The transcription factor Krox20 is an E3 ligase that sumoylates its Nab coregulators

Pablo García-Gutiérrez[1], Francisco Juárez-Vicente[1], Francisco Gallardo-Chamizo[1], Patrick Charnay[2] and Mario García-Domínguez[1+]

[1]CABIMER (CSIC) Av. Americo Vespucio, E-41092, Seville, Spain.

[2]IBENS, Inserm U1024, CNRS UMR 8197, Paris Cedex 75230, France

[+]Corresponding author. Tel: +34 954 468201; Fax: +34 954 461664; E-mail: mario.garcia@cabimer.es

Count: 28,702

Krox20 mediates sumoylation of Nab proteins

ABSTRACT

Covalent attachment of sumo to proteins regulates multiple processes in the eukaryotic cell. This reaction is similar to ubiquitination and usually requires an E3 ligase for substrate modification. However, only a few sumo ligases have been described to date, which frequently facilitate sumoylation by bringing together the sumo-conjugating enzyme Ubc9 and the target protein. Ubc9 is an interaction partner of the transcription factor Krox20, a key regulator of hindbrain development. Here, we show that Krox20 acts as a sumo ligase for its coregulators -the Nab proteins- and that Nab sumoylation negatively modulates Krox20 transcriptional activity in vivo.

Krox20, Nab, sumo ligase, Ubc9

INTRODUCTION

Sumoylation consists in the post-translational modification of proteins by the small ubiquitin-like modifier (sumo), and regulates many processes in the eukaryotic cell (Gareau and Lima, 2010). Sumo is a 100-amino acids polypeptide that is covalently attached to the ε-amino group of a Lys residue of target proteins, commonly included in the consensus ΨKxE/D (Ψ: large hydrophobic residue). Reaction, similar to ubiquitination, involves transfer from the conjugating enzyme Ubc9 to the target protein and frequently requires the concourse of a sumo ligase. They often facilitate transfer by simultaneously binding Ubc9 and target protein. In comparison with ubiquitination, very few sumo ligases have been described so far (Gareau and Lima, 2010). Indeed, identification of new sumo ligases still remains an open challenge, and will shed light on the mechanisms and regulation of the sumoylation process.

From a previous two-hybrid screening we identified the sumo-conjugating enzyme Ubc9 as a partner of the transcription factor Krox20 (Garcia-Dominguez *et al*, 2006), a key regulator of hindbrain development (Schneider-Maunoury *et al*, 1993), where it participates in the control of the expression of a variety of genes, including its own and genes encoding its coregulators Nab and the tyrosine kinase receptor EphA4 (Desmazieres *et al*, 2009). Development of the vertebrate hindbrain involves a transient segmentation process that generates 7 to 8 segments or rhombomeres (r) along the anterior-posterior axis (Lumsden and Krumlauf, 1996). *Krox20* is expressed in r3 and r5 and is required for the formation and maintenance of these segments (Schneider-Maunoury *et al*, 1993; Voiculescu *et al*, 2001). Besides three zinc fingers for DNA binding, Krox20 contains the R1 motif, the surface for interaction with Nab1 and Nab2, initially identified as corepressors (Russo *et al*, 1995; Svaren *et al*, 1996). It has been

reported that the repressive activity of Nab2 is due in part to interaction with CHD4 (Srinivasan *et al*, 2006).

In this report we show that Krox20 functions as a ligase for the sumoylation of its coregulators, the Nab proteins. Sumo modification of Nab2 negatively modulates Krox20 transcriptional activity. Thus, sumoylation adds to the list of mechanisms involved in Krox20 autoregulation.

RESULTS

Krox20 interacts with Ubc9

From a previous two-hybrid screening based on Krox20 (Garcia-Dominguez *et al*, 2006) we isolated 7 clones corresponding to the sumo-conjugating enzyme Ubc9 (Fig 1A). Pull-down experiments with purified GST or a GST-Ubc9 fusion, and in vitro translated Krox20, demonstrated direct and specific interaction between Krox20 and Ubc9 (Fig 1B). We mapped the interaction surface in Krox20 by yeast two-hybrid. Analysis indicated that the zinc finger domain was necessary and sufficient for Ubc9 binding (Fig 1C).

Krox20 acts as a sumo ligase

Protein interaction with Ubc9 often leads to sumoylation of the interacting protein. However, we have previously reported that Krox20 is not sumoylated (Garcia-Dominguez *et al*, 2006). Ubc9 also interacts with sumo ligases to facilitate sumoylation of target proteins. We therefore speculated that Krox20 might recruit Ubc9 to function as a ligase in the sumoylation of other proteins, and the Krox20 coregulators Nab1 and Nab2 (Russo *et al*, 1995; Svaren *et al*, 1996) represent good candidates. Indeed amino acid sequence analysis revealed two conserved sumoylation consensus sites in each protein. A two-hybrid assay did not reveal direct interaction between Nab2 and Ubc9 (not shown).

To test sumoylation of Nab proteins we performed sumoylation assays in 293T cells transfected with flag-tagged Nab expression constructs and analyzed the cell extracts by Western blot. Transfection of Nab2 resulted in detection of a single band. However, when Nab2 and Krox20 expression vectors were cotransfected, up to 3 additional bands were observed, consistent with the presence of two sumoylation

consensus sites (Fig 2A). Nab1 was also sumoylated in the presence of Krox20 (supplementary Fig S1A online). Since Nab1 and Nab2 are highly homologous and have been shown to display similar functions (Svaren *et al*, 1996), we restricted the following experiments to Nab2, referred to as Nab. In the cell, sumo1 is mostly bound to proteins, resulting in a reduced free sumo1 pool (Gareau and Lima, 2010). The addition of low amounts of a histidin-tagged sumo1 (His-sumo1) expression vector in cotransfections resulted in increased modification of Nab (Fig 2A). Overexpression of the different PIAS proteins did not significantly modify sumoylation (data not shown). GFP-sumo1 was also efficiently conjugated to Nab (Fig 2B), but not His-sumo2 (supplementary Fig S1B online). In addition, Nab sumoylation was prevented by a dominant negative version of Ubc9 (C93S) (Fig 2C). Nab was specifically modified in the presence of Krox20 since a non-related transcription factor (neuroM) had no effects (Fig 2C). As expected, double mutant of putative target Lys (K379RK517R (KR2)) was not sumo-modified by Krox20 (Fig 2D). The mutation did not affect Nab nuclear localization nor its interaction with Krox20 (supplementary Fig S2 online).

We next investigated the involvement of Krox20-Nab interaction in Nab sumoylation. The single mutation I268F in Krox20 and the double mutation Q64RH95Q in Nab have been shown to abrogate Krox20-Nab interaction (Svaren *et al*, 1998). We found that both mutations prevented sumoylation (Fig 2E). In contrast, neither Krox20 nor the I268F mutant showed any effect in general sumoylation as monitored by modification of the C-terminal part of RanGAP1 (RanGAP1-C-ter), as a control (Fig 2F).

To investigate sumoylation of endogenous Nab we used P19 cells, as they express Krox20 and Nab. In this line, constitutive levels of Nab protein were detected under normal growth conditions, whereas *Krox20* expression required serum

stimulation (Fig 2G and supplementary Fig S3 online). We were not able to observe sumoylation of endogenous Nab with endogenous sumo1. Then, we decided to transfect P19 cells with low amounts of the His-sumo1 expression vector to analyze Nab sumoylation after pull-down of the His-tagged products. As shown in Fig 2G, whereas Nab sumoylated forms could not be detected in the input (1.5% of the total), they were observed in the precipitates. Moreover, the presence of these bands was strongly reinforced upon serum stimulation, i.e. upon induction of Krox20 (Fig 2G). Sumoylation of endogenous Nab was also observed upon transfection of a Krox20 expression vector without serum stimulation (Fig 2H). Finally, knock-down of induced Krox20 by a combination of two siRNA molecules also prevented sumoylation of endogenous Nab (Fig 2I).

To definitively demonstrate that Krox20 acts as a sumo ligase for Nab, we performed in vitro sumoylation assays with purified proteins produced in bacteria. As illustrated in Fig 2J, sumoylation of Nab occurred in vitro and was indeed dependent on the presence of Krox20. Furthermore, comparison of the number of molecules between input Krox20 ($0.3\ 10^{-12}$ mol) and sumoylated Nab ($2.78\ 10^{-12}$ mol, as estimated by measure of chemiluminescence from western blots and comparing to the amount of loaded protein, Fig 2J) indicated a much larger number of sumoylated Nab molecules, suggesting that Krox20 was acting in a catalytic manner.

Nab sumoylation contributes to recruit sumo to chromatin

We next investigated whether sumo can be recruited to a Nab-regulated sequence in the context of chromatin. We chose to examine the *Id4* promoter because it has been previously shown to be regulated by the Krox20-Nab complex (Mager *et al*, 2008). In P19 cells, serum addition led to a 3-fold increase in sumo levels associated to the *Id4*

promoter (Fig 3A). To investigate whether Nab sumoylation was involved, cells were transfected with wild type Nab or the KR2 mutant immediately after serum deprivation. Whereas in the presence of wild type Nab the increase of sumo levels were similar to those observed in the absence of transfection, with the KR2 mutant serum-mediated accumulation of sumo on the *Id4* promoter was impaired, presumably due to competition with the endogenous Nab (Fig 3A).

Nab sumoylation modulates Krox20 activity in vivo

To evaluate whether Nab sumoylation has an impact on Krox20 transcriptional control, we took advantage of a *lacZ*-based reporter of Krox20 activity (Garcia-Dominguez *et al*, 2006). This construct was transfected in P19 cells together with *Krox20*, sumo1 and *Nab* expression constructs. The analysis demonstrated that Nab efficiently repressed Krox20 transcriptional activity in the presence of sumo, whereas the KR2 mutant resulted in increased reporter activity, suggesting a dominant negative effect (Fig 3B).

To investigate the possible modulation of Krox20 activity by Nab sumoylation in vivo, we turned to the hindbrain, where Krox20 regulates a variety of genes, including itself and *Nab* genes, in r3 and r5 (Desmazieres *et al*, 2009; Mechta-Grigoriou *et al*, 2000; Chomette *et al*, 2006; Giudicelli *et al*, 2001). Furthermore, in gain-of-function experiments, Nab was shown to repress Krox20 activity, suggesting the existence of a negative feedback regulatory loop (Mechta-Grigoriou *et al*, 2000).

Electroporation of chick embryos allows gain-of-function analysis in the hindbrain. We first confirmed the expression of *Ubc9* in this structure by in situ hybridization and immunofluorescence analyses (Fig 4A,B). We analyzed the consequences of Nab and sumo misexpression on the expression of the r3/r5 marker EphA4, whose gene is under direct control of Krox20 (Theil *et al*, 1998). Since

electroporation only affects one side, the other constitutes a control. Limited repression of *EphA4* upon electroporation of Nab expression vectors has been previously reported (Desmazieres *et al*, 2009) and was confirmed in our experiments (Fig 4C,D,K). We observed that coelectroporation with Nab and sumo expression vectors led to more severe repression (Fig 4C-H,K). In contrast, electroporation of the KR2 mutant led to upregulation of *EphA4* (Fig 4I-K). Analysis of the expression of the r4 marker *Hoxb1* together with *EphA4* by double in situ hybridization showed that electroporation of Nab KR2 also resulted in reduced r4 size (Fig 4L,N), possibly associated with r3 and/or r5 expansion. Finally, expression of *Krox20* itself appeared upregulated upon KR2 electroporation, similar to *EphA4* (Fig 4M,N). Together these data support an involvement of Nab sumoylation in the repression of Krox20 activity in the hindbrain.

DISCUSSION

In this work we report that Krox20 acts as a ligase in the sumoylation reaction of its coregulators, the Nab proteins. Ligase activity of Krox20 is based on the following observations: i) over-expression of Krox20 under limiting sumo availability leads to sumoylation of transfected Nab; ii) Krox20 is able to recruit Ubc9 and Nab through different domains; iii) physical interaction between ligase and target is critical for modification; iv) sumoylation of endogenous Nab can also be observed in cultured cells and is dependent on Krox20 expression; v) Krox20 promotes Nab sumoylation in vitro and acts in a catalytic manner. To our knowledge, this constitutes the first example of a transcription factor acting as a ligase for the sumoylation of its own coregulators. Our results support a role of Krox20 in locally recruiting Ubc9 for sumoylation of other components in its transcriptional complex, the Nab proteins, contributing to explain how specificity of target sumoylation may be achieved. This raises the possibility that other factors might be sumoylated using Krox20 as a ligase, an exciting hypothesis considering the role of Krox20 in various developmental systems (Schneider-Maunoury *et al*, 1993; Topilko *et al*, 1994). Our findings also represent a stimulus for the identification of new ligases. Different types of ligase do not share significant homology. Similarly, no significant homology was observed between Krox20 and previously described ligases. These have in common the ability to interact with Ubc9, in many cases through apparently unrelated domains. The zinc finger domain of Krox20 binds Ubc9. Intriguingly, different zinc-based structures have been reported to be involved in Ubc9 binding and ligase function (Garcia-Dominguez *et al*, 2008).

Several pieces of evidence are consistent with a role of Nab sumoylation in vivo: i) Nab sumoylation modulates Krox20 transcriptional activity in a reporter assay in cultured cells; ii) sumo recruitment to the *Id4* promoter is dependent on Nab

sumoylation sites; iii) altered Nab sumoylation affects the expression of Krox20 target genes in the hindbrain. Despite the numerous roles attributed to sumo in eukaryotic cells, a function in transcriptional repression stands out (Garcia-Dominguez and Reyes, 2009). Accordingly, our results support a role of Nab sumoylation in transcriptional repression. Srinivasan and collaborators have reported that CHD4 participates in but does not account for full repression activity associated to Nab2 (Srinivasan *et al*, 2006), supporting the involvement of additional mechanisms, for instance sumoylation. Indeed, the fact that both wild type Nab and the KR2 mutant equally interact with CHD4 (supplementary Fig S1C online) suggests that sumoylation is not involved in CHD4 recruitment.

It has been proposed that Krox20 mediates expansion of r3 and r5 territories by recruiting cells from adjacent even-numbered territories (Giudicelli *et al*, 2001). Subsequently, other mechanisms should limit Krox20 activity to restrict expansion of r3 and r5. Induction by Krox20 of its own corepressors, the Nab proteins, has been proposed as one of these mechanisms (Mechta-Grigoriou *et al*, 2000). In agreement with this hypothesis, interference with the interaction between Krox20 and Nab proteins leads to delayed downregulation of Krox20 target genes (Desmazieres *et al*, 2009; Desmazieres *et al*, 2008). In this report we show that interfering with Nab sumoylation also leads to altered expression of Krox20 target genes and to modifications in the size of rhombomeres. Together, our data are consistent with Nab sumoylation limiting Krox20 activity and the extension of Krox20-positive territories, in agreement with the proposed role of Nab proteins. However, experiments performed in the mouse have shown that double *Nab* knock-out or knock-in of the I268F mutation in *Krox20* do not lead to major defects in hindbrain patterning (Desmazieres *et al*, 2008; Le *et al*, 2005).

This suggests the existence of redundant mechanisms for the limitation of the expansion of Krox20-positive territories.

In conclusion, we have revealed an intriguing novel activity of the Krox20 transcription factor, as a sumo ligase for its coregulators Nab. Nab sumoylation affects Krox20 transcriptional activity, establishing an additional loop in the complex control of its own activity and expression by Krox20.

METHODS

Plasmid constructs, protein production and purification, yeast two-hybrid and pull-down assays. Details for plasmid constructs and protein production and purification are provided as supplementary information online. Yeast two-hybrid was previously described (Garcia-Dominguez *et al*, 2006). Pull-down experiments were carried out with GST or GST-Ubc9 proteins loaded on Glutathione Sepharose 4B beads (GE, Healthcare) and in vitro translated Krox20, as previously described (Garcia-Dominguez *et al*, 2006), or from 2 10^7 His-sumo1 transfected cells under denaturating conditions (6 M urea) using His-Select Nickel Affinity Gel (Sigma) as indicated by manufacturer.

Cell culture, transfection, reporter and sumoylation assays and Western blot. 293T and P19 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum and α-modified Eagle's medium supplemented with 7.5% calf and 2.5% fetal bovine sera (PAA), respectively. For serum stimulation, P19 cells were deprived of serum for 48 h and harvested 2 h after serum re-addition. Transient transfections of plasmids or siRNA molecules were performed with Lipofectamine 2000 or Oligofectamine (Invitrogen), 36 or 48 h before harvesting the cells, respectively. The sequences of the siRNAs are provided in supplementary Table S1 online. For sumoylation assays in cells, we used 0.5 µg of RSV-flag-Nab2, 0.15 µg of RSV-His-sumo1/2 and 1 µg of other constructs. ß-galactosidase activity of reporter construction was determined using a chemiluminescent assay (Roche). The CMV-driven luciferase expression vector pGL4.51 (Promega) was used for normalization. For Western blot, cell extracts were prepared in 8 M urea, 10 mM Tris-HCl pH8.0, and analyzed with the ECL procedure (GE Healthcare). A ChemiDocXRS apparatus (Bio-Rad) was used for

chemiluminescence measurement. Antibodies and in vitro sumoylation assay are detailed in supplementary information online.

Chromatin immunoprecipitation and quantitative PCR. Chromatin immunoprecipitation (ChIP) experiments were performed on P19 cells. 10^7 cells fixed in 1% formaldehyde for 10 min at 37 ºC were used in each experiment. The D-11 anti-sumo1 antibody (sc-5308, Santa Cruz Biotechnology) was used for chromatin precipitation. Quantitative PCR was used for analysis of the *Id4* promoter and determination of gene expression levels, as detailed in supplementary information online. Sequence of primers is provided in supplementary Table S1 online.

In ovo electroporation, immunofluorescence and in situ hybridization. Electroporation, preparation of embryos for immunofluorescence and in situ hybridization were conducted as previously described (Giudicelli *et al*, 2001). Eggs were incubated at 38 ºC for 30 h (stage HH8-HH9) for electroporation and embryos were recovered 24 h after. For electroporation monitoring, the GFP expression vector pEGFP-N1 (Clontech) was used at 0.3 µg/µl. Other constructs were electroporated at 1 µg/µl. Protocols for immunofluorescence and in situ hybridization have been previously described (Garcia-Dominguez *et al*, 2006). Probes and antibodies are detailed in supplementary information on line. Fluorescent images were acquired on a Leica confocal microscope.

Supplementary information is available at *EMBO reports* online.

ACKNOWLEDGEMENTS

We thank A. Rojas and P. Gilardi-Hebenstreit for critical reading of the manuscript. We also thank personnel in the microscopy facility at CABIMER for assistance. P. G.-G. and F. J.-V. were recipients of JAE predoc (CSIC) fellowships. F. G.-C. was recipient of a JAE intro (CSIC) fellowship. This work was supported by BFU2009-10986/BMC (MICIN) and Intramural 200920I085 (CSIC) grants.

REFERENCES

Chomette D, Frain M, Cereghini S, Charnay P, Ghislain J (2006) Krox20 hindbrain cis-regulatory landscape: interplay between multiple long-range initiation and autoregulatory elements. *Development* **133**: 1253-1262

Desmazieres A, Charnay P, Gilardi-Hebenstreit P (2009) Krox20 controls the transcription of its various targets in the developing hindbrain according to multiple modes. *J Biol Chem* **284**: 10831-10840

Desmazieres A, Decker L, Vallat JM, Charnay P, Gilardi-Hebenstreit P (2008) Disruption of Krox20-Nab interaction in the mouse leads to peripheral neuropathy with biphasic evolution. *J Neurosci* **28**: 5891-5900

Garcia-Dominguez M, Gilardi-Hebenstreit P, Charnay P (2006) PIASxbeta acts as an activator of Hoxb1 and is antagonized by Krox20 during hindbrain segmentation. *Embo J* **25**: 2432-2442

Garcia-Dominguez M, March-Diaz R, Reyes JC (2008) The PHD domain of plant PIAS proteins mediates sumoylation of bromodomain GTE proteins. *J Biol Chem* **283**: 21469-21477

Garcia-Dominguez M, Reyes JC (2009) SUMO association with repressor complexes, emerging routes for transcriptional control. *Biochim Biophys Acta* **1789**: 451-459

Gareau JR, Lima CD (2010) The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat Rev Mol Cell Biol* **11**: 861-871

Giudicelli F, Taillebourg E, Charnay P, Gilardi-Hebenstreit P (2001) Krox-20 patterns the hindbrain through both cell-autonomous and non cell-autonomous mechanisms. *Genes Dev* **15**: 567-580

Le N, Nagarajan R, Wang JY, Svaren J, LaPash C, Araki T, Schmidt RE, Milbrandt J (2005) Nab proteins are essential for peripheral nervous system myelination. *Nat Neurosci* **8**: 932-940

Lumsden A, Krumlauf R (1996) Patterning the vertebrate neuraxis. *Science* **274**: 1109-1115

Mager GM, Ward RM, Srinivasan R, Jang SW, Wrabetz L, Svaren J (2008) Active gene repression by the Egr2.NAB complex during peripheral nerve myelination. *J Biol Chem* **283**: 18187-18197

Mechta-Grigoriou F, Garel S, Charnay P (2000) Nab proteins mediate a negative feedback loop controlling Krox-20 activity in the developing hindbrain. *Development* **127**: 119-128

Russo MW, Sevetson BR, Milbrandt J (1995) Identification of NAB1, a repressor of NGFI-A- and Krox20-mediated transcription. *Proc Natl Acad Sci U S A* **92**: 6873-6877

Schneider-Maunoury S, Topilko P, Seitandou T, Levi G, Cohen-Tannoudji M, Pournin S, Babinet C, Charnay P (1993) Disruption of Krox-20 results in alteration of rhombomeres 3 and 5 in the developing hindbrain. *Cell* **75**: 1199-1214

Srinivasan R, Mager GM, Ward RM, Mayer J, Svaren J (2006) NAB2 represses transcription by interacting with the CHD4 subunit of the nucleosome remodeling and deacetylase (NuRD) complex. *J Biol Chem* **281**: 15129-15137

Svaren J, Sevetson BR, Apel ED, Zimonjic DB, Popescu NC, Milbrandt J (1996) NAB2, a corepressor of NGFI-A (Egr-1) and Krox20, is induced by proliferative and differentiative stimuli. *Mol Cell Biol* **16**: 3545-3553

Svaren J, Sevetson BR, Golda T, Stanton JJ, Swirnoff AH, Milbrandt J (1998) Novel mutants of NAB corepressors enhance activation by Egr transactivators. *Embo J* **17**: 6010-6019

Theil T, Frain M, Gilardi-Hebenstreit P, Flenniken A, Charnay P, Wilkinson DG (1998) Segmental expression of the EphA4 (Sek-1) receptor tyrosine kinase in the hindbrain is under direct transcriptional control of Krox-20. *Development* **125**: 443-452

Topilko P, Schneider-Maunoury S, Levi G, Baron-Van Evercooren A, Chennoufi AB, Seitanidou T, Babinet C, Charnay P (1994) Krox-20 controls myelination in the peripheral nervous system. *Nature* **371**: 796-799

Voiculescu O, Taillebourg E, Pujades C, Kress C, Buart S, Charnay P, Schneider-Maunoury S (2001) Hindbrain patterning: Krox20 couples segmentation and specification of regional identity. *Development* **128**: 4967-4978

FIGURE LEGENDS

Fig 1. The zinc finger domain of Krox20 mediates interaction with Ubc9. (A) Growth of yeast transformed with the indicated constructs was tested on non-selective and selective media. Bait and prey constructs were based on Gal4 DNA binding domain (G4DB) and Gal4 activation domain (G4AD) vectors, respectively. (B) Pull-down experiments were carried out with immobilized purified GST or GST-Ubc9 fusion and in vitro translated radioactively labeled Krox20. (C) Deletion constructs of Krox20 were tested for interaction with Ubc9 by yeast two-hybrid as indicated in (A).

Fig. 2. Krox20 mediates Nab sumoylation. (A-F) 293T cells were transfected with flag-Nab or flag-RanGAP1-C-ter (flag-Ran) expression vectors and the constructs indicated at the top of each panel. Flag-tagged proteins were detected by Western blot. Black arrowheads indicate non-modified proteins and other arrowheads indicate modified proteins. Bottom panels correspond to inputs of the indicated proteins. (A,B) Nab is sumoylated in the presence of Krox20 and sumoylation is enhanced if low amounts of His-sumo1 or GFP-sumo1 are transfected. (C) Nab is specifically sumoylated by Krox20, as the addition of neuroM does not affect Nab sumoylation state; however, dominant negative Ubc9 (C93S) interferes with Nab sumoylation. (D) The Nab K379RK517R (KR2) protein is not sumoylated. (E) Krox20 I268F and Nab Q64RH95Q mutants, affected in their ability to interact with each other, are ineffective as ligase and target in the sumoylation reaction, respectively. (F) Sumoylation of RanGAP1-C-ter is not altered by the presence of Krox20 or the I268F mutant. (G-I) Sumoylation of endogenous Nab was analyzed in P19 cells in pull-down experiments with anti-Nab antibodies. (G) Endogenous Nab is sumoylated following His-sumo1 transfection and serum stimulation, which results in the expression of *Krox20*. (H) Sumoylation of endogenous Nab was also observed upon transfection of an HA-Krox20 expression

construct under normal growth conditions (H). Nab sumoylation was prevented by transfection of Krox20 siRNA (si), and not by a control GFP siRNA, under serum stimulation conditions (I). Upper panel in (G-I) shows pulled His-sumo1-Nab, revealed with anti-Nab antibodies, while lower panels show 1.5% input of the indicated proteins. Note that anti-Krox20 antibodies also reveal a non-specific upper band. (J) In vitro sumoylation assays with 300 ng of purified flag-tagged Nab were carried out in the presence or the absence of mature sumo (sumo1GG) and 15 ng of Krox20 as indicated. Sumoylated products corresponded to 52,96% ± 2,74 (mean ± s.d.) of loaded protein, as determined by chemiluminescence measurement from 3 independent western blots.

Fig. 3. Sumoylation of Nab contributes to sumo recruitment to chromatin and modulates Krox20 transcriptional activity. (A) Sumo levels associated to the *Id4* promoter were determined by ChIP experiments in P19 cells transfected with flag-tagged wild type Nab or the KR2 mutant or not transfected (-). Sumo levels were determined on cells normally growing (white bars) or subjected to serum stimulation (black bars). Fold-increase in sumo levels were normalized to the value determined in non-transfected untreated cells. Flag levels were also determined as a control (grey bars) and fold-increase was normalized to the value of Nab transfected cells. (B) A *lacZ* reporter construct responsive to Krox20 was tested in P19 cells cotransfected with the indicated expression constructs. Relative units of ß-galactosidase were normalized to the value of cells cotransfected with Krox20 alone (100%). Values are means of three independent experiments ± s.d.

Fig. 4. Nab sumoylation regulates Krox20 target genes in vivo. Flat mounted chick hindbrains were analyzed by in situ hybridization using an *Ubc9* antisense RNA probe (A) or by immunofluorescence using Ubc9 and EphA4 antibodies (B). (C-J) The neural tube of chick embryos was electroporated with constructs expressing the proteins

indicated at the top of each panel. GFP was used to monitor electroporation. Electroporated hindbrains were processed for immunofluorescence using EphA4 antibodies (C-J), or in situ hybridization using *EphA4*, *Hoxb1* and *Krox20* RNA probes (L,M). Arrowheads in (L) delimit r4. Electroporations were performed on the left side of embryos. (K) Fluorescence signals of EphA4 hybridization in C, E, G and I were measured using the MetaMorph software. For that, regions of the same area encompassing rhombomeres 3 to 5 were defined on both electroporated and control sides. (N) Size of r4 was determined by measurement of the *Hoxb1* positive area using the ImageJ application and intensity of the *Krox20* hybridization signal was measured with the MetaMorph software on inverted grey scale-converted images. Relative levels were normalized in respect to those on the control side (-). Values correspond to the mean ± s.d. of 5 to 8 samples from three independent experiments. Statistical significance was analyzed using the Student's t-test: (K) $*p=0.13$, $**p<0.025$, $***p<0.005$, $****p<0.001$; (N) $*p<0.025$, $**p<0.005$.

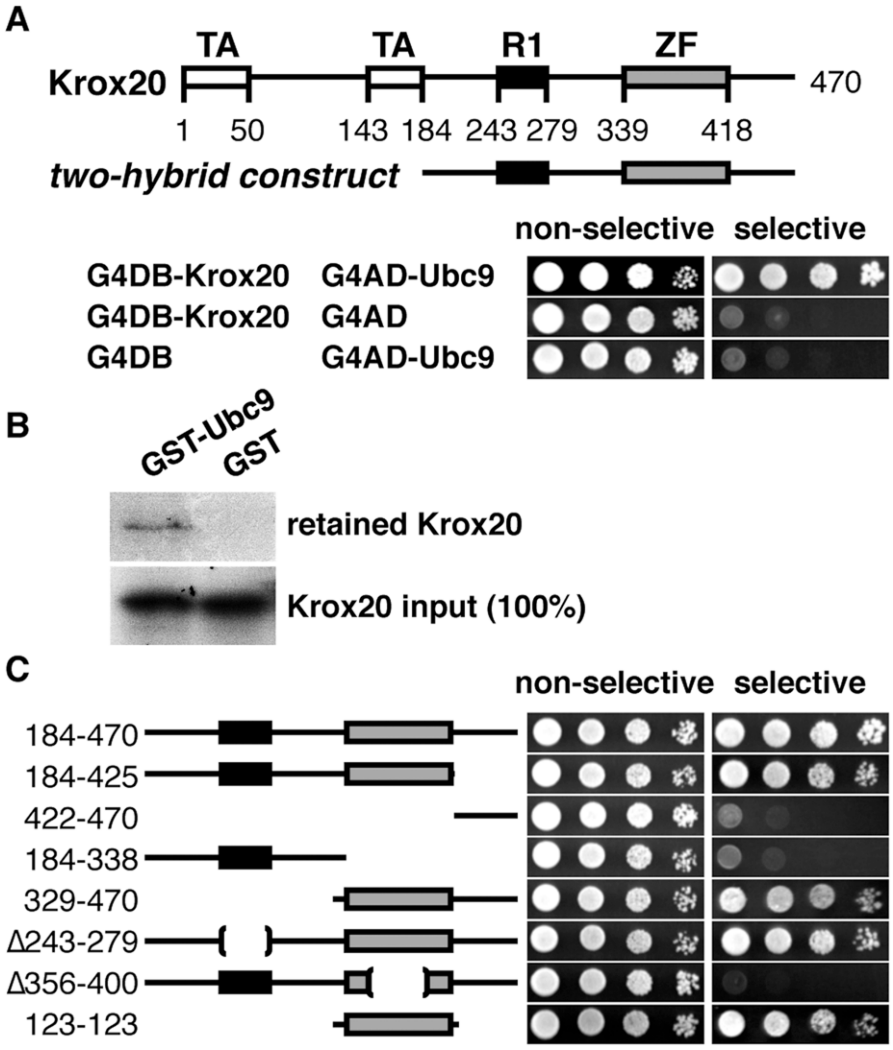
A
TA
TA
R1
ZF
Krox20
470
1
50
143
184
243
279
339
418
two-hybrid construct
non-selective
selective
G4DB-Krox20
G4AD-Ubc9
G4DB-Krox20
G4AD
G4DB
G4AD-Ubc9
B
GST-Ubc9
GST
retained Krox20
Krox20 input (100%)
C
non-selective
selective
184-470
184-425
422-470
184-338
329-470
Δ243-279
Δ356-400
123-123

Figure 1

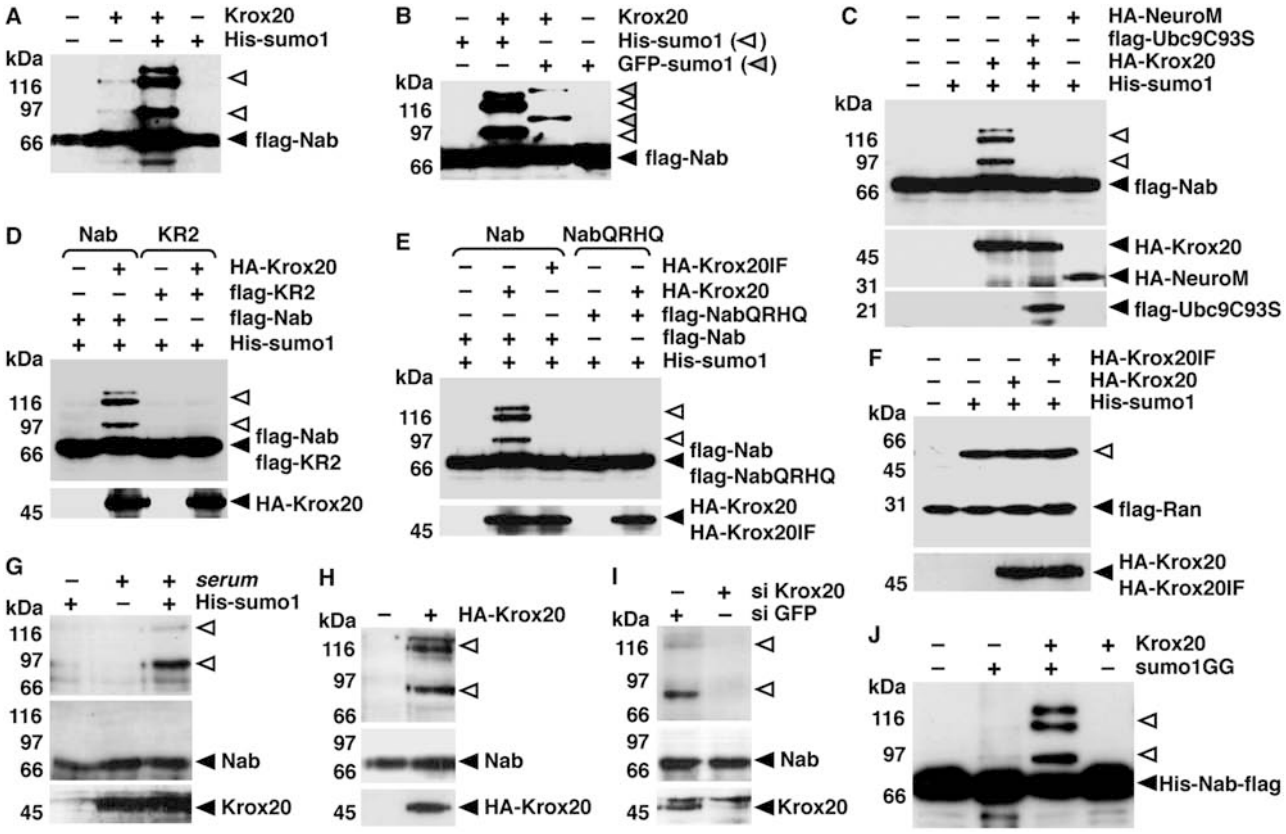
A
Krox20
His-sumo1
kDa
116
97
66
flag-Nab
B
Krox20
His-sumo1 (◁)
GFP-sumo1 (◁)
kDa
116
97
66
flag-Nab
C
HA-NeuroM
flag-Ubc9C93S
HA-Krox20
His-sumo1
kDa
116
97
66
flag-Nab
45
31
21
HA-Krox20
HA-NeuroM
flag-Ubc9C93S
D
Nab
KR2
HA-Krox20
flag-KR2
flag-Nab
His-sumo1
kDa
116
97
66
flag-Nab
flag-KR2
45
HA-Krox20
E
Nab
NabQRHQ
HA-Krox20IF
HA-Krox20
flag-NabQRHQ
flag-Nab
His-sumo1
kDa
116
97
66
flag-Nab
flag-NabQRHQ
45
HA-Krox20
HA-Krox20IF
F
HA-Krox20IF
HA-Krox20
His-sumo1
kDa
66
45
31
flag-Ran
45
HA-Krox20
HA-Krox20IF
G
serum
His-sumo1
kDa
116
97
66
116
97
66
Nab
45
Krox20
H
HA-Krox20
kDa
116
97
66
97
66
Nab
45
HA-Krox20
I
si Krox20
si GFP
kDa
116
97
66
97
66
Nab
45
Krox20
J
Krox20
sumo1GG
kDa
116
97
66
His-Nab-flag

Figure 2

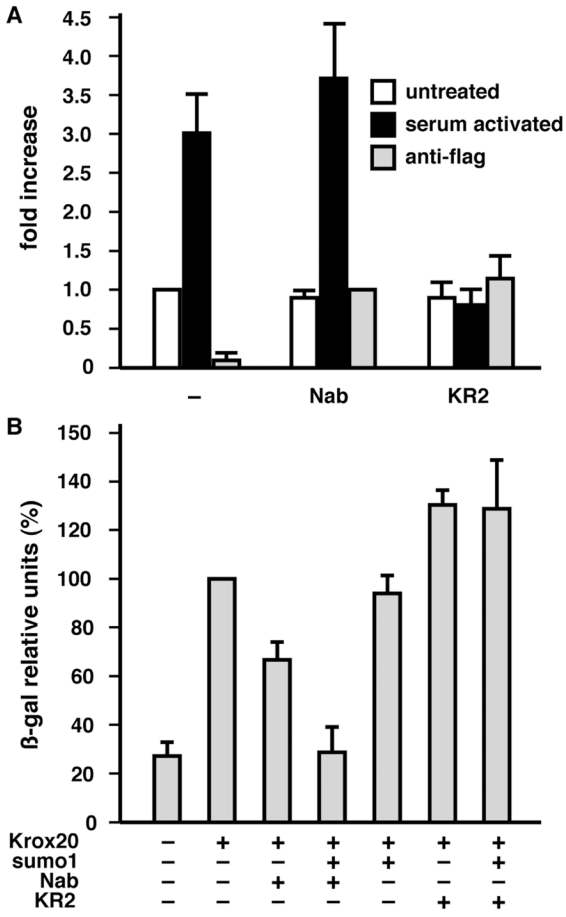
A
fold increase
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
untreated
serum activated
anti-flag
–
Nab
KR2
B
β-gal relative units (%)
150
140
120
100
80
60
40
20
0
Krox20 – + + + + + +
sumo1 – – – + + – +
Nab – – + + – – –
KR2 – – – – – + +

Figure 3

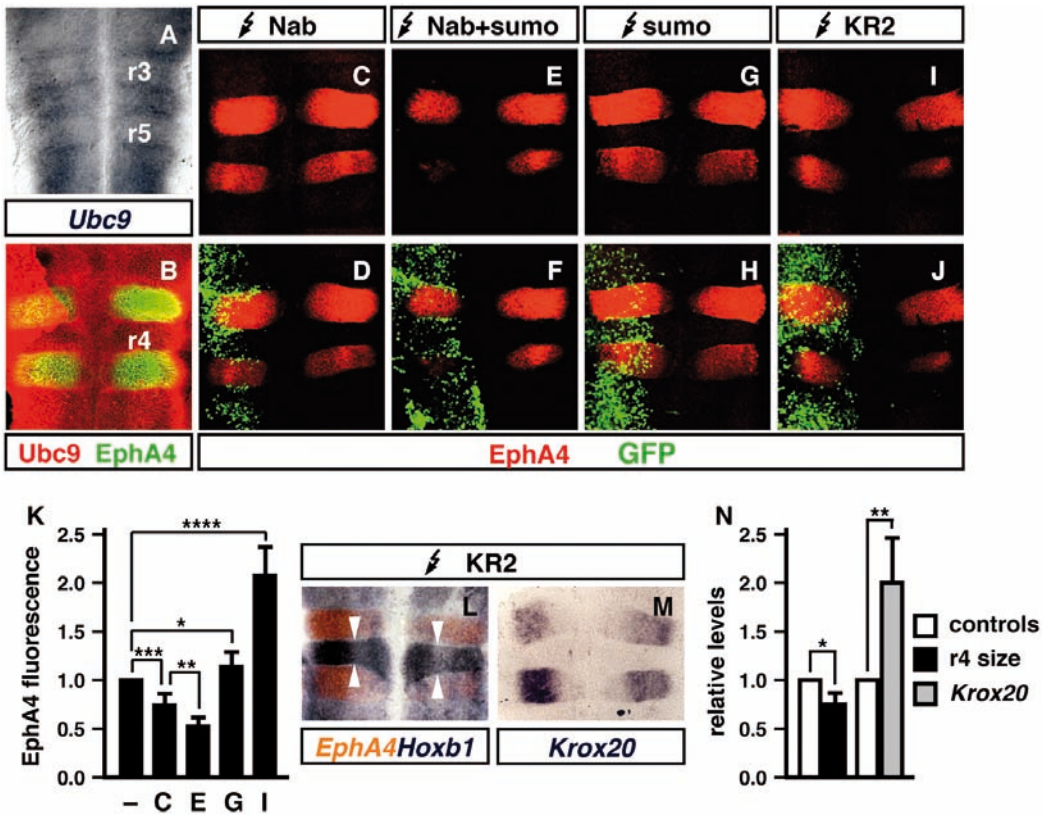
A
r3
r5
Ubc9
B
r4
Ubc9 EphA4
Nab
Nab+sumo
sumo
KR2
C
E
G
I
D
F
H
J
EphA4 GFP
K
EphA4 fluorescence
2.5
2.0
1.5
1.0
0.5
0.0
****
*
***
**
– C E G I
KR2
L
M
EphA4Hoxb1
Krox20
N
relative levels
2.5
2.0
1.5
1.0
0.5
0.0
*
**
controls
r4 size
Krox20

Figure 4

Supplementary Information

METHODS

Plasmid constructs. Deletion constructs of Krox20 were performed by standard PCR techniques. All expression constructs except GFP-sumo1 were derived from vector pAdRSV-Sp (RSV) (Giudicelli *et al*, 2003), with flag, HA or His tail tags. Mouse *Nab1*, *Nab2*, *Ubc9*, *CHD4, Aos1,* and *Uba2*, and human *sumo1* and *sumo2* were cloned by RT-PCR reactions with RNA isolated from mouse P19 or human 293T cells. The Krox20 I268F, Nab2 Q64RH95Q and the Ubc9 C93S mutations, and the HA-neuroM expression and *EphA4-lacZ* reporter constructs have been described elsewhere (Garcia-Dominguez *et al*, 2006; Garcia-Dominguez *et al*, 2003; Giorgino *et al*, 2000; Svaren *et al*, 1998). The RanGAP1-C-ter expression construct, corresponding to the C terminus of RanGAP1, was derived from plasmid pET28RanGAP1-C2 (Uchimura *et al*, 2004), and was used as a control for sumoylation (Lee *et al*, 1998). Mutation of Lys379 and Lys517 of Nab2 to Arg was conducted by standard PCR techniques. The GFP-sumo1 construct was based on the pEGFP-C2 plasmid (Clontech). GST constructions were derived from plasmid pGEX-6P-3 (GE Healthcare). The pET28a plasmid (Novagen) was used for bacterial production of Nab2.

Protein purification and in vitro sumoylation assay. Mouse Krox20, Ubc9, Aos1 and Uba2, and matured human sumo1, were produced in *E. coli* DH5α at 20 °C as GST fusions and purified with Glutathione Sepharose 4B beads (GE, Healthcare) as indicated by manufacturer. Except for Aos1 and Uba2, the GST moiety was excised by using the PreScission protease (GE, Healthcare). His and flag-tagged mouse Nab2 was produced in *E. coli* BL21(DE3) at 4 °C and purified with His-Select Nickel Affinity Gel (Sigma) as indicated by manufacturer. In vitro sumoylation assays with purified Nab2 and Krox20 were performed at 30 °C for 2 h in 20 µl of 20 mM Hepes pH 7.5, 50 mM NaCl, 4 mM $MgCl_2$ and 1 mM DTT buffer, containing 0.2 µg of Aos1/Uba2 mix (E1), 0.25 µg of Ubc9 and 1 µg of sumo1. Reactions were initiated with 250 µM ATP and stopped with SDS and ß-mercaptoethanol-containing Laemmli buffer.

Western blot. Antibodies and dilutions were as follows: mouse monoclonal anti-flag M2 (Sigma-Aldrich, 1:2000), rat monoclonal anti-HA (Roche, 1:2000), rabbit anti-Krox20 (Covance, 1:1000), mouse anti-Nab2 1C4 (Santa Cruz Biotecgnology sc-23867, 1:1000), goat anti-mouse HRP, anti-rat HRP and anti-rabbit HRP (Sigma-Aldrich, 1:10,000).

Quantitative PCR. For gene expression analysis total RNA was isolated with the RNAsy kit (QIAGEN). For retrotranscription of RNA we used the Superscript III enzyme (Invitrogen). Quantitative PCR reactions, in triplicate, were performed with the SensiMix SYBR Low-ROX kit (BIOLINE) in the Applied Biosystems 7500 FAST Real-Time PCR System. *GAPDH* was used for normalization. Primers for gene expression analysis and ChIP are described in Table S1. Algorisms for calculation of relative units and normalization of values according to oligonucleotide efficiencies are described in (Pfaffl, 2001).

Immunofluorescence and in situ hybridization. Antibodies and dilutions were as follows: rabbit anti-EphA4 S-20 (sc-921, Santa Cruz biotechnology, 1:100), mouse anti-Ubc9 (BD Bioscience, 1:100), donkey anti-rabbit DyLight549 and DyLight488, and donkey anti-mouse DyLight649 (Jackson ImmunoResearch, 1:800). Full-length *Ubc9* was cloned in pBluescript SK+ for synthesis of antisense RNA probe. cDNA was obtained by PCR with primers based on available EST sequences. Other probes were previously described: *Krox20* (Giudicelli *et al*, 2001), *EphA4* (Sajjadi and Pasquale, 1993), *Hoxb1* (Guthrie *et al*, 1992).

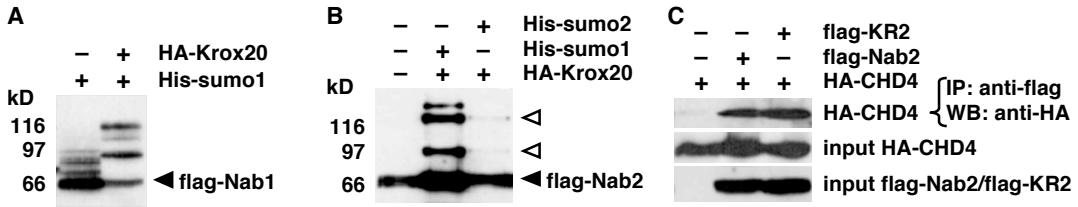

Fig S1. Analysis of Nab sumoylation. 293T cells were transfected with flag-Nab1 (A) or flag-Nab2 (B) and the constructs indicated at the top of each panel. Flag-tagged proteins were detected by Western blot. Black arrowheads indicate non-modified proteins while open arrowheads indicate modified proteins. Nab1 was sumoylated in 293T cells (A). Nab2 was more efficiently sumoylated by sumo1 than by sumo2 (B). (C) To test interaction of Nab and the KR2 mutant with CHD4, 293T cells were transfected with expression constructs of these molecules as indicated. For CHD4 we expressed the Nab-interacting domain tagged to the HA epitope (Srinivasan *et al*, 2006). Cell extracts were subjected to immunoprecipitation experiments with anti-flag antibodies and precipitates were analyzed by Western blot with anti-HA antibodies. 10% of input proteins are also shown.

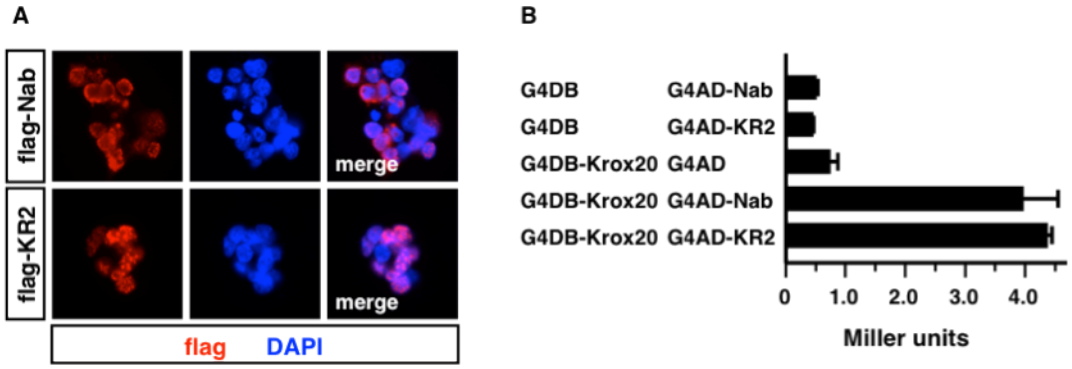

Fig S2. The KR2 mutant localizes to the nucleus and interacts with Krox20. (A) 293T cells transfected with expression constructs corresponding to flag-tagged wild type Nab (Fl-Nab) and the KR2 mutant were analyzed by immunofluorescence using an anti-flag antibody (red). DNA was visualized by DAPI staining (blue). (B) ß-galactosidase assays were used to analyze the interaction between the indicated proteins by yeast two-hybrid. Bait and prey constructions were based on Gal4 DNA binding domain (G4DB) and Gal4 activation domain (G4AD) vectors, respectively. Activities are indicated in Miller units and correspond to means of three independent experiments ± s.d.

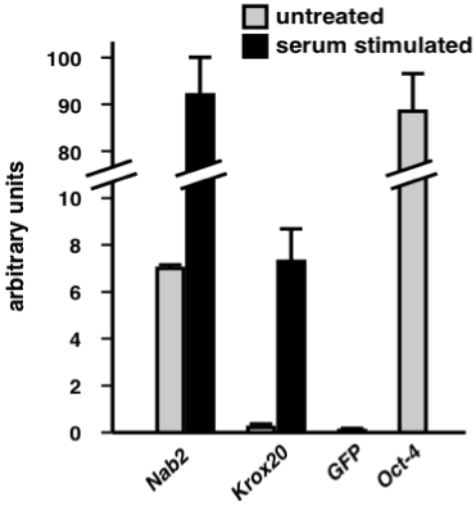

Fig S3. Expression levels of *Nab2* and *Krox20* genes in P19 cells. Expression levels of *Nab2* and *Krox20* in proliferating P19 cells (grey bars) were determined by quantitative PCR after retrotranscription of the isolated RNA. Levels of well-expressed and not expressed genes such as *Oct-4* and *GFP*, respectively, were determined as controls. Levels of *Nab2* and *Krox20* were also measured after serum stimulation (black bars). Means of arbitrary units from three independent experiments ± s.d. are represented.

REFERENCES

Garcia-Dominguez M, Gilardi-Hebenstreit P, Charnay P (2006) PIASxbeta acts as an activator of Hoxb1 and is antagonized by Krox20 during hindbrain segmentation. *Embo J* **25**: 2432-2442

Garcia-Dominguez M, Poquet C, Garel S, Charnay P (2003) Ebf gene function is required for coupling neuronal differentiation and cell cycle exit. *Development* **130**: 6013-6025

Giorgino F, de Robertis O, Laviola L, Montrone C, Perrini S, McCowen KC, Smith RJ (2000) The sentrin-conjugating enzyme mUbc9 interacts with GLUT4 and GLUT1 glucose transporters and regulates transporter levels in skeletal muscle cells. *Proc Natl Acad Sci U S A* **97**: 1125-1130

Giudicelli F, Gilardi-Hebenstreit P, Mechta-Grigoriou F, Poquet C, Charnay P (2003) Novel activities of Mafb underlie its dual role in hindbrain segmentation and regional specification. *Dev Biol* **253**: 150-162

Giudicelli F, Taillebourg E, Charnay P, Gilardi-Hebenstreit P (2001) Krox-20 patterns the hindbrain through both cell-autonomous and non cell-autonomous mechanisms. *Genes Dev* **15**: 567-580

Guthrie S, Muchamore I, Kuroiwa A, Marshall H, Krumlauf R, Lumsden A (1992) Neuroectodermal autonomy of Hox-2.9 expression revealed by rhombomere transpositions. *Nature* **356**: 157-159

Lee GW, Melchior F, Matunis MJ, Mahajan R, Tian Q, Anderson P (1998) Modification of Ran GTPase-activating protein by the small ubiquitin-related modifier SUMO-1 requires Ubc9, an E2-type ubiquitin-conjugating enzyme homologue. *J Biol Chem* **273**: 6503-6507

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45

Sajjadi FG, Pasquale EB (1993) Five novel avian Eph-related tyrosