3X, 9X, GST-DPC) used in GST-pull-downs.

Supplemental Data for Figure 7. *Left* panels: Control Western blots for PIAS expression: anti-FLAG immunoblot for FLAG tagged PIAS1, PIASxa and PIAS3 (*top*) and anti-T7 tag immunoblot for T7 tagged PIASg (*bottom*). Equal amounts of total protein were loaded. *Right* panel: Anti-HA immunoblot of lysates from COS-7 cells co-transfected with wild type HA-SF-1, SUMO1 (1μg each) and PIASxa alone (0.15 μg) with increasing amounts of DP103 (0, 0.15, 0.5, 1.5 mg). Sumoylated SF-1 (*SF-1) and non-sumoylated SF-1 (SF-1) are indicated.

Figure 1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LBD (38%)
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mSF-1
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Hinge
R
LBD (85%)
AFH1
AF2
1
105
221
462
mLRH-1
DBD
Hinge
R
LBD
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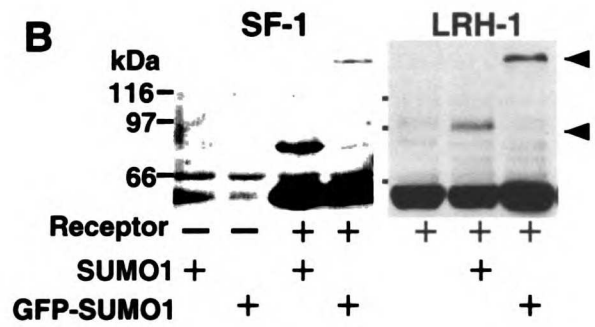
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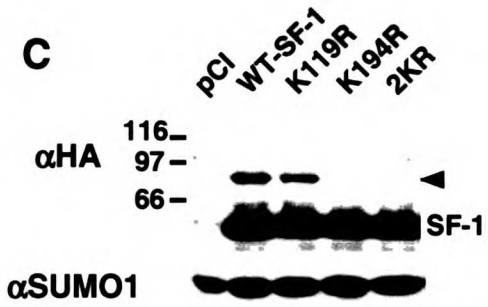
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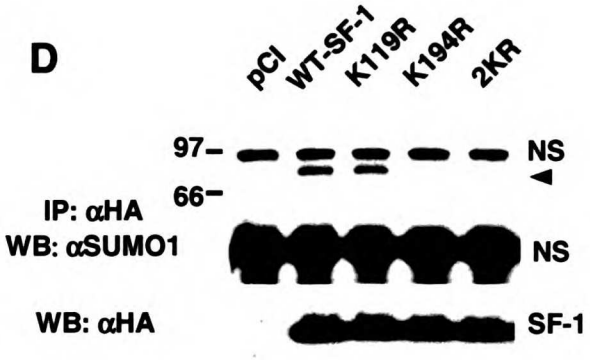
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WB: αSUMO1
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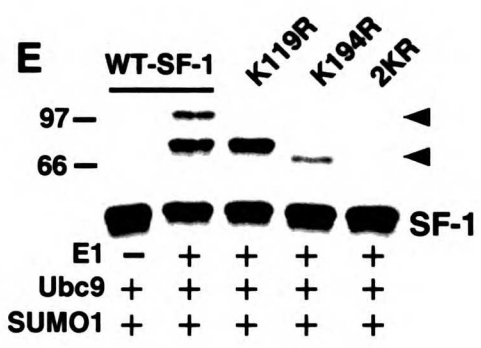
SF-1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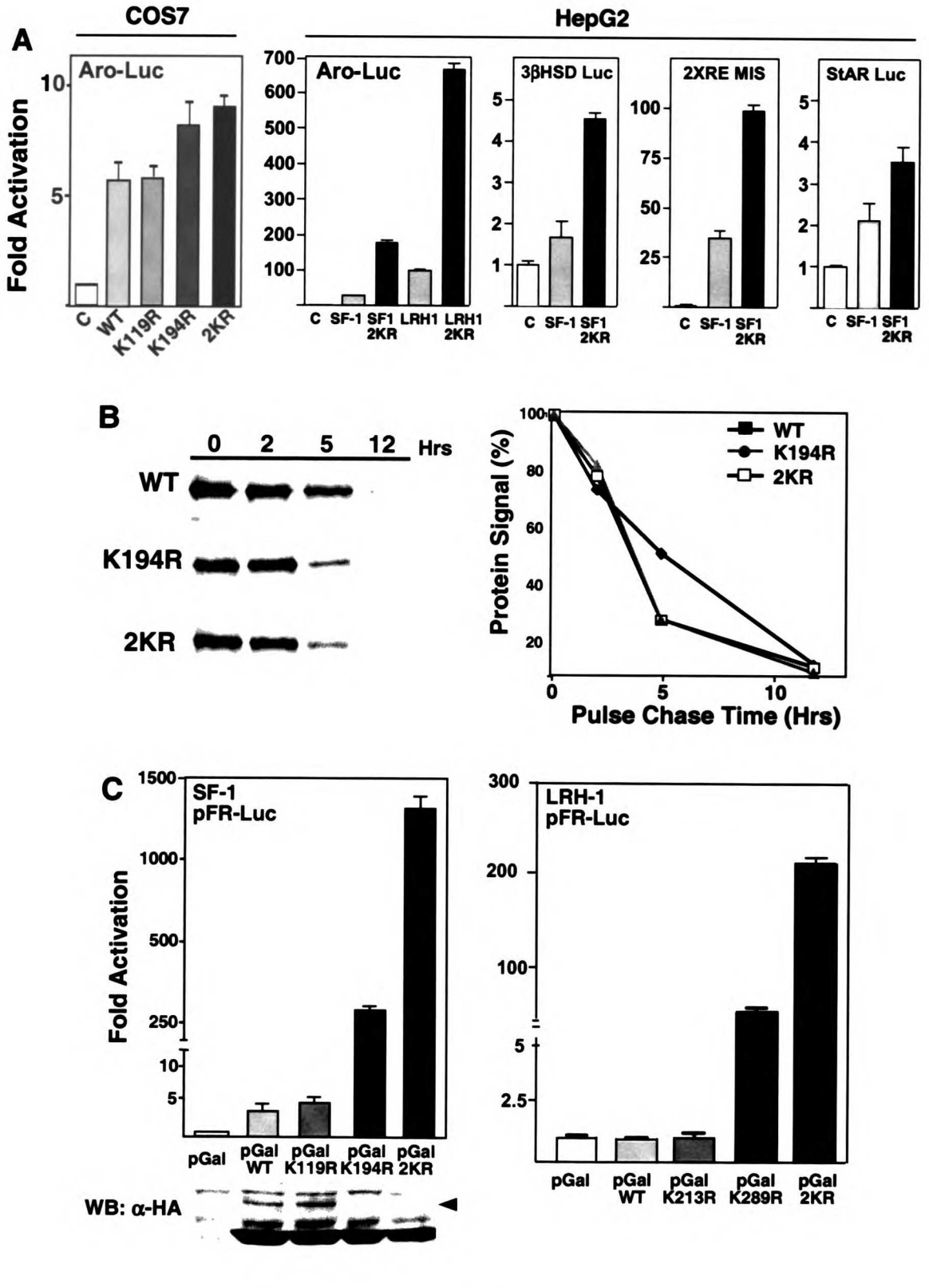
SUMO1

Figure 2



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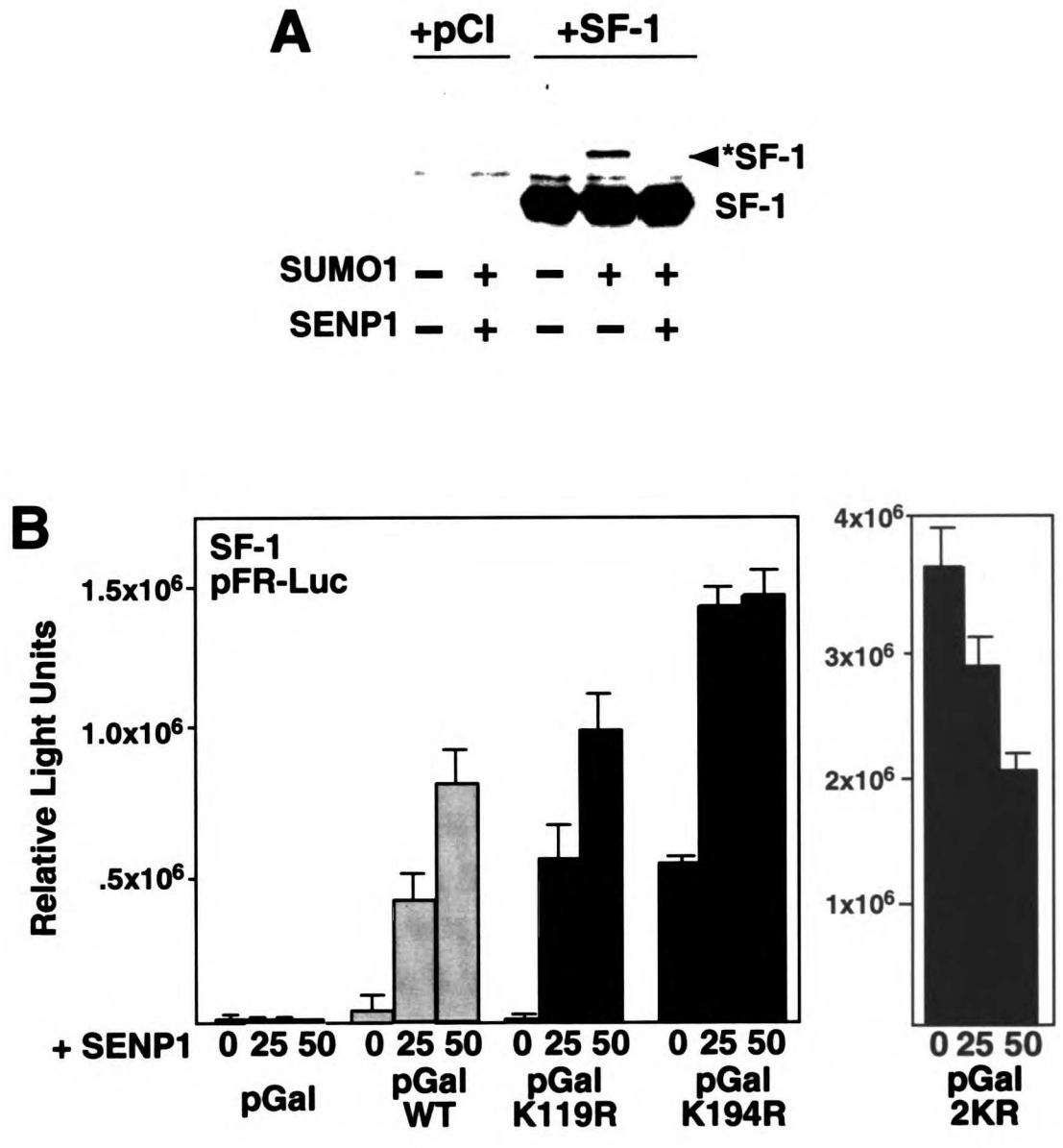

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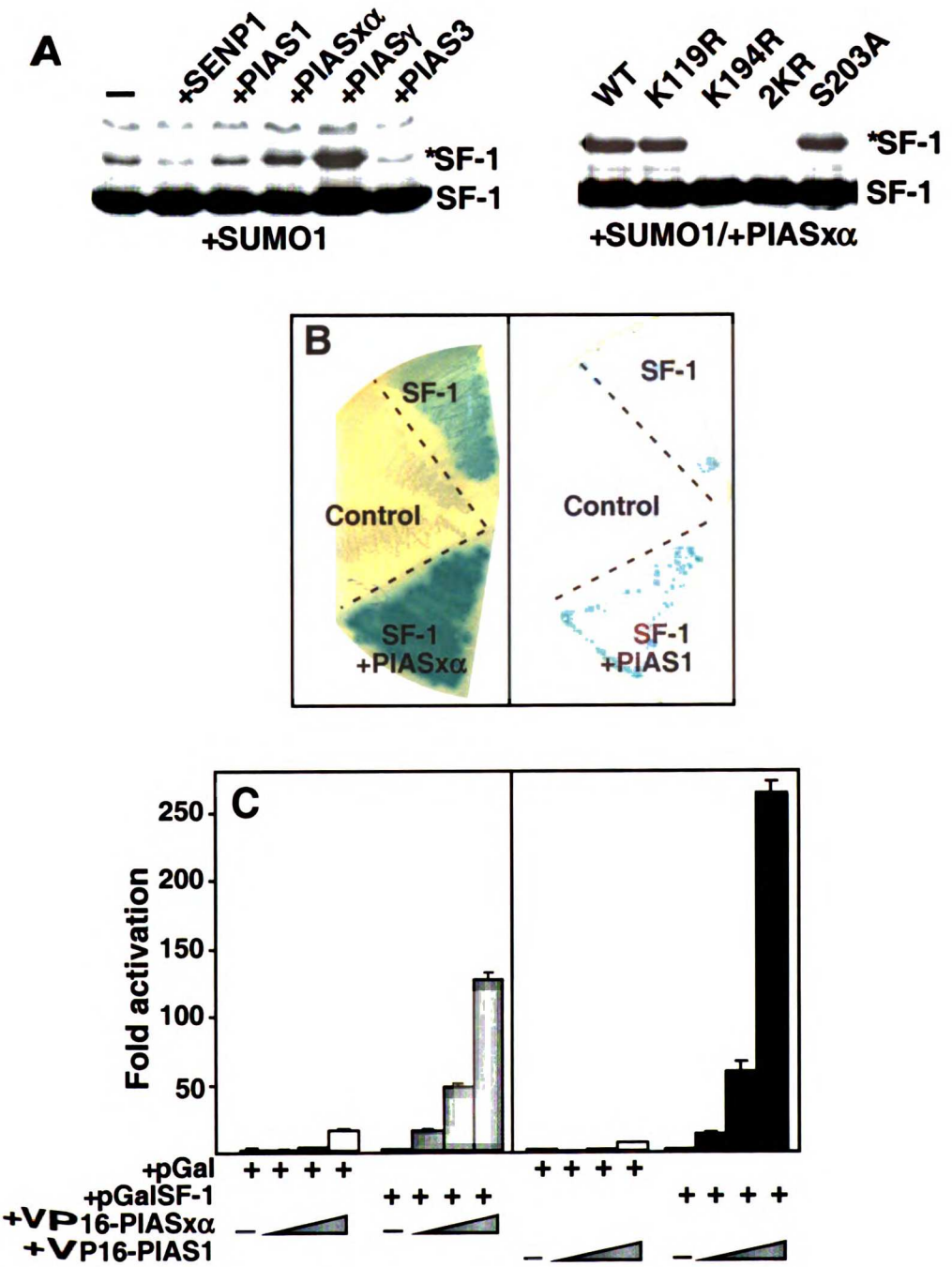
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–
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+PIAS1
+PIASxα
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*SF-1
SF-1
+SUMO1
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K119R
K194R
2KR
S203A
*SF-1
SF-1
+SUMO1/+PIASxα
B
SF-1
Control
SF-1
+PIASxα
SF-1
Control
SF-1
+PIAS1
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200
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100
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+VP16-PIASxα
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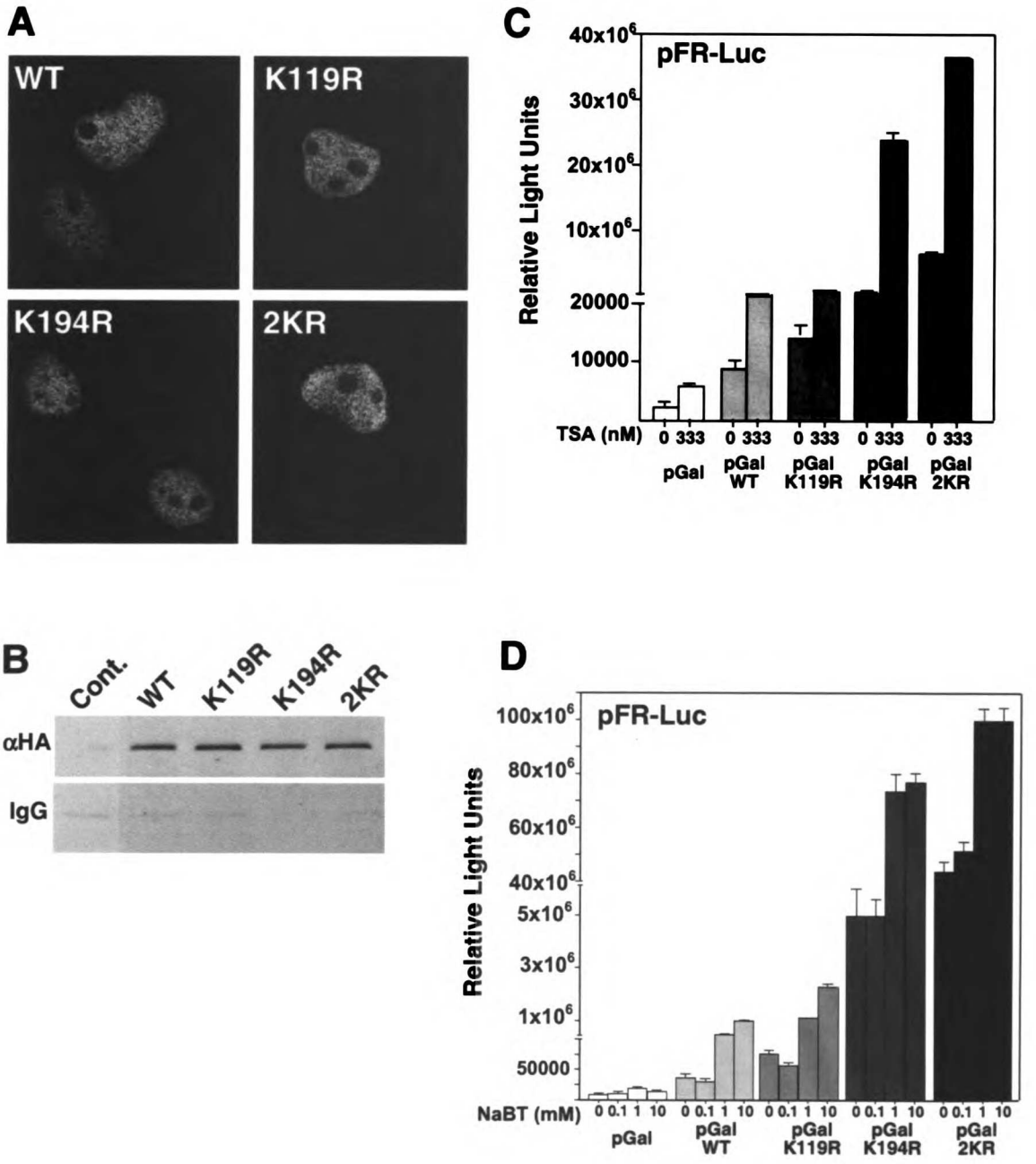
Figure 5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10000
TSA (nM)
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pGal WT
pGal K119R
pGal K194R
pGal 2KR
B
Cont.
WT
K119R
K194R
2KR
αHA
IgG
D
pFR-Luc
Relative Light Units
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80x10^6
60x10^6
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5x10^6
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1x10^6
50000
NaBT (mM)
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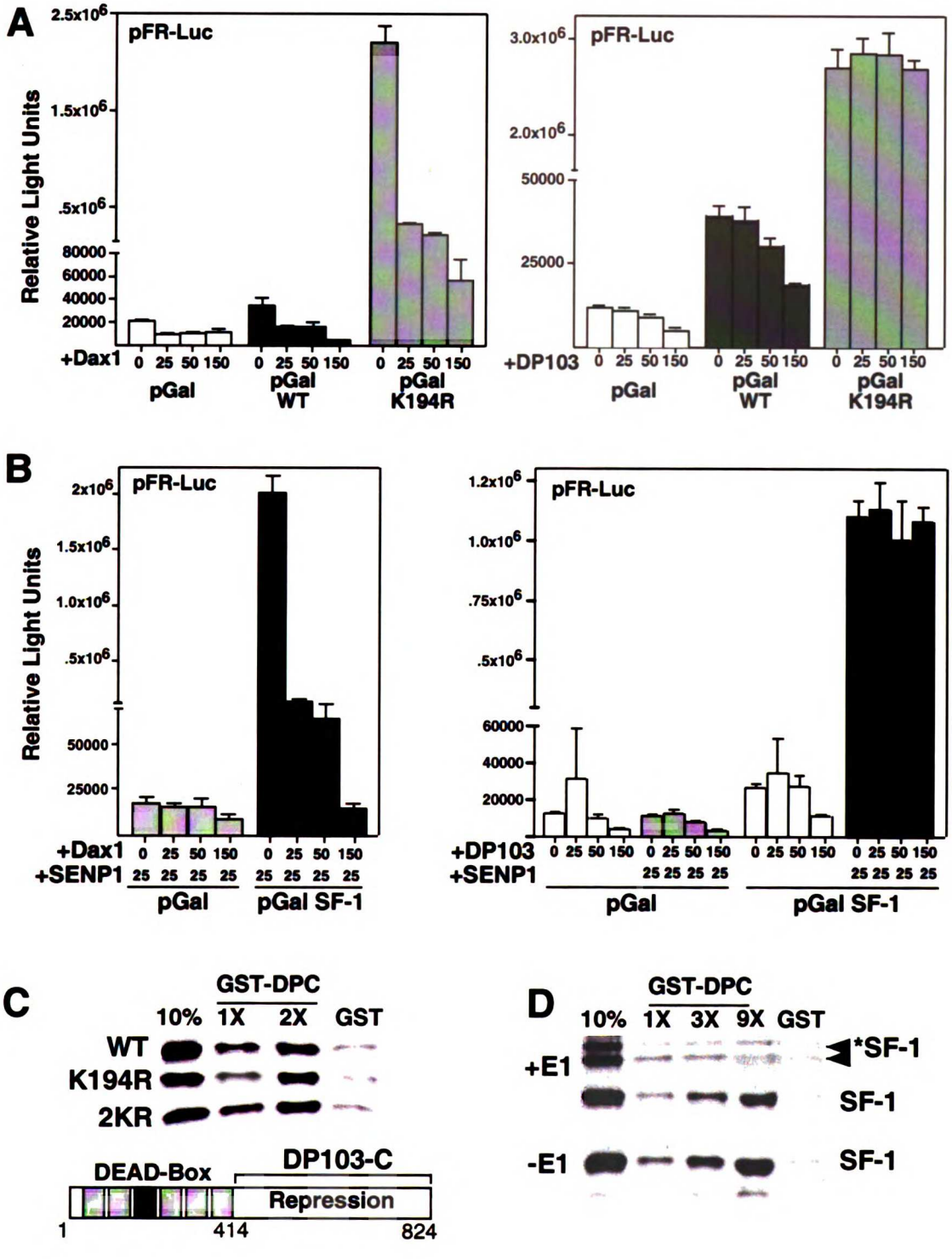
Figure 6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pGal WT
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.5x10^6
60000
40000
20000
+DP103
+SENP1
0 25 50 150 0 25 50 150 0 25 50 150 0 25 50 150
25 25 25 25 25 25 25 25
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pGal SF-1
C
GST-DPC
10% 1X 2X GST
WT
K194R
2KR
DEAD-Box
DP103-C
Repression
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+E1
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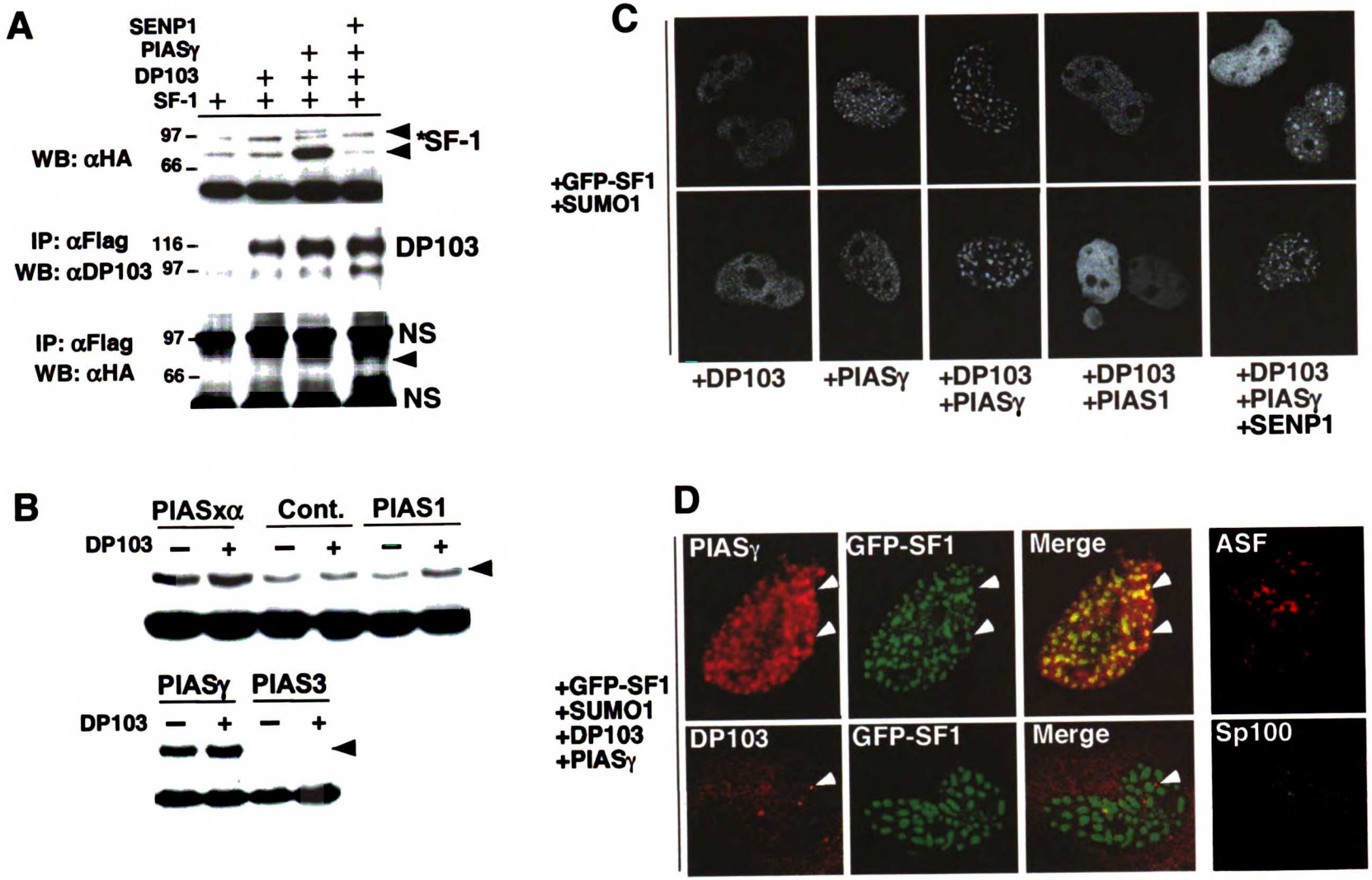
*SF-1
SF-1
SF-1

Figure 7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97
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NS
NS
B
PIASxα
Cont.
PIAS1
DP103
PIASγ
PIAS3
DP103
C
+GFP-SF1
+SUMO1
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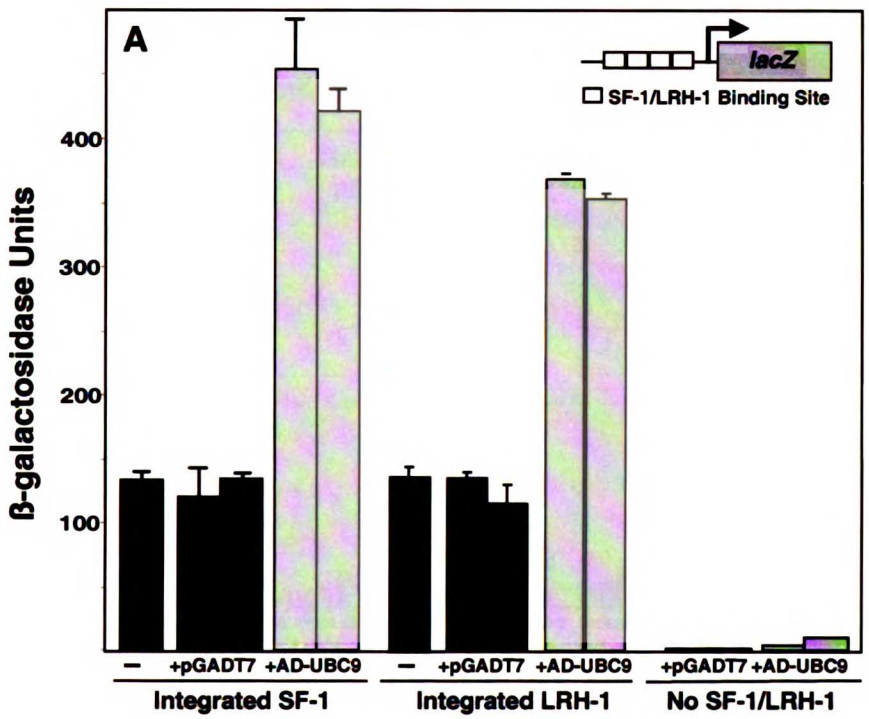
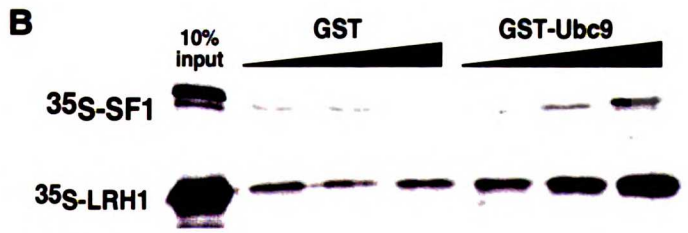
Supplemental Data P1





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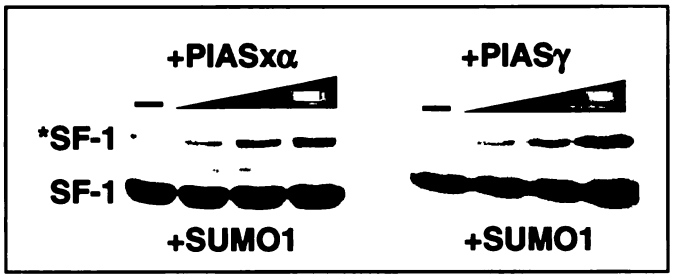
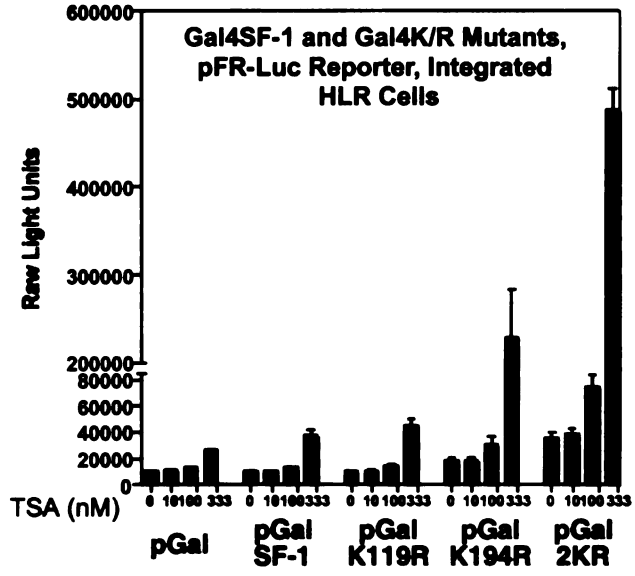
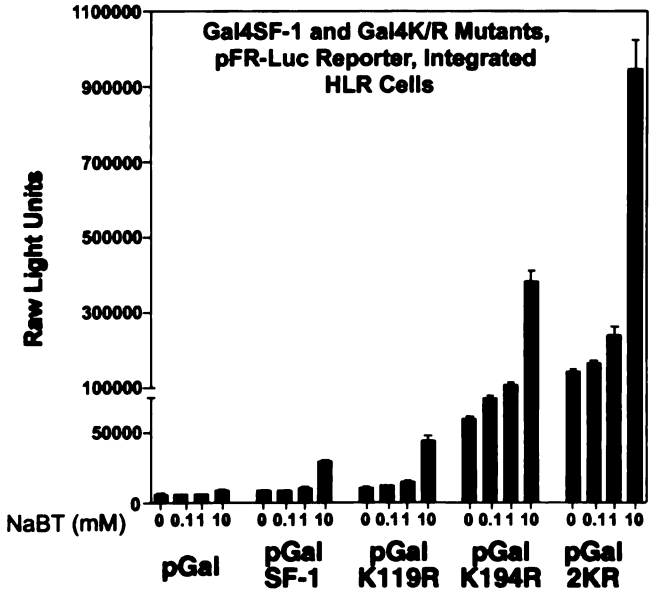
Supplemental Data for Figure 4



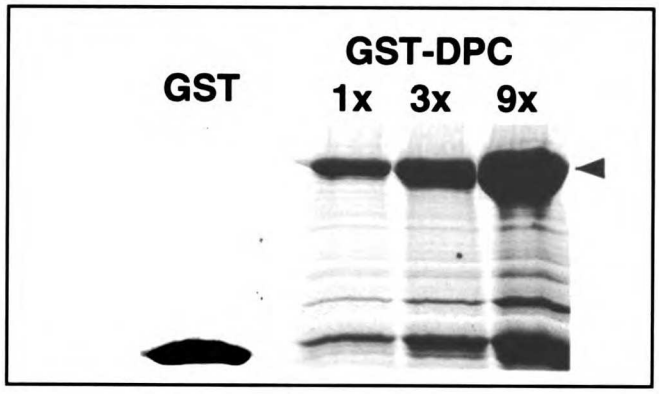
Supplemental Data for Figure 5





Supplemental Data P3

Supplemental Data for Figure 6



Supplemental Data for Figure 7

CHAPTER 3

REQUIREMENT OF THE ORPHAN NUCLEAR RECEPTOR SF-1 IN TERMINAL DIFFERENTIATION OF VENTROMEDIAL HYPOTHALAMIC NEURONS

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REQUIREMENT OF THE ORPHAN NUCLEAR RECEPTOR SF-1 IN TERMINAL DIFFERENTIATION OF VENTROMEDIAL HYPOTHALAMIC NEURONS

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Running title: SF-1 Required for Differentiation of Hypothalamic Neurons

ABSTRACT

The ventromedial hypothalamic nucleus (VMN) is known to mediate autonomic responses in feeding and reproductive behaviors. To date the most definitive molecular marker for the VMN is the orphan nuclear receptor steroidogenic factor-1 (SF-1). However, it is unclear whether SF-1 functions in the VMN as it does in peripheral endocrine organ development where loss of SF-1 results in organ agenesis due to apoptosis. Here we provide evidence that SF-1 has a distinct role in later stages of VMN development by demonstrating the persistence of VMN precursors, the misexpression of an early marker (NKX2-1) concomitant with the absence of a late marker (BDNF neurotrophin), and the complete loss of projections to the bed nucleus of stria terminalis and the amygdala in *sf-1* null mice. Our findings demonstrate that SF-1 is required for terminal differentiation of the VMN and suggest that transcriptional targets of SF-1 mediate normal circuitry between the hypothalamus and limbic structures in the telencephalon.

INTRODUCTION

Physical and chemical ablation studies of the hypothalamic ventromedial nucleus (VMN) suggest that this region of the hypothalamus controls a number of homeostatic and autonomic behavioral responses. For instance, the VMN has been implicated in regulating reproductive cycles, sexual and feeding behaviors, diurnal rhythm of glucocorticoid secretion, body temperature, and possibly locomotor behavior (Chateau *et al.*, 1987; Egawa *et al.*, 1991; Cohen and Pfaff, 1992; Choi *et al.*, 1998; Flier and Maratos-Flier, 1998; Choi and Dallman, 1999; Majdic *et al.*, 2002). The VMN is located in the mediobasal region of the diencephalon and is recognized morphologically as a dense bilateral aggregate of cell bodies surrounded by a cell-free neuropil zone. Based on different projections, possible functions, and histochemical properties, the VMN can be organized into dorsomedial, central, and ventrolateral regions (Altman and Bayer, 1986; Canteras *et al.*, 1994; Canteras, 2002). VMN neurons send projections to adjacent hypothalamic regions and to other brain regions, including the bed nucleus of stria terminalis and the amygdala of limbic system (Altman and Bayer, 1986; Luiten *et al.*, 1987; Swanson, 1987; Canteras *et al.*, 1994). Although the precise molecular and chemical nature of the VMN circuitry remains to be defined, recent studies have proposed that the orphan nuclear receptor steroidogenic factor 1 (SF-1) is required for proper VMN development and function (Ikeda *et al.*, 1995; Shinoda *et al.*, 1995; Majdic *et al.*, 2002).

SF-1 belongs to the hormone nuclear receptor gene family, and remains an orphan member because a cognate ligand has yet to be identified (Mangelsdorf *et al.*, 1995). It is

well established that SF-1 is an essential regulator in endocrine tissue and organ development. Indeed, targeted disruption of *sf-1* in mice results in gonadal and adrenal agenesis, as well as the complete loss of pituitary gonadotropes (Ingraham *et al.*, 1994; Luo *et al.*, 1995; Sadovsky *et al.*, 1995; Shinoda *et al.*, 1995). SF-1 also regulates multiple targets that mediate normal adult endocrine physiology, reviewed in (Parker, 1998; Hammer and Ingraham, 1999), and controls male sexual differentiation by regulating three important male hormones, including the Müllerian inhibiting substance (MIS), steroidogenic enzymes for testosterone synthesis and Insl-3, which is required for testicular descent, for review see (Roberts *et al.*, 1999). Consistent with SF-1's role in male development, *sf-1* null (-/-) mice have female external genitalia regardless of their sex chromosomal complement (Luo *et al.*, 1995; Sadovsky *et al.*, 1995; Shinoda *et al.*, 1995). More recently, phenotypic sex reversal of XY genotypes in humans has been associated with a partial loss of SF-1 function (Achermann *et al.*, 1999; Achermann *et al.*, 2002). Although male sexual development in mice does not require a full dosage of SF-1, as shown in humans, two fully functional *sf-1* alleles are required for proper adrenal development and function (Bland *et al.*, 2000). *Sf-1* heterozygous (+/-) mice exhibit symptoms of adrenal insufficiency due to the hypoplastic and disorganized nature of the organ (Bland *et al.*, 2000; Babu *et al.*, 2002), consistent with adrenal insufficiency in humans due to partial loss of SF-1 function (Achermann *et al.*, 1999; Biason-Lauber and Schoenle, 2000; Achermann *et al.*, 2002). These studies suggest that a threshold level of SF-1 activity must be maintained for optimal growth and differentiation of the adreno-gonadal primordium.

The precise role of SF-1 in VMN development is less clear, in part because SF-1 targets in the hypothalamus have not been identified and because of the early postnatal lethality in *sf-1* -/- mice due to the complete lack of adrenal function. Previous analyses of *sf-1* -/- mice report an apparent change in the cytoarchitecture of the VMN as assessed by the absence of a prominent condensed nucleus, an indistinct cell-free neuropil zone, and an increased number of neuroblasts in the paraventricular zone (Ikeda *et al.*, 1995; Shinoda *et al.*, 1995). Impaired VMN development in *sf-1* -/- mice was explained as either a failure of VMN aggregation and organization or the regression of VMN neurons at late stages of development, analogous to the prominent cell death accounting for adrenal and gonadal agenesis in *sf-1* -/- mice (Ikeda *et al.*, 1995; Shinoda *et al.*, 1995). More recently, detailed histological analyses reported an expansion of glutamic acid decarboxylase (GAD) 67 and estrogen receptor (ER)α positive neurons in the presumptive embryonic *sf-1* -/- VMN, leading to the suggestion that VMN precursors either fail to aggregate, survive, or differentiate properly (Dellovade *et al.*, 2000). Collectively, these studies suggest that SF-1 could participate in multiple phases of VMN development; early phases would include the proliferation, survival, and migration of VMN precursors from the ventricular zone, while later stages would involve the aggregation and condensation of the VMN nucleus. At both early and late phases, SF-1 could also function to specify VMN cell fate.

Here we investigated prenatal VMN development in the *sf-1* +/+ and -/- mice using birth dating analysis, expression profiles of early and late VMN markers, and DiI neuronal

tracing. Our collective results provide evidence that functional SF-1 protein is needed for terminal differentiation of VMN precursors.

RESULTS

Cell proliferation is normal in the VMN of *sf-1* -/- mice.

VMN precursors are derived from neuronal stem cells located within the third ventricular zone of the diencephalon (Altman and Bayer, 1986). Consistent with this fact, we find that most BrdU pulse-labeled cells were SF-1 negative and were localized to the neuroepithelial layer lining the third ventricle (Fig. 1A). Further labeling for mitotic cells using an anti-phospho-histone H3 antibody confirmed that cell proliferation is restricted to the third ventricle neuroepithelium (data not shown). By contrast, all SF-1 positive cells resided immediately outside of the ventricular zone (Fig. 1A). We conclude that SF-1 is expressed in post-mitotic cells of the embryonic hypothalamus and is therefore unlikely to participate in the proliferative phase of VMN development. These findings contrast the role of SF-1 in adrenal development, where SF-1 is expressed in mitotically active cells and is required for normal proliferation (unpublished data and (Bland *et al.*, 2000)).

To assess the numbers and distribution of VMN precursors born at different ages in *sf-1* -/- mice, BrdU birth dating analysis was carried out on +/+ and -/- littermates. Previous work established that in mice VMN neurons are born between embryonic day (E) 10 and E15, peaking at E13 (Shimada and Nakamura, 1973). BrdU-positive VMN neurons were quantified by colocalization with SF-1 positive cells in +/+ mice and in the comparable

hypothalamic region for *sf-1* -/- mice, as shown in Fig. 1C, D. Equivalent numbers of BrdU labeled neurons were observed in the mediobasal hypothalamus for both genotypes at all stages of labeling, with the peak number of VMN neurons born between E11.5 and E13 (Fig. 1B), suggesting that the birth rate of VMN precursors is unaffected by the loss of SF-1. We also noted that VMN neurons were born in a ventrolateral progression during development; for example, neurons born early (E11) reside in the ventrolateral VMN, while those born later (E14) reside in the dorsomedial VMN (data not shown). Furthermore, the distribution of BrdU-labeled cells appeared normal in mutant mice implying that their migration is unaffected by the loss of SF-1. Taken together, these results show that SF-1 is not required for normal proliferation of VMN precursors, consistent with expression of SF-1 in post-mitotic cells.

VMN precursors persist in *sf-1* -/- neonates.

Previous studies proposed that the altered hypothalamic cytoarchitecture observed in *sf-1* -/- mice might arise from the complete loss of VMN neurons due to cell death (Ikeda *et al.*, 1995; Dellovade *et al.*, 2000). However, TUNEL analysis revealed no apparent cell death in the presumptive VMN of both +/+ and -/- mice from E11 through postnatal day (P) 0 despite our ability to detect TUNEL-positive cells in the developing olfactory epithelium, as previously reported ((Pellier and Astic, 1994; Voyron *et al.*, 1999), and data not shown). Based on these observations, we conclude that programmed cell death is unable to explain the apparent altered VMN morphology in *sf-1* -/- mice.

These findings predict that VMN precursors may be present in *sf-1* -/- mice. To study the fate of SF-1 positive cells, we analyzed expression of the *sf-1* mutant transcript targeted

in the *sf-1* -/- mice, which includes the first two exons and a modified third exon that contains the selectable marker gene, *neomycin* (Ikeda *et al.*, 1995). In situ hybridization studies revealed that *sf-1* expression was maintained in the *sf-1* -/- mice, and largely recapitulated the pattern observed in wild type mice (Fig. 2). However, no SF-1 protein is observed in the *sf-1* -/- mice due to disruption of third exon, which encodes the second zinc finger of the DNA binding domain (see insets). Although the boundaries of *sf-1* transcript expression were maintained in *sf-1* -/- mice, expression of the mutant transcripts decreased, especially in the ventrolateral VMN. Expression of the neuronal specific marker NeuN confirmed that the cells populating the presumptive VMN retain a neuronal identity. No labeling was detected with glial specific marker, GFAP in either wild type or mutant mice (data not shown). Thus, these data provide evidence that the absence of SF-1 does not preclude the birth, migration and condensation of VMN neurons.

Altered expression of early and late VMN markers in *sf-1* -/- mice.

Previous reports suggested that GABAergic neurons are expanded into the VMN as early as E15 in *sf-1* -/- mice (Dellovade *et al.*, 2000). Therefore, to examine a potential change in cell fate of VMN neurons, we analyzed *GAD67* expression by in situ hybridization in the VMN of *sf-1* -/- mice. Indeed, we noted increased expression of *GAD67* in the mutant VMN; however, this increased expression was still low relative to expression in neighboring parts of the hypothalamus (Fig. 3). Interestingly, the lowest expression of *GAD67* was found in the dorsomedial VMN of mutant mice, where we also noted the strongest expression of the *sf-1* mutant transcript (see above).

To examine more closely VMN differentiation in the *sf-1* mutant we used two molecular markers; the first is the homeobox gene *Nkx2-1*, which is expressed in basal telencephalic and ventral hypothalamic neurons (Kimura *et al.*, 1996; Sussel *et al.*, 1999; Marin *et al.*, 2002). The second marker is brain derived neurotrophic factor (BDNF), which was found previously to mark ventrolateral VMN neurons in adult mice (Kernie *et al.*, 2000). Using mutant mice that harbor *lacZ* in the *BDNF* locus ($BDNF^{lacZ}$), we observed prominent expression of $BDNF^{lacZ}$ (β-galactosidase) in the ventrolateral region of the anterior and medial VMN of adult mice that overlapped significantly with SF-1, especially in the anterior VMN (Fig. 4). During prenatal development, $BDNF^{lacZ}$ expression followed an anterior to posterior progression in the presumptive VMN and was not apparent until E17.5 (Fig. 5A). $BDNF^{lacZ}$ expression was markedly decreased in the VMN of *sf-1* -/- mice, as shown by the loss of β-galactosidase expression (Fig. 5B). However, $BDNF^{lacZ}$ was maintained in the substantia nigra in *sf-1* -/- mice (data not shown), implying that BDNF expression in the VMN is dependent on SF-1.

In contrast to the late onset of BDNF expression, NKX2-1 expression was observed early in the ventral hypothalamus and overlapped with SF-1 expression until E18.5, at which time its expression was greatly reduced in the VMN (Fig. 5A). At this stage, NKX2-1 expression was restricted to the ventral neuroepithelium and the ventrolateral area of lateral hypothalamus (vlLH) (Fig. 5A). Therefore, NKX2-1 is an early marker of VMN development that is downregulated as the VMN precursors differentiate into a morphologically distinct nucleus. In *sf-1* mutant mice, NKX2-1 expression persisted in

the presumptive VMN concomitant with reduced expression in the vlLH (Fig. 5B). Interestingly, SF-1 positive cells persist in *Nkx2-1* null embryos (O. Marin, unpublished). These preliminary results could imply that some and perhaps all VMN precursors arise from a distinct lineage other than NKX2-1. Alternatively, the presence of SF-1 positive cells in *Nkx2-1* null mice could also result from possible compensation activities of other NKX homeobox proteins, such as NKX2-2 and NKX2-4. Whether NKX2-1 and SF-1 function in the same or separate genetic pathways that govern VMN development remains to be determined. In summary, the expansion of *GAD67*, the persistence of NKX2-1, and the corresponding loss of BDNF in *sf-1* -/- mice suggest that SF-1 participates in terminal differentiation of VMN neurons.

VMN projections are lost in SF-1 -/- neonates.

The hypothesis that VMN neurons in *sf-1* -/- mice fail to differentiate properly was further tested by tracing afferent projections of this nucleus to other regions of the brain; these include projections to the paraventricular hypothalamus (PVN), bed nucleus of stria terminalis (BST), and amygdala (A) (Swanson, 1987). Consistent with previous reports, all of these known projections were identified in wild type mice using the neuronal tracer DiI (Fig. 6A-C) with a large number of fibers extending through the stria terminalis to innervate the BST and the amygdala (Fig. 6A-C). In contrast, *sf-1* -/- littermates showed a complete loss of VMN projections to both the BST and amygdala, but maintained limited projections to the anterior hypothalamus (Fig. 6D-F). Closer examination of these projections in mutant mice showed that these are bona fide afferent fibers that terminate at axonal junctions (Fig. 6G-I). Collectively, these data provide strong

evidence that SF-1 is required for VMN precursors to establish their respective neuronal connections.

DISCUSSION

SF-1 regulates multiple genes in the endocrine system and is essential for peripheral endocrine organ development. Although the function of SF-1 in adrenal, gonadal and pituitary function has been studied extensively, the role of SF-1 in VMN development is less well understood. Here, unexpectedly, our studies provide evidence that early stages of VMN development, which commence when neuronal precursors are born and migrate from the third ventricle neuroepithelium, are independent of SF-1 function. Two additional observations are consistent with this hypothesis: first, SF-1 expression is restricted to post-mitotic cells and, second, neurons in the presumptive VMN of *sf-1* -/- mice continue to express *sf-1* mutant transcripts. Instead, our findings showing misexpression of early and late molecular markers and a loss of neuronal projections in the *sf-1* -/- VMN, suggest strongly that SF-1 is required in late stages of VMN development. Moreover, no overt differences were noted between wild type and heterozygous *sf-1* mice for all parameters examined, including cell proliferation, cell death, expression of molecular markers, and neuronal projections (data not shown). Thus, the function of SF-1 in VMN development appears to be independent of gene dosage and is therefore distinct from its role in gonadal and adrenal development, where cell survival and cell proliferation required a full complement of SF-1 activity.

While our data suggest that VMN precursors fail to undergo terminal differentiation, it is unclear whether they are blocked from undergoing full differentiation or adopt an alternative neuronal cell fate. In wild type mice, normal repression of NKX2-1 and the subsequent expression of BDNF coincide with initial stages of VMN condensation. Therefore, the persistence of NKX2-1 and the loss of BDNF expression in *sf-1* -/- mice may indirectly imply that the final phases of VMN development are blocked. The loss of VMN afferent projections in *sf-1* -/- mice also implies that establishing the definitive VMN neuronal phenotype requires SF-1 activity. However, the presence of limited afferent projections from the mutant VMN area suggests that either terminal differentiation of a limited number of VMN neurons is not entirely dependent on SF-1 function, or alternatively, VMN precursors in mutant mice adopt neuronal fates of neighboring hypothalamic nuclei that also send axonal projections to the anterior hypothalamus. In this regard, Tobet and colleagues noted an expansion of neurons expressing ERα, galanin and neuropeptide Y in the presumptive VMN of *sf-1* -/- neonates (Dellovade *et al.*, 2000). Normally, these peptides are selectively expressed in the neurons of LH, DMN and ARC, respectively. Identification of additional markers for later stages of VMN differentiation should help to clarify the molecular events and regional specification underlying VMN development.

SF-1 and BDNF in Feeding and Locomotor Behavior

Our findings suggest that the normal BDNF expression in mature vlVMN neurons is dependent on SF-1. Whether SF-1 actively participates in regulating BDNF expression is not known at this time. Elucidating the exact mechanisms governing BDNF expression

has proven difficult given that four distinct promoter regions are found in the BDNF upstream genomic sequences (Timmusk *et al.*, 1993; Nakayama *et al.*, 1994) and it is not clear which of the four BDNF transcripts participates in the differentiation of vlVMN neurons. However, we have noted two potential SF-1 binding sites within the 5' regulatory region of transcript 4 (Liu *et al.*, 2001). Nevertheless, BDNF has been established to promote neuronal differentiation and survival both in vitro and in vivo (Jones *et al.*, 1994; Schwartz *et al.*, 1997), and thus it is possible that BDNF functions to promote the maturation of VMN neurons.

Previous ablation studies implicate the VMN as one of the homeostatic centers of energy expenditure. Although the precise molecular circuitry for this function has not been elucidated as for other centers, such as the arcuate and paraventricular nuclei, it is known that reduced locomotor activity leading to an obesity phenotype is observed in rats with reversible chemical VMN lesions (Choi *et al.*, 1998; Choi and Dallman, 1999). Parker and colleagues observed a similar phenotype in adrenal rescued *sf-1* -/- mice; these animals exhibit late onset obesity associated with a significant reduction in locomotor activity and normal overall food intake (Majdic *et al.*, 2002). Similarly, *BDNF* heterozygous mice also display abnormal locomotor activity and late onset obesity. However, the obesity phenotype observed in *BDNF* +/- mice results from hyperphagia and shows incomplete penetrance. Obese *BDNF* +/- mice exhibit normal locomotor activity, whereas non-obese *BDNF* +/- mice are thought to counteract this hyperphagia through hyperactivity (Kernie *et al.*, 2000). More recently, conditional inactivation of BDNF in adult mice also resulted in hyperactivity associated with increased anxiety-like

behavior (Rios *et al.*, 2001). BDNF is not expressed in the arcuate nucleus (Fig. 4A), suggesting that this hypothalamic center is unlikely to be involved in BDNF regulation of feeding and locomotive behavior. Although the functional significance of an altered BDNF expression pattern in the vlVMN of *sf-1*-/- mice remains to be established, the obvious links between the VMN to energy homeostasis and to conditioned responses is intriguing. Further studies are needed to decipher the possible interplay in the VMN between SF-1 and the other molecular determinants of energy homeostasis found in the VMN, such as BDNF, the leptin receptor, the tubby peptide and the ghrelin receptor (Kleyn *et al.*, 1996; Mercer *et al.*, 1996; Guan *et al.*, 1997; Wren *et al.*, 2000).

Orphan Nuclear Receptors in CNS Development

Based on our findings presented here, we can now add SF-1 to the subset of orphan nuclear receptors that control specific aspects of CNS development. For example, Nurr1, a member of the Nurr1/Nur77/Nor1 sub-family of receptors, appears to be essential for the migration, differentiation and survival of dopaminergic neurons of the ventral mesencephon (Zetterstrom *et al.*, 1997; Saucedo-Cardenas *et al.*, 1998; Le *et al.*, 1999; Wallen *et al.*, 1999). Similarly, the orphan nuclear receptor RORα is crucial for maturation and survival of Purkinje cells in the cerebellum (Dussault *et al.*, 1998; Chu and Zingg, 1999; Vogel *et al.*, 2000). Finally, the inhibitory orphan receptor, COUP-TFI is known to mediate neocortex identity as suggested by the loss of regional organization and specific gene expression in the absence of COUP-TFI function (Zhou *et al.*, 2001). In the absence of bona fide ligands, potential mechanisms for regulating these orphan receptors, include posttranslational phosphorylation and selective recruitment of

coactivator proteins, as shown for SF-1 (Hammer and Ingraham, 1999; Ito *et al.*, 2000; Desclozeaux *et al.*, 2002). Further insights into SF-1's function in the hypothalamus should be directly relevant to these other orphan nuclear receptors that function in neural development. In addition, our findings are complementary to the known role of the bHLH-PAS transcription factor, SIM1 in the development of several hypothalamic nuclei, including the PVN, supraoptic nucleus (SON), and anterior periventricular nucleus (aPV). All of these nuclei share a common precursor and SIM1 function is required for the terminal differentiation of PVN/SON/aPV precursors (Michaud *et al.*, 1998; Michaud, 2001). In the absence of SIM1, PVN/SON/aPV common precursors persist initially, but are subsequently lost upon terminal differentiation. Whether they undergo embryonic cell death or fail to fully differentiate has yet to be established. Nonetheless, these data suggest that the morphological identification of distinct hypothalamic nuclei will arise from distinct developmental genetic pathways in a cell autonomous manner.

In summary, we provide evidence that SF-1 regulates late stages of VMN development by showing that terminal differentiation and establishment of normal afferent projections in the VMN are dependent on SF-1 activity. Our findings in the VMN illustrate the versatility of this orphan nuclear receptor in regulating distinct developmental programs in multiple tissues of the neuroendocrine reproductive and stress axes. Future studies aimed at delineating the circuitry of the VMN by genetic tracing, and in eliminating the VMN by conditional ablation to avoid the early post natal lethality, should help to

elucidate how this hypothalamic region regulates complex homeostatic behavioral responses.

EXPERIMENTAL METHODS

Animals

The *sf-1* +/- mice (obtained from the Jackson Laboratory) were maintained on a C57BL/6J X FVB background and kept on a 12 hours light-dark cycle. *sf-1* +/- mice were bred to generate +/+, +/- and -/- embryos and were designated E0.5 on the morning when sperm plug was found. Newly born pups were collected immediately after birth and were designated P0. Embryos, P0 and adult mice were genotyped using genomic DNA isolated from tail tissue. Genotyping was determined by PCR using the following oligos: *sf-1*For (ACAAGCATTACACGTGCACC), *sf-1*Rev (TGACTAGCAACCACCTTGCC), and *neo*Rev (AGGTGAGATGACAGGAGATC). $BDNF^{lacZ}$ mice were maintained on a FVB background. The BDNF*lacZneo* ($BDNF^{lacZ}$) mouse strain was constructed by replacing the BDNF coding region, beginning at the initial methionine codon, with the *Escherichia coli* lacZ gene and the PGK*neo* selectable marker, as previously described (Bennett *et al.*, 1999). Transcription from the targeted genomic locus is predicted to produce active ß-galactosidase (ß-gal) protein rather than the neurotrophin. Genotyping of $BDNF^{lacZ}$ mice was determined using primers GTGCTGCAAGGCGATTAAGT (lacZN5-For) and GTGGAGTTCTGCTAATGAGA (MBDSA10-Rev) to detect the presence of LacZ insertion. $BDNF^{lacZ}$ heterozygous (+/-) mice were mated to *sf-1* +/- mice to generate compound heterozygous, which are viable and fertile. These mice were mated to *sf-1* +/- to generate both $BDNF^{lacZ}$ +/-; *sf-1* +/+

and *BDNF*^{lacZ} +/-; *sf-1* -/- mice. Newly born pups were collected and perfused with 4% paraformaldehyde (PFA) fixative for all subsequent analyses. All research with animals was performed according to guidelines of the UCSF Committee on Animal Research.

Histology and Immunohistochemistry

Dissected postnatal brains and embryonic heads were fixed in 4% PFA overnight at 4°C. For cryosectioning, fixed tissues were cryoprotected by infusion with 15% PBS-sucrose and followed by 30% PBS-sucrose overnight at 4°C. Tissues were embedded in OCT compound (Tissue Tek) and sectioned at 12 μm or 20 μm. For all vibratome sections, fixed tissues were rinsed once in PBS and embedded in 4% low melt agarose and sectioned at 100 μm using a Leica VT1000S vibratome.

Immunohistochemistry analyses were performed on sections equilibrated at room temperature, rinsed in PBS for 10 minutes, and permeabilized in PBT (PBS + 0.2% Triton X-100) for one hour. All sections were first incubated in blocking solution (10 mg/ml BSA in PBT) for 30 minutes, followed by primary antibody incubation overnight at 4°C. Sections were rinsed in PBT (3x) for 10 minutes and then incubated with fluorescence-conjugated secondary antibody for one hour at room temperature. Excess antibody was removed by rinsing sections in PBT for 10 minutes (3x), following by DNA staining with DAPI for 5 minutes. Finally, sections were rinsed again in PBS for 10 minutes (3x) and mounted. The following antibodies were used: rabbit anti-mouse SF-1 (Kindly provided by Dr. K. Morohashi 1:1000), rabbit anti-mouse NKX2-1 (1:1000, Biopat Biotechnologies), rat anti-BrdU (1:50, Harlan Sera-lab), rabbit anti-β-

galactosidase (1:1000, ICN Pharmaceuticals), mouse anti-neuronal nuclei NeuN (1: 500, Chemicon International) and mouse monoclonal GFAP (1:10000, Cymbus Biotech). Secondary antibodies used in this study include Alexa 488 conjugated goat anti-rabbit (1:200), Alexa 546 conjugated goat anti-rat (1:200, Molecular Probes).

TUNEL-positive cells were detected using a modified version of a method originally developed by (Gavrieli *et al.*, 1992). Briefly, brain sections (12 μm) were equilibrated to room temperature and rehydrated in PBS. Sections were permeabilized in PBT for one hour, rinsed twice in TdT buffer (30 mM Tris pH7.6, 0.024% $CoCl_2$, 30 mg/ml sodium cacodylate), and labeled with biotinylated dUTP using terminal transferase for one hour at 37°C. The labeling reaction was stopped by incubation in termination buffer (300 mM NaCl, 30 mM sodium citrate). Tissues were rinsed twice in PBT and incubated in blocking solution (10 mg/ml BSA in PBS) for 10 minutes. Biotin labeled DNA was detected by incubating tissue with fluorescence-conjugated streptavidin (Molecular Probes) diluted in PBS containing 1 mg/ml BSA for 30 minutes. Slides were then rinsed three times in PBT and mounted. All data was collected using a Bio-Rad confocal microscope.

BrdU labeling and neuronal birth-dating

For bromo-deoxyuridine (BrdU) labeling, BrdU solution (10 mg/ml in H_20, Sigma) was injected into the peritoneal cavity of pregnant mice (40 μg per gram of body weight). For birth-dating, injected pregnant mice were allowed to give birth and newly born pups were collected immediately and perfused with 4% paraformaldehyde (PFA) fixative. For shorter pulse labeling experiments, pregnant mice were sacrificed one hour after BrdU

injections, and embryos were collected and fixed in 4% PFA. Fixed tissues were sectioned and treated with 2N HCl at 37°C for 20 minutes to denature DNA prior to staining with BrdU antisera. BrdU labeled cells were counted using NIH Image software and inverted images. The number of digitally counted positive cells was confirmed by visual assessment to ensure appropriate parameter settings.

In situ hybridization

In situ hybridization was performed as described (Erskine *et al.*, 2000). Briefly, PFA fixed newborn brains were dissected from the cranium and sectioned using a vibratome. Sections were mounted (Superfrost plus, Fisher Scientific), dried overnight, and rehydrated in PBT. Sections were dehydrated followed by rehydration in a series of methanol-PBT (25%, 50%, and 100%) and rinsed in PBT followed by bleaching in 6% H_2O_2-PBT for one hour. Hybridization conditions followed exactly the protocol by Erskine et al. (2000) using DIG-labeled probes (Roche Chemical) made from the full length cDNAs encoding mouse *sf-1* and rat *GAD67*.

DiI implantation and labeling

Experiments were performed as described (Marin *et al.*, 2002) with minor modifications. In brief, sf-1 +/+ and -/- P0 littermates were cold anesthetized and perfused with 4% PFA. Brains were dissected and stored in 4% PFA. Crystals of the axonal tracer 1,1'–dioctodecyl-3,3,3',3'–tetramethyl-indocarbocyanine perchlorate (DiI, Molecular Probes) were inserted into the VMN of fixed brains by light dissecting microscopy using visual landmarks. Brains were kept in fixative at room temperature for 6 or 7 weeks to

allow sufficient diffusion of the DiI tracer. Brains were then embedded into 4% low melt agarose and sectioned at 100 μm using a vibratome. All sections were counterstained with Cytox Green (Molecular Probes). Data were collected and analyzed using a Nikon compound microscope equipped with a CCD camera.

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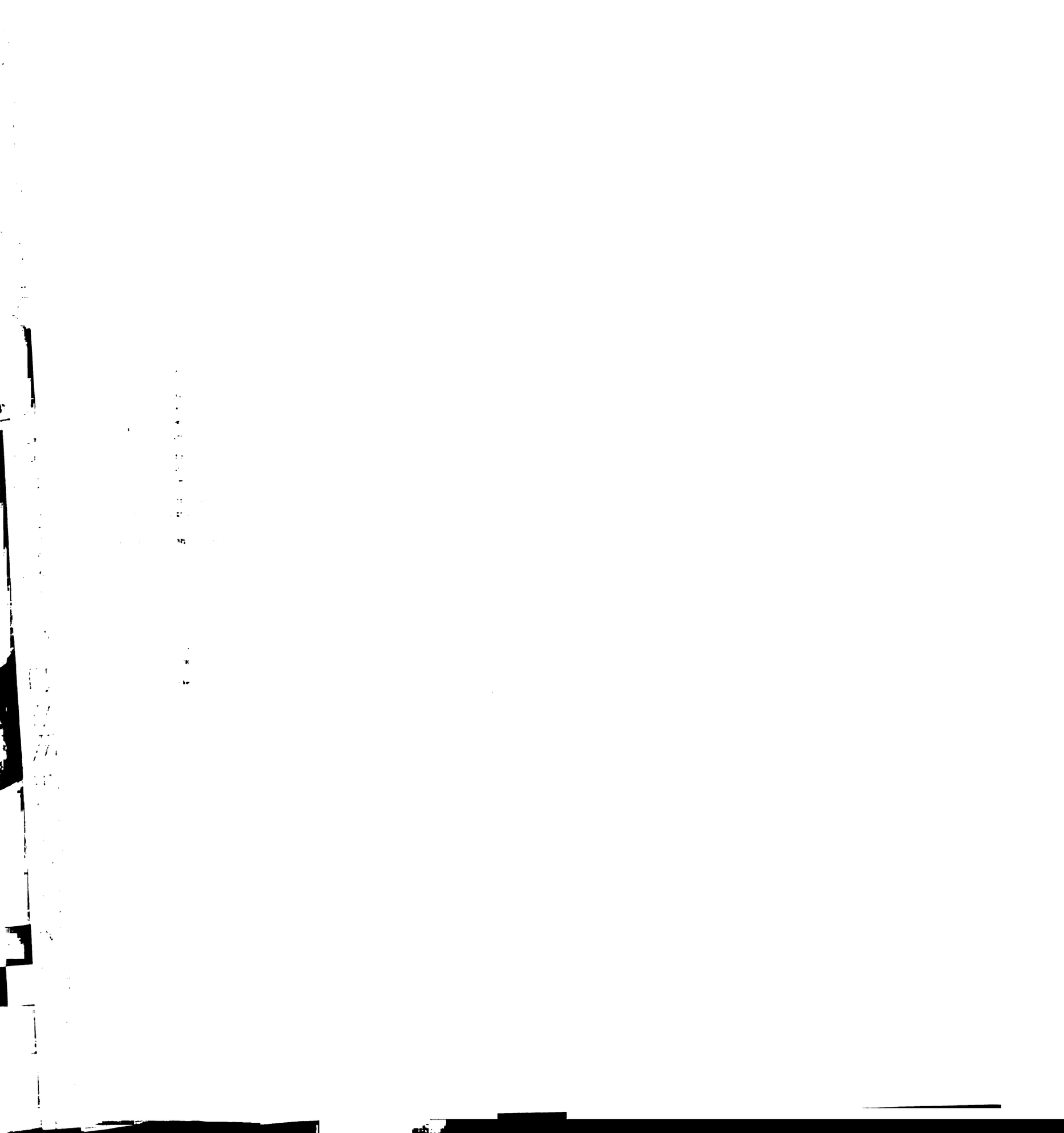
FIGURES

Figure 1. Normal cell proliferation is detect in SF-1 -/- mice

Coronal sections from embryonic day (E) 14.5 mouse embryos were stained with both anti-SF-1 and anti-BrdU antisera (panel A). SF-1 and BrdU signals were developed with the secondary goat anti-rabbit Alexa 488 conjugate (green) and secondary goat anti-rat Alexa 546 (red), respectively using confocal microscopy. BrdU labeled cells are mostly restricted to the third ventricle (3V) neuroepithelium (ne, arrowhead), whereas SF-1 positive cells are found outside of the neuroepithelium (arrow). Panel B shows the number of BrdU pulse-labeled neurons in the VMN plotted for different embryonic stages, spanning E10.5 to E15.5. The number of BrdU labeled cells was determined from comparable coronal sections obtained from both *sf-1* +/+ (yellow bars) and -/- (blue bars) mice. Equivalent sections were selected based on morphological landmarks surrounding the hypothalamus, including the fornix, the fasciculus retroplexus, the cerebral peduncle, and the arcuate nucleus. Four independent sections were counted for each developmental time point; data shown here represent the most medial VMN sections. A representative P0 section of each genotype is shown for BrdU-labeled cells (red) at stage E12.5 in



panels C, D. The VMN outlined with a white dashed circle, based on SF-1 expression (green) in the +/+ mice. Scale bars = 100 μm.

Figure 2: Mutant *sf-1* transcripts are present in the VMN of *sf-1* -/- mice.

In situ hybridization of *sf-1* mRNA is shown for both coronal (panels A, D) and sagittal whole brain sections (panels B, E). Expression of *sf-1* mRNA was restricted to the VMN of both wild type (+/+) and *sf-1* null (-/-) mice as indicated in whole brain sections or in enlarged sections of sagittal VMN regions (panels C, F). SF-1 protein staining is shown for corresponding sections as insets with the VMN region indicated (arrowhead). Abbreviations for anatomical landmarks are as follows: anterior commissure (ac), cerebellum (Cb), hippocampus (H), neocortex (NCx), olfactory bulb (ob), superior colliculus (SC), and the third ventricle (3V).

Figure 3. Increased *GAD67* expression in the VMN of *sf-1* -/- mice.

In situ hybridization of *GAD67* mRNA is shown for 100 μm coronal (panels A, C) and sagittal sections (panels B, D) of P0 mice. The VMN is indicated (arrow) in all panels of sections obtained from either wild type (+/+) or *sf-1* null (-/-) mice. Enlargements of the VMN area for both genotypes are shown as insets in panels B and D. Abbreviations for anatomical landmarks are as follows: cerebellum (Cb), hippocampus (H), neocortex (NCx), olfactory bulb (ob), superior colliculus (SC), and third ventricle (3V).

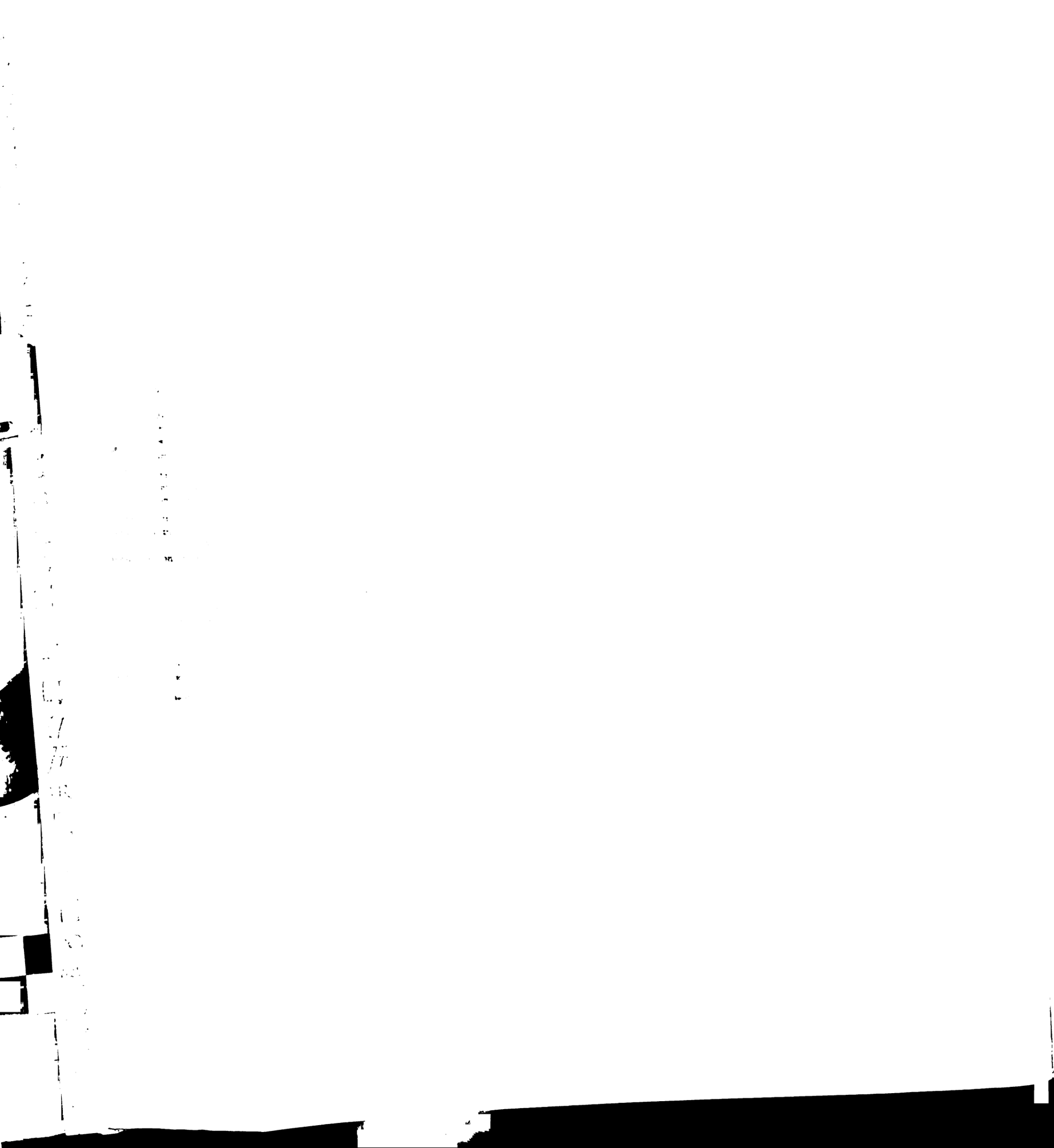


Figure 4. BDNF expression overlaps with SF-1 in the adult VMN.

Anterior (panel A) and posterior sections (panel B) of adult mouse mediobasal hypothalamus show both SF-1 (brown) and *BDNF*^{lacZ} (blue), as described in the Material and Methods. A dashed line outlines the area of SF-1 positive cells. Other hypothalamic regions indicated include the third ventricle (3V), the lateral hypothalamus (LH) and arcuate nucleus (ARC).

Figure 5. NKX2-1 and BDNF mark early and late VMN development, respectively.

NKX2-1 staining was carried out on coronal brain sections using rabbit anti-mouse NKX2-1 antibody (panel A). Staining is shown for wild type (+/+) mice at different stages of development (top panels). BDNF expression was examined using mice carrying a *lacZ* insertion at the *BDNF* locus (see Materials and Methods). Expression of ß-galactosidase was detected in *BDNF*^{lacZ} +/- mice using rabbit anti-ß-gal and developed with the secondary goat anti-rabbit Alexa 488 at different stages of development, beginning at E16.5 (middle panels). β-gal expression was found in the ventrolateral portion of both anterior and medial VMN as indicated by the dash line in (A) and (B). SF-1 staining in wild type embryos is shown for adjacent sections at each stage (bottom panels). Panel B shows NKX2-1 and BDNF^{lacZ} staining (red) for *sf-1* null (-/-) neonates (P0) and wild type littermates. A similar boundary for the VMN is assumed in both the wild type and mutant mice. By P0 NKX2-1 is completely excluded from the VMN in +/+ mice, while persisted in the VMN of -/- mice. In contrast, BDNF^{lacZ} is expressed in +/+ VMN, but is markedly lost in *sf-1* -/- VMN. For all panels, the relative position of

the VMN (dashed circle), as judged by SF-1 expression, and the arcuate nucleus (ARC) are indicated. Scale bar = 100 μm.

Figure 6. Loss of VMN projections in *sf-1* -/- neonates.

Coronal sections of brains implanted with DiI crystals are shown for either wild type mice (+/+, panels A-C) or *sf-1* null mice (-/-, panels D-F). Sections are shown in an anterior (left) to posterior (right) arrangement with nuclei stained with Cytox Green (green, Molecular Probes), and the DiI neuronal tracer (red). Stained projections from the VMN to the amygdala are indicated with arrows. Panels C and F show the central site where DiI crystals were implanted in the VMN (white arrowhead). Schematics illustrating the afferent projections emanating from the VMN in wild type and SF-1 mutant brains are shown on the far right hand side of panels A-F; the corresponding plane of sectioning for panels A-F are indicated above each schematic. Abbreviations for anatomical landmarks include the amygdala (A), anterior hypothalamus (AH), bed nucleus of stria terminalis (BST), caudoputamen (CPu), cerebral peduncle (cp), fasciculus retroflexus (fr), fimbria (fi), fornix (f), globus pallidus (GP), hippocampus (H), neocortex (NCx), paraventricular hypothalamus (PVN), stria terminalis (st).. Afferent fibers terminating on axons within the anterior hypothalamus are shown in Panels G-I (white arrowheads); each panel represents a higher magnification of the white-boxed area from Panel D. Scale bar = 10μm or 100x magnification

Figure 1

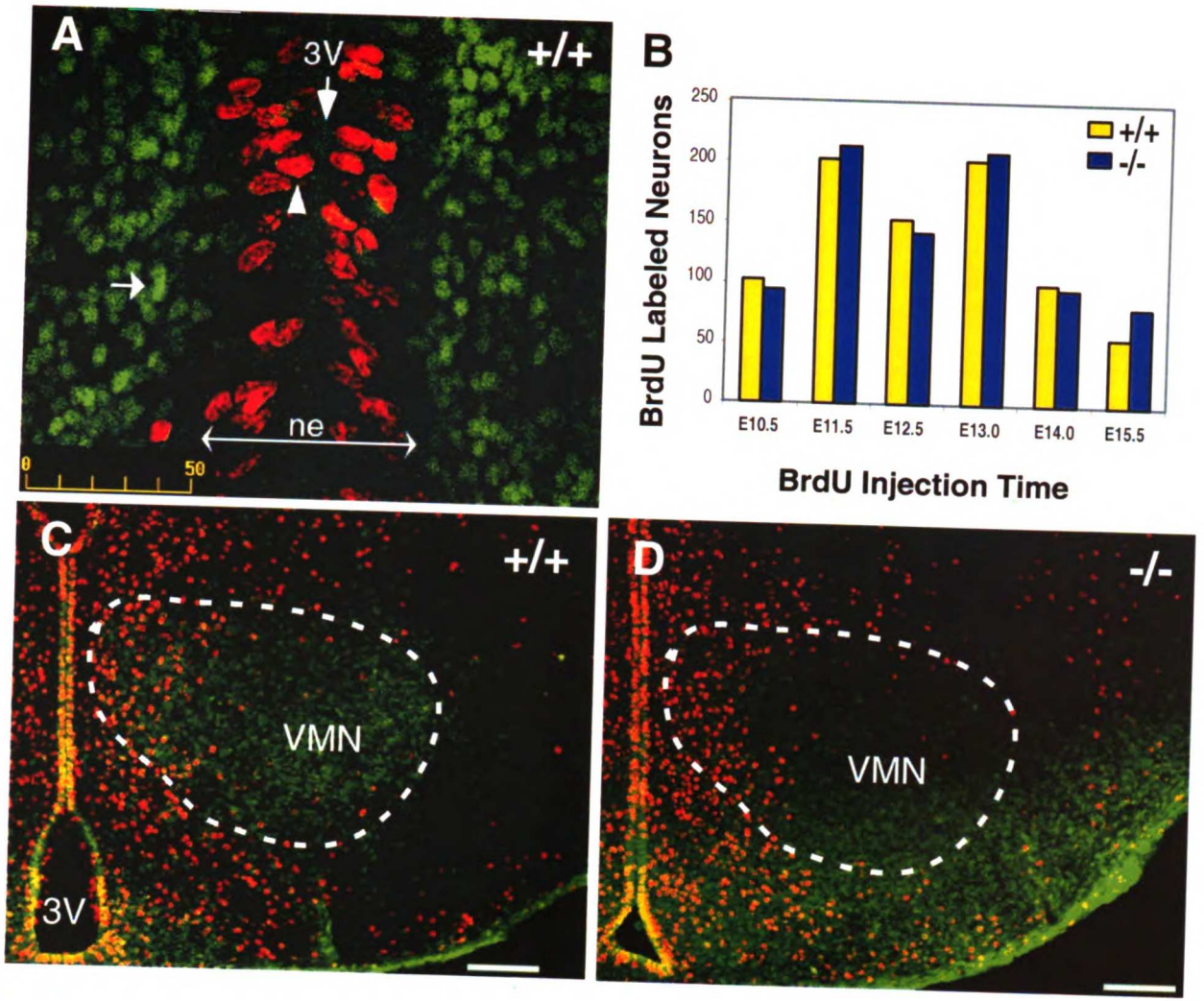

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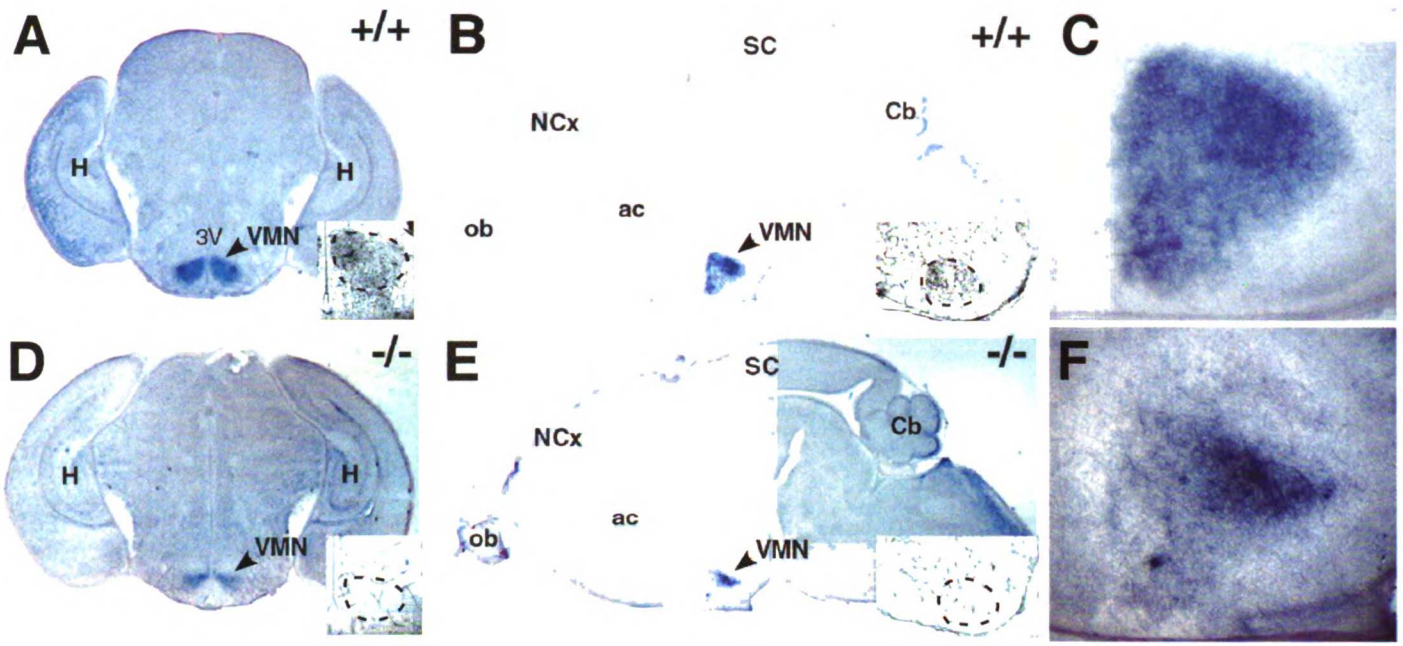
Figure 2



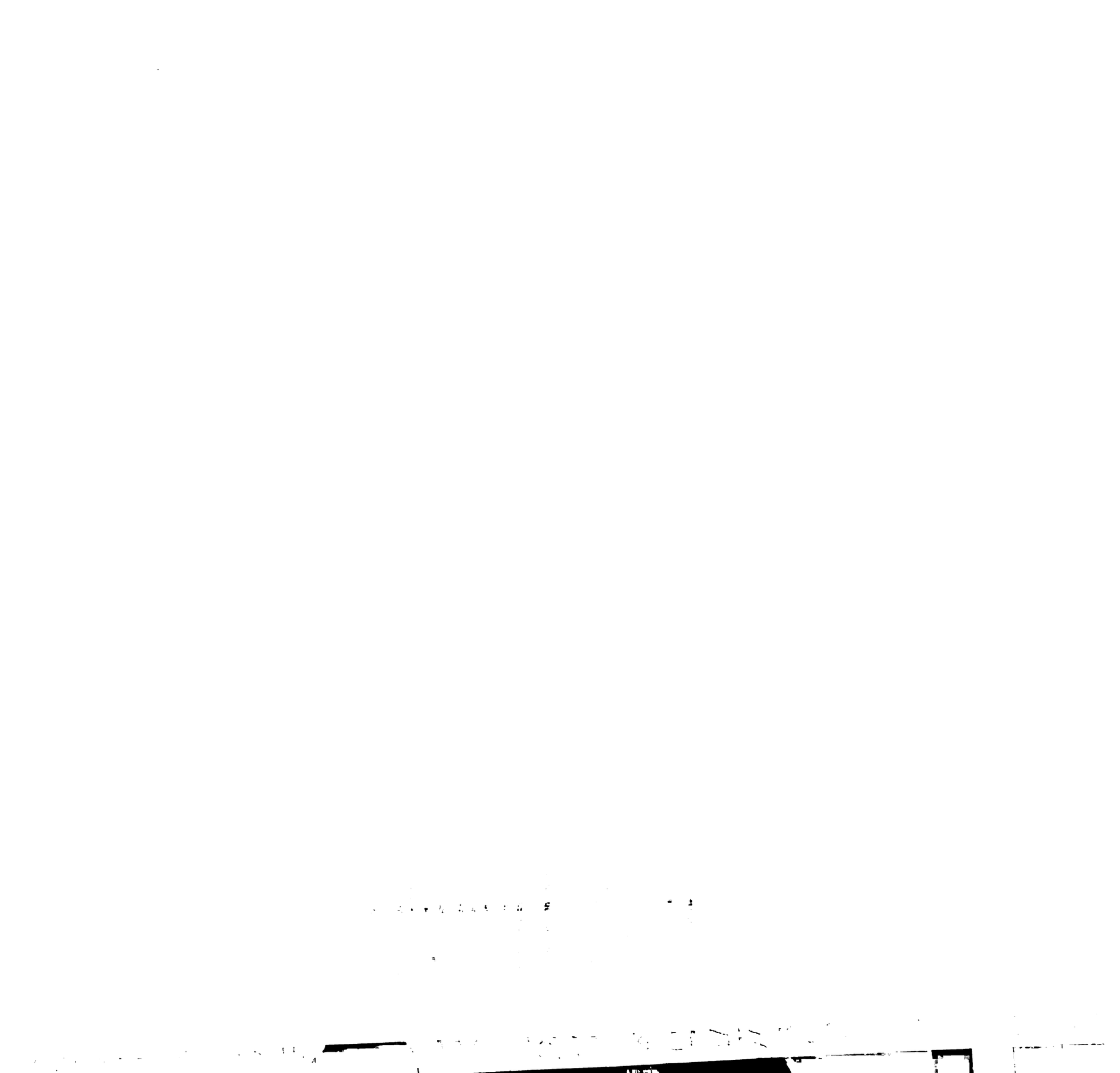


Figure 3

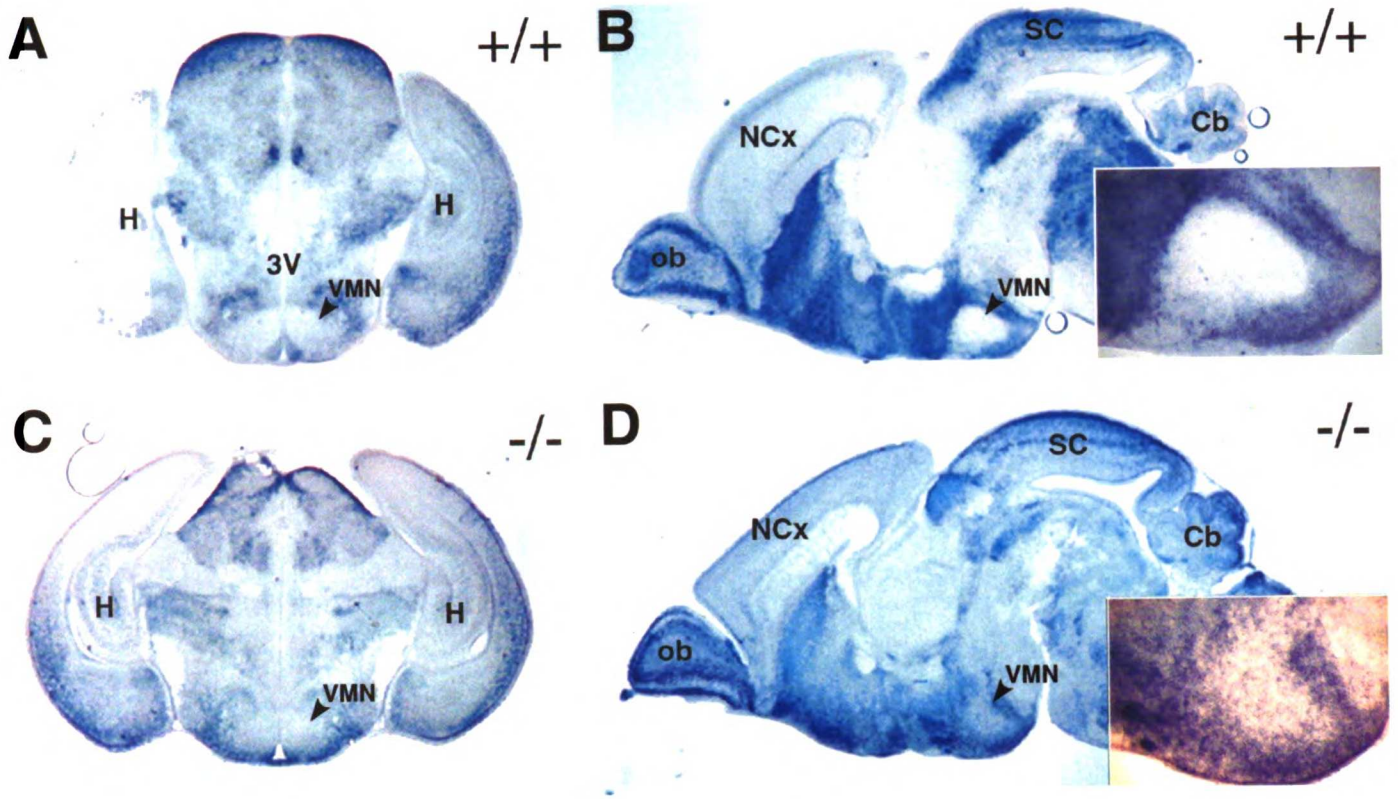


Figure 4

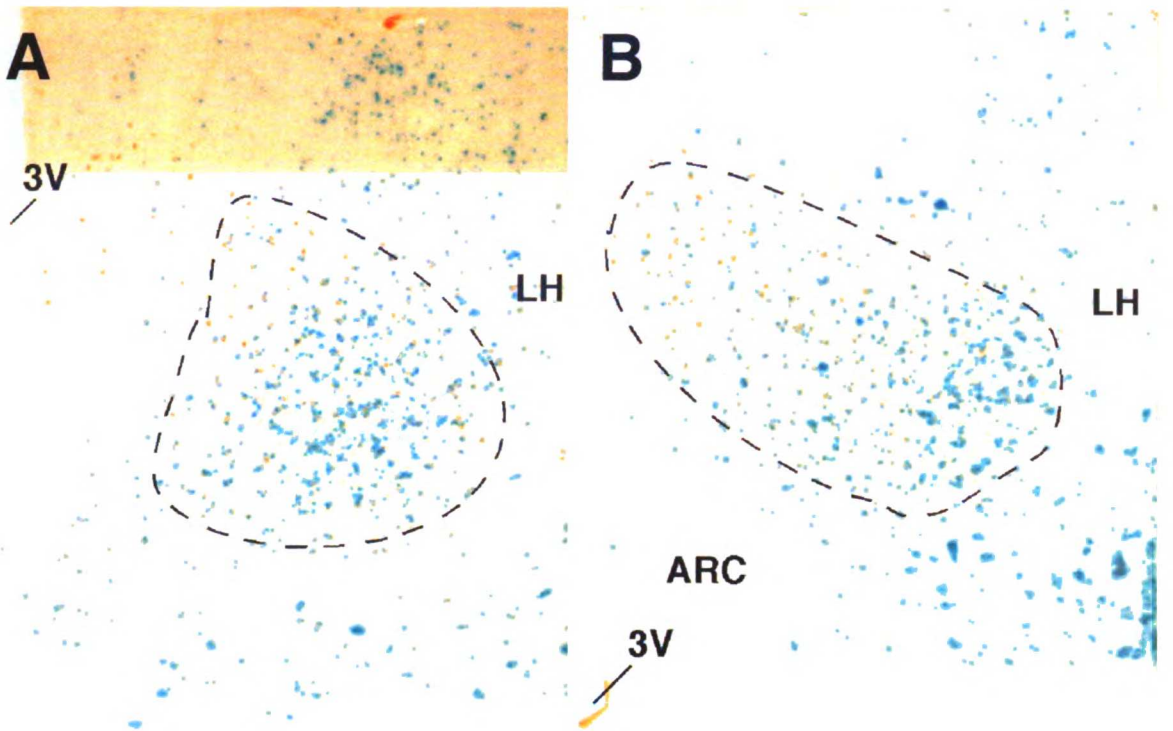


Figure 5

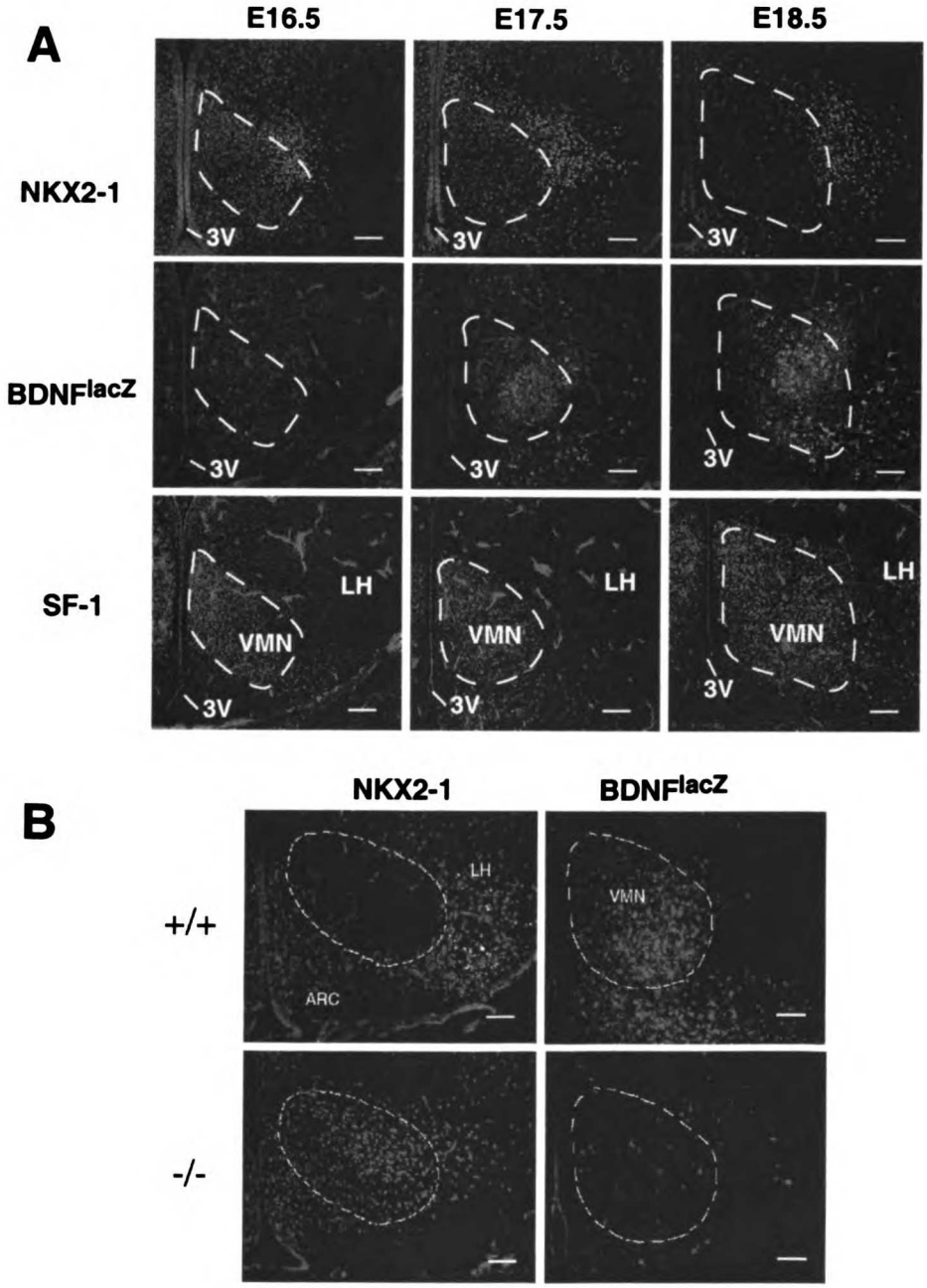

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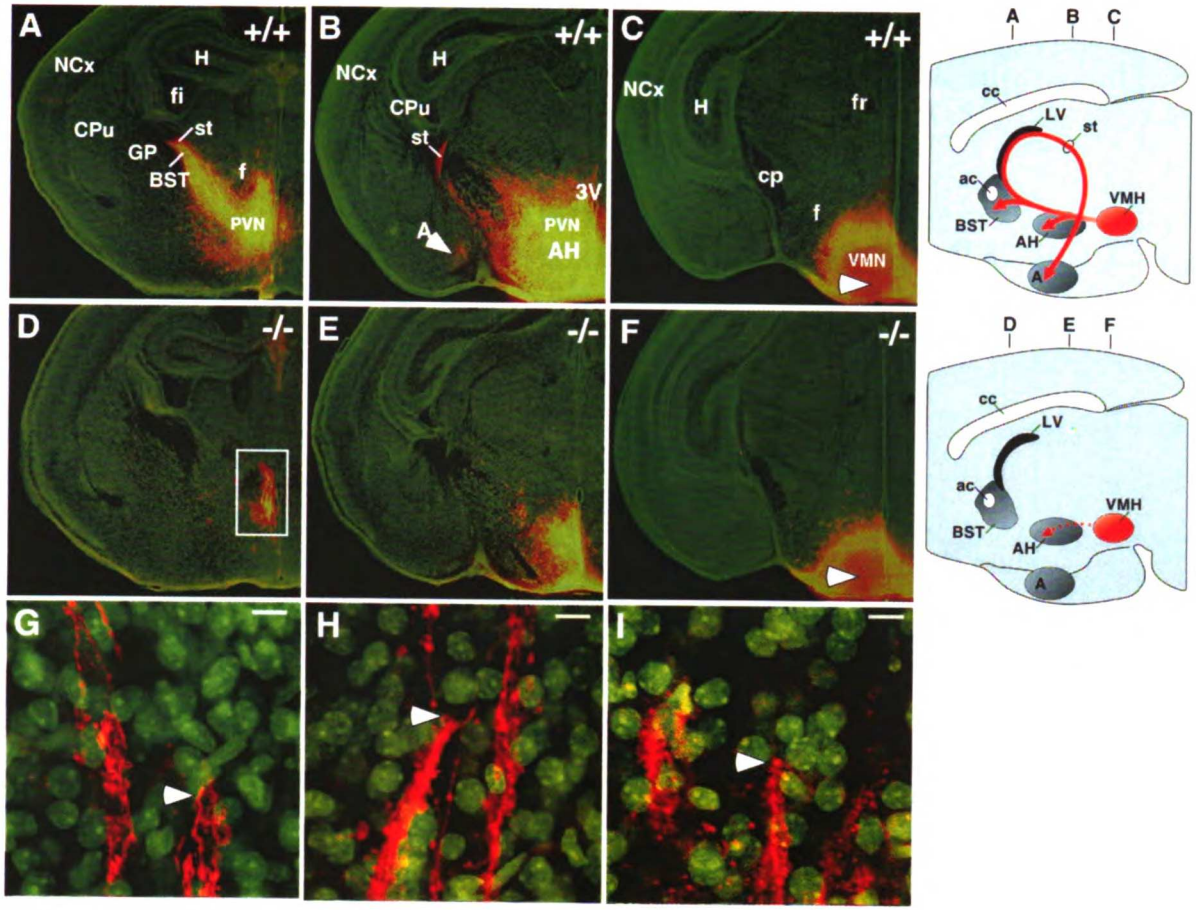
Figure 6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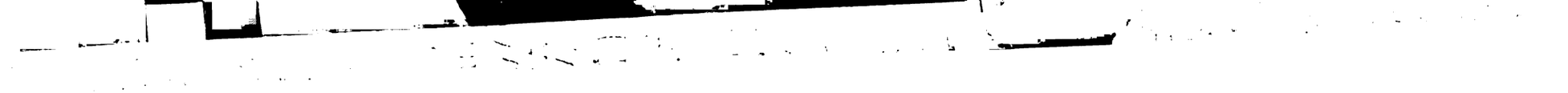
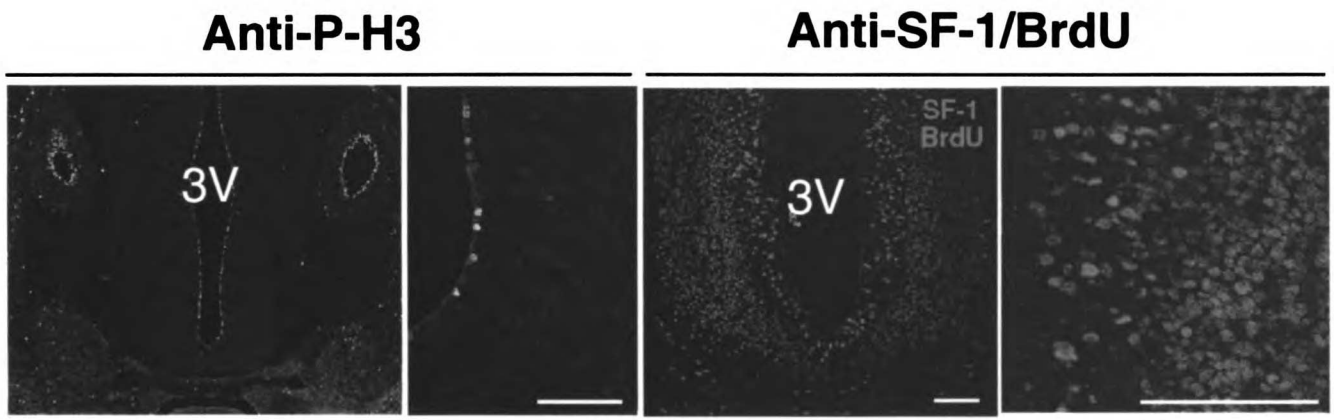
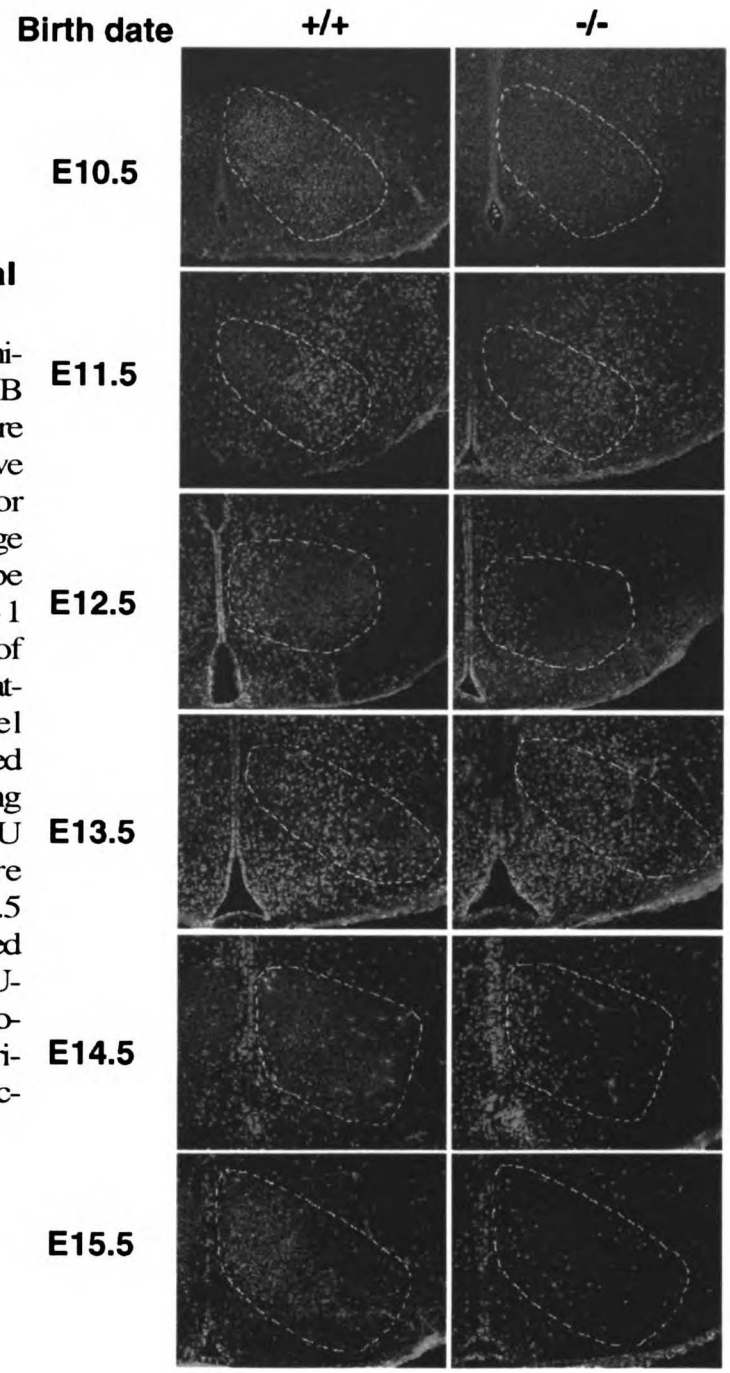


Supplemental Figure A

Staining of an E12.5 coronal section including the ventricular zone and the presumptive VMN is shown for both phosphorylated histone H3 (anti-P-H3) and for SF-1 (anti-SF-1). Comparison between BrdU labeled cells and phospho-Histone H3 shows that mitotically active cells are confined to the third ventricle neuroepithelium. An enlargement of each panel is shown to the right. Scale bars are 100 μm.



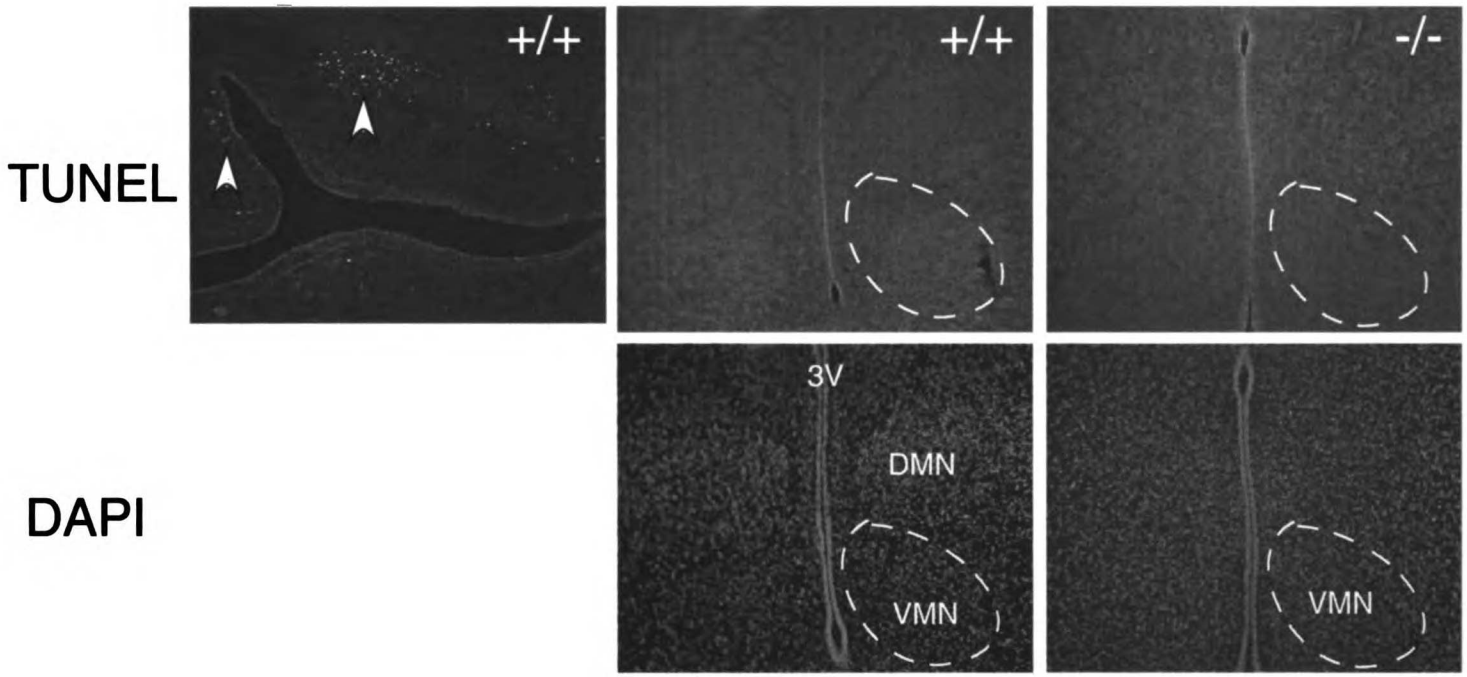


Supplemental Figure B

Data shown graphically in Figure 1B are also shown here with a representative section shown for each embryonic age for both wild type and mutant *sf*-1 mice. The area of the VMN is indicated in each panel with a white dashed area. SF-1 staining (green) and BrdU staining (red) are shown from E10.5 to E15.5. A detailed description of BrdU-pulse labeling is provided in the Materials and Method section.

Supplemental Figure C

TUNEL staining for the olfactory epithelium in wild type mice (left panel) shows positive cells (white arrowheads) and serves as a positive control for the absence of TUNEL-positive cells in E18.5 VMN as shown for both wild type *sf-1* (middle panel) and mutant *sf-1* mice (right panel). DAPI staining shows the approximate location of the VMN and dorsomedial nucleus (DMN), as indicated.



CHAPTER 4

DISCUSSION AND FUTURE DIRECTIONS

DISCUSSION AND FUTURE DIRECTIONS

I SUMO-modification of Subfamily V receptors

Recent literature has demonstrated the SUMO-modification of PPARgamma and all steroid receptors except ER. The work in this thesis, demonstrates that the Subfamily V orphan receptors SF-1 and LRH-1 are repressed by sumoylation at conserved SUMO sites in the distal hinge region,. In both these systems, attachment of SUMO1 to SF-1 displayed a clear preference for Lys194 over Lys119 despite the fact that both lysines resided within canonical SUMO motifs. In LRH-1, five SUMO sites are found. Two sites located in the hinge region (Lys213 and Lys289) are highly conserved within Subfamily V, and correspond to Lys119 and Lys194 of SF-1. The third site is at Lys329 (LKCE) in the LBD, and overlaps the C-terminal end of helix 1. The two remaining sites are in the N-terminal region (Lys34, PKRE and Lys65, PKVE) and are not conserved. Not unexpectedly, preliminary data indicate that Lys289 is the major sumoylation site for LRH-1 in overexpression and functional studies in COS-7 cells, recapitulating the role of Lys194 for SF-1. Experiments are underway to identify the acceptor lysines in LRH-1 for SUMO conjugation, and it is expected that only some of these five potential SUMO sites will prove to be utilized in vivo. Nevertheless, it is worth speculating that sumoylation at minor sites in SF-1 and LRH-1 may occur in vivo and may function in cross-talk between SUMO sites (Iniguez-Lluhi and Pearce, 2000; Poukka et al., 2000).

Our experiments demonstrated sumoylation of SF-1 and LRH-1 in cellular systems where both receptor and SUMO1 were overexpressed, as well as in an in vitro sumoylation assay using purified proteins, raising the question of whether endogenous SF-1 and LRH-

1 are sumoylated. Slower migrating bands consistent with sumoylated SF-1 were detected on Western blots of lysates from Y1 and aT3 cells prepared with NEM, using anti-SF1 and anti-phospho-Ser203 SF-1 antibodies. Confirmation that these slower migrating species represent sumoylated SF-1 requires an antibody suitable for immunoprecipitating endogenous SF-1, while the identification of sumoylation sites in endogenous receptor requires antibodies to SF-1 sumoylated at specific Lys residues, or construction of SF-1 knock–in mice containing Lys point mutations. Interestingly, preliminary immunohistochemical staining of the VMH of P0 wild-type mice with anti-SF1 antibody (from K. Morohashi) reveals localization of SF-1 in nuclear bodies in almost all SF-1 expressing neurons (Dr Phu Tran, unpublished observations). This observation may indicate sumoylation of endogenous SF-1 in the VMH, given that subnuclear localization of SF-1 in COS-7 cells correlated strongly with sumoylation.

Another issue is the role of DNA in SF-1 sumoylation and repression. It would be interesting to determine the effect of DNA on SF-1 sumoylation by adding SF-1 response element oligomers or SF-1 target promoter fragments to our in vitro sumoylation assays, and we can further extend these experiments to test the potential role of the helicase domain of DP103. We know from our ChIP analyses that the inability to sumoylate Gal4-SF-1 does not change DNA-binding, as evidenced by comparable binding of wild-type and all KR mutants of Gal4-hinge-LBD SF-1 to integrated Gal4 binding sites. However, we have not determined if the same holds true for the full-length receptor on native promoters. A recent study has shown that in gel-shift assays, sumoylated SF-1 is able to bind to oligomeric DNA containing SF-1 response elements (Komatsu et al.,

2004). It is possible that sumoylation may occur on DNA-bound SF-1 in order to shut down gene expression, or that sumoylated SF-1 might be targeted to promoters of genes that are to be silenced, perhaps by DEAD-box proteins. It is tempting to speculate that SUMO-mediated receptor repression may be promoter specific, as a means to modulate the spectrum of target gene expression within a larger physiological or developmental program. Such a hypothesis could be tested using both in vitro and in vivo sumoylation assays by including SF-1 target reporter constructs. The controls would include unrelated promoters and SF-1 target promoters containing mutated SF-1 response elements. Another approach would be to compare SF-1 target gene expression profiles generated from SF-1 2KR versus wild-type mice to identify candidate target genes that may be regulated via sumoylation.

Finally, there is the question of what impact the SUMO conjugate might have on receptor conformation. The main SUMO site at Lys194 of SF-1 lies within what is thought to be an unstructured hinge region, although it has been suggested that the region immediately adjacent to the SUMO site may possess some degree of ordered structure (R. Fletterick, personal communication). Nevertheless, it is plausible that SUMO conjugates at Lys 194 of SF-1 or Lys289 of LRH-1 in the distal hinge may form a composite interface with the adjacent highly ordered LBD, to recruit proteins that function in SUMO-mediated repression. If so, it would be interesting to determine if the SUMO conjugate affects helix 12 and recruitment of co-activators. Answering these questions will be facilitated by knowledge of the crystal structure of sumoylated hinge-LBD of SF-1.

II Sumoylation and phosphorylation.

Given the proximity of the major sumoylation site at Lys194 to the main phosphorylation site at Ser203, it was somewhat surprising that sumoylation and phosphorylation of SF-1 proved to be interdependent in COS7 cells. Whether this is true for endogenous SF-1 is unknown. It is clear that SF-1 can be simultaneously sumoylated and phosphorylated, but whether sumoylation or phosphorylation enhance each other remains to be seen. In connection with this, the K194R, but not the 2KR mutant of SF-1 displayed a significantly reduced phospho-Ser203 signal upon detection with anti-phospho-Ser203 antibody. Expression of wild-type and all KR variants in COS7 cells was comparable, as shown in Fig 1 of Chapter 2. These data suggest that the reduced phospho signal of the K194R mutant required Lys119. It is tempting to speculate that the SUMO conjugate at Lys194 protects Ser203 from dephosphorylation by a phosphatase that docks via a second SUMO conjugate at Lys119. We also need to exclude contributions from acetylation and methylation of Lys119 in this potential interplay between sumoylation and phosphorylation.

III Mechanism of SUMO-mediated repression by DP103

It is clear from the functional data that repression by DP103 required sumoylation at Lys194. However, the temporal and spatial mechanisms underlying SUMO-mediated repression by DEAD-box proteins remain unclear. Our interaction studies show that a GST fusion of the C-terminal fragment of DP103 (GST-DP103-C) binds to both sumoylated and unsumoylated SF-1, with a slight preference for the sumoylated form. This finding is in agreement with published data showing that the C-terminal fragment is

sufficient for repression of SF-1 (Yan et al., 2003). In addition, GST-DP103-C interacts comparably with both wild-type SF1 as well as all KR mutants, suggesting that Lys194 and its associated sumoylation are not essential, and that some of the interactions occur outside of the SUMO site at Lys194. Because of the size of DP103 (103 kDa), it is likely that its interaction with sumoylated SF-1 involves at least some contacts with the SUMO conjugate, even though preliminary pull-down experiments with His-SUMO1 did not support a strong interaction. This point needs to be clarified. It is possible that additional factors stabilize the DP103/SUMO-SF-1 interaction, one candidate being the E2 enzyme Ub9, which has been shown to bind to SF-1 as well as PIAS proteins. Experiments are underway to determine if DP103 and Ubc9 interact.

Lys194 may be dispensable for interaction between DP103 and SF-1, but it is clearly essential for repression by DP103, suggesting a key role for the SUMO conjugate in mediating repression. Exactly how the SUMO conjugate does so is not clear. Our experiments did not support a role for recruitment of HDACs, the AF2-dependent repressor Dax1, or the SF-1 repressors PSF and NonO. It is possible that the PIAS E3 ligase forms a platform with sumoylated receptor for assembling a repression complex. Indeed, PIASxa binds strongly to SF-1 and SUMO, and PIAS proteins are known to have scaffolding functions by virtue of their N-terminal SAP domain. Repressors that bind to PIAS proteins should therefore be tested for a potential role in SUMO-mediated repression by DP103.

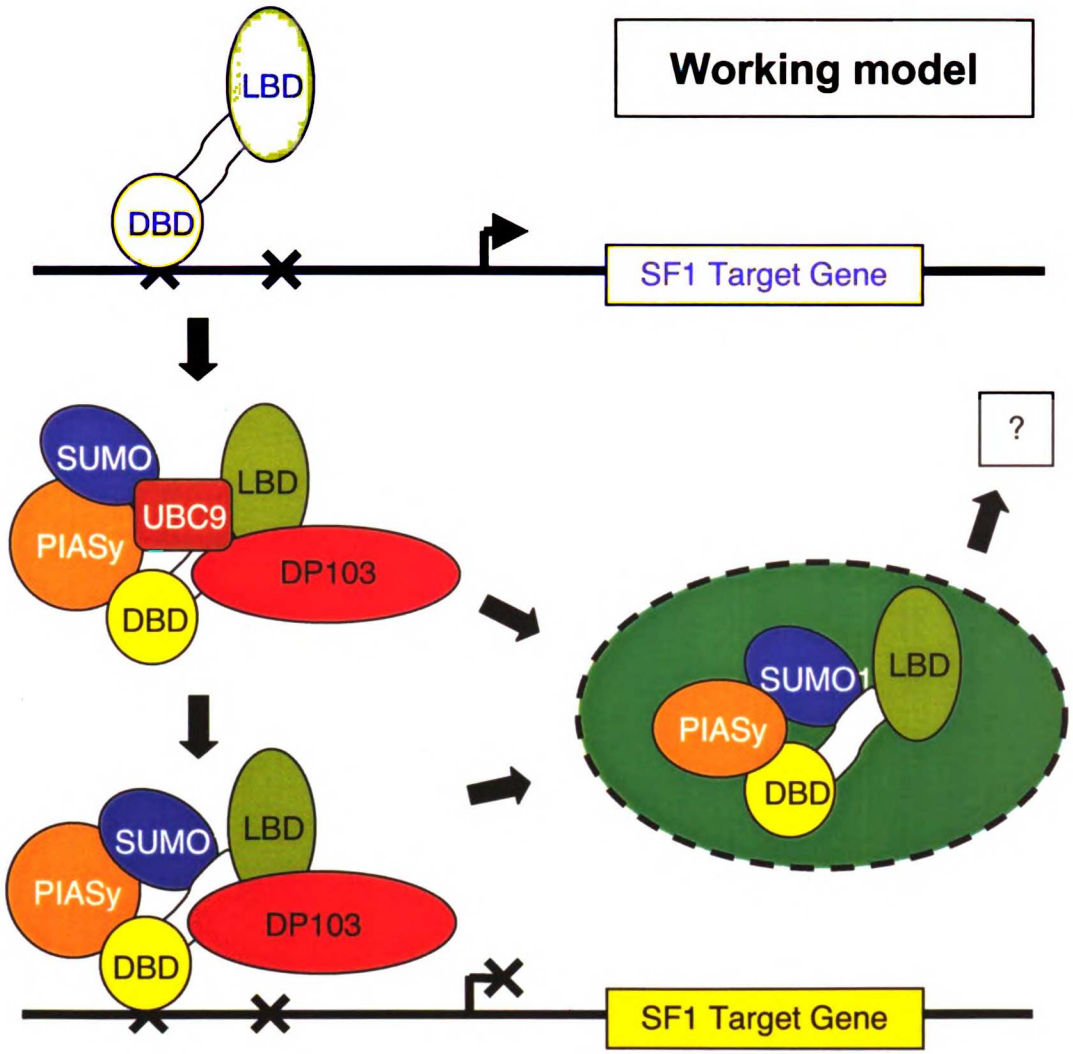
The data further showed that although DP103 was incapable of acting as an E3 SUMO ligase, it was able to increase the levels of sumoylated SF-1 when coexpressed with PIASxα and PIASy, which act as E3 SUMO ligases for SF-1. A similar but smaller effect of DP103 was also observed for PIAS1, which did not possess intrinsic E3 ligase activity for SF-1, suggesting that DP103 is able to facilitate such activity for competent E3 ligases. However, mammalian two-hybrid studies using Gal4-DP103 and VP16-PIASxα and VP16-PIAS1 failed to demonstrate interaction between DP103 and these PIAS proteins, while interaction with PIASy has yet to be tested. In these interaction studies, SF-1 was not co-expressed with Gal4-DP103 and VP16-PIAS; it is conceivable that SF-1, DP103 and PIAS form a complex that is stabilized by the receptor itself. This hypothesis is consistent with the fact that sumoylated SF-1 coimmunoprecipitated with DP103 in the presence of PIASy. The simplest explanation for the ability of DP103 to increase the level of sumoylated receptor is that DP103 protects the SUMO conjugate from cleavage by SUMO isopeptidases. Whether DP103 binds to SF-1 prior to sumoylation, or is recruited by sumoylated receptor is not known. Another possibility is that DP103 may be enhancing the E3 SUMO ligase activity of PIAS proteins indirectly through a bridging factor, such as the receptor itself, Ubc9, or an unidentified factor.

We turn now to the effect of DP103 to enhance PIASy-dependent subnuclear relocalization of GFP-SF-1 to nuclear bodies. We can summarize the data as follows: First, sumoylation is not required for relocalization and the sumoylation status of SF-1 in nuclear bodies is unknown. Second, DP103 is not sufficient for relocalization, even when excess SUMO1 is present. Third, PIASy is sufficient for partial relocalization, but

requires DP103 for full relocalization of GFP-SF-1 into discrete nuclear bodies. Finally, PIASy and GFP-SF-1, but not DP103, colocalize to nuclear bodies. Instead, DP103 has been observed to colocalize with SF-1 prior to relocalization, when SF-1 is more diffusely distributed throughout the nucleus (data not shown). This result is consistent with the weak interaction between DP103 and SF-1, and suggests that DP103 and SF-1 interact transiently, perhaps targeting SF-1 for PIASy–dependent relocalization. A major caveat is that these relocalizations were obtained in overexpression studies in COS-7 cells, where it is possible that high levels of DP103 or PIASy change the kinetics of intranuclear SF-1 distribution, revealing prominent nuclear body formation that may never occur under physiological conditions. It is therefore important to verify the preliminary observations of SF-1 nuclear relocalization in VMH neurons mentioned previously, and to determine if similar relocalization occurs in other SF-1 target tissues.

Despite these reservations, the data clearly show a strong correlation between subnuclear relocalization of SF-1 and conditions that favor sumoylation. However, relocalization does not require sumoylation, as evidenced by relocalization of all KR variants of SF-1 into prominent nuclear bodies. This result recapitulates that seen with Lef1 where relocalization is PIASy-dependent, but independent of sumoylation of Lef1 (Sachdev et al., 2001). The data raise several questions: What is the relationship between relocalization and sumoylation? Is sumoylation occurring within or in transit to nuclear bodies? This question would be best addressed by immunohistochemical studies with an antibody to sumoylated SF-1, or by detecting sumoylated SF-1 using a bipartite fluorescent protein assembly assay where one half of the fluorescent protein is fused to

SUMO and the other is fused within the receptor hinge. Since sumoylation confers repression, one wonders if relocalization and repression are correlated. It is noteworthy that DP103 is sufficient to repress SF-1 in transient transfection assays, but is not sufficient for relocalization. Furthermore, only 10-15% of GFP-SF-1 expressing cells in a given transfection show prominent relocalization in the presence of DP103. The remaining cells display a range of phenotypes ranging from diffusely distributed receptor to prominent nuclear bodies, suggesting that relocalization is dynamic and that DP103 changes the kinetics of relocalization to accentuate nuclear body formation. It would therefore be interesting to correlate the extent of relocalization with the transcriptional activity of GFP-SF-1 on a single cell basis. Finally, how does DP103 enhance relocalization and is the helicase domain involved? Experiments are underway to test ATPase/helicase mutants of DP103 for their ability to enhance relocalization and to repress receptor transcriptional activity.


Working model
LBD
DBD
SF1 Target Gene
SUMO
LBD
UBC9
PIASy
DP103
DBD
?
SUMO1
LBD
PIASy
DBD
SUMO
LBD
PIASy
DP103
DBD
SF1 Target Gene

IV Model

One working model that satisfies the data is that DP103, SF-1, PIASy and possibly Ubc9-SUMO form a stable complex that facilitates receptor sumoylation. DP103 then detaches, leaving PIASy to shuttle sumoylated SF-1 to nuclear bodies. In agreement with this model, DP103 did not colocalize with PIASy and SF-1 in nuclear bodies. As mentioned earlier, it is not known whether SF-1 is sumoylated before, in transit to, or after relocalization into nuclear bodies; all three possibilities are consistent with this model. In this model, the helicase domain of DP103 acts as an RNPase, catalyzing DP103 detachment by dissociating protein interactions and rearranging the complex. Activation of the ATPase/helicase/RNPase function may well be RNA-dependent, and it is tempting to propose that SF-1 target gene transcripts act as the RNA signal, thus linking target gene transcription and receptor silencing in an autoregulatory feedback loop.

V Biological significance

Our ultimate goal is to understand the function of sumoylation within the living organism. Numerous studies have examined the role of sumoylation in yeast, but to date few studies in higher eukaryotes have been published. Recently, Zhang et al showed in *C. elegans* that sumoylation of the RNA-binding PcG protein SOP-2 resulted in Hox gene repression (Zhang et al., 2004). Two recent studies used *Drosophila* as a model to study the role of sumoylation in Huntington's disease (Steffan et al., 2004), and in polyglutamine toxicity of the androgen receptor in spino-bulbar muscular atrophy (Chan et al., 2002). These studies illustrate the utility of animal systems for investigating

sumoylation of proteins implicated in the pathogenesis of human disease. The next few years will likely see the development of mice expressing sumoylation defective proteins and tissue specific inducible defects in the sumoylation pathway. For Subfamily V receptors, a knock-in mouse model expressing K194R or 2KR point mutants of SF-1, if viable, would clarify the in vivo role of SF-1 sumoylation. From the transient transfection data in this thesis, the straightforward prediction is that these would be gain-of-function mutants. Conversely, mice expressing SUMO-SF-1 chimeric fusion proteins are expected to be loss-of-function mutants, assuming that the chimeric proteins are stable. Microarray analysis of such mutant mice may identify SF-1 target genes that are regulated via sumoylation and provide starting points for unraveling SF-1 dependent processes such as VMH differentiation, body weight regulation and adrenal morphogenesis.

Assuming that sumoylation of Subfamily V receptors is physiologically relevant, a major question would be how receptor sumoylation is regulated. Our data show that receptor sumoylation levels can be modulated, and that SENP1 is a potent SUMO isopeptidase for SF-1, suggesting that receptor sumoylation in vivo must be a highly dynamic and tightly regulated process. Are there SF-1 or LRH-1 specific E3 SUMO ligases or SUMO isopeptidases? Interestingly, although DP103 is not itself an E3 ligase for SF-1, its ability to enhance PIAS-mediated SF-1 sumoylation suggests that one of its functions may be as an accessory factor for PIAS E3 SUMO ligase activity. It would not be surprising if other DEAD-box proteins were identified that perform this function for Subfamily V receptors. As alluded to in the Overview, GRTH is one such candidate

within the testes. As a first step, it would be useful to determine if GRTH and SF-1 expression colocalize during gonadal development and whether GRTH expression is sexually dimorphic. Finally, it might be worthwhile performing homology searches using the very C-terminal end of DP103 (aa721-825) that has been shown to repress SF-1 (Yan et al., 2003), in order to identify potential DEAD-box candidates for Subfamily V repression.

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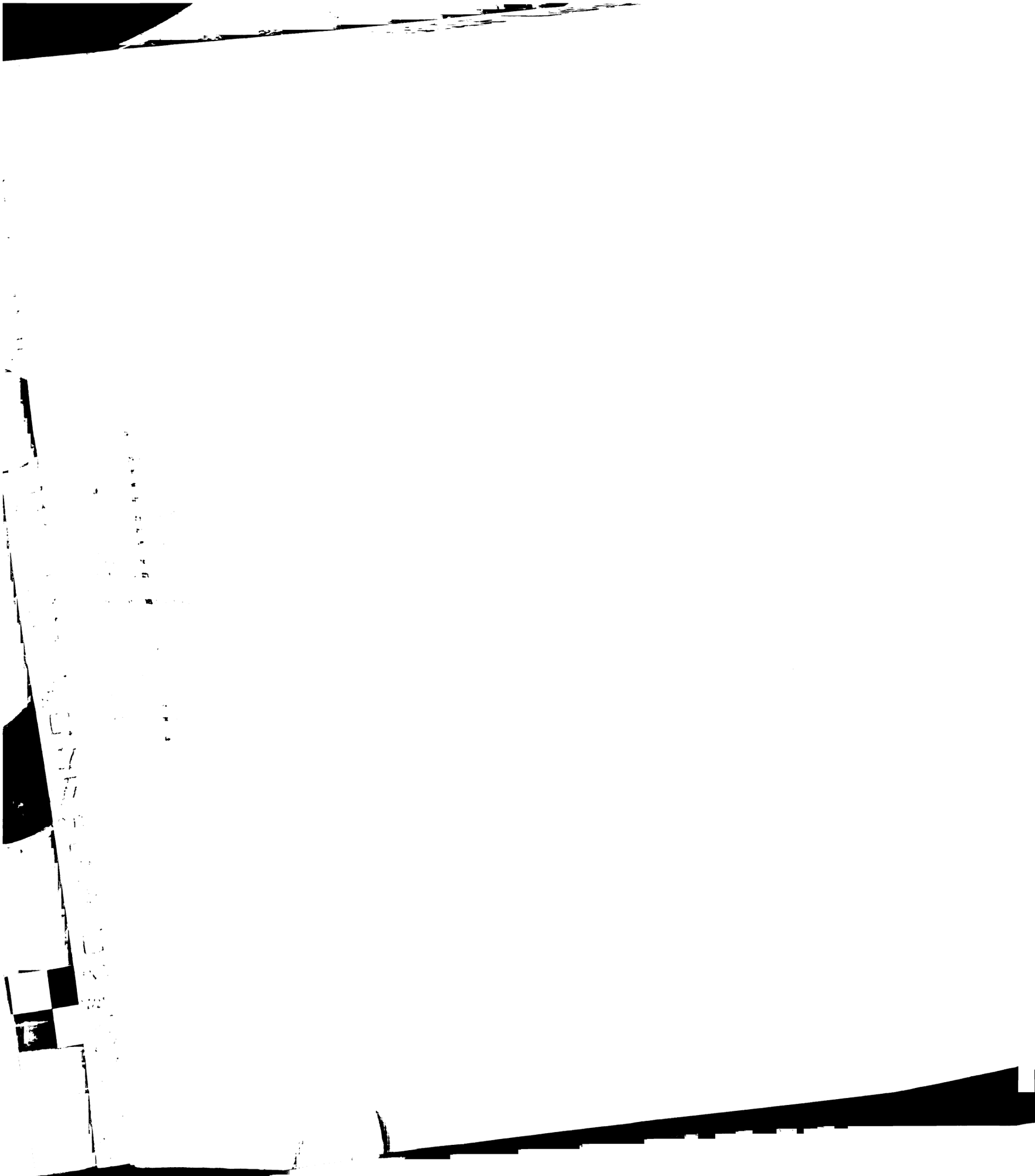

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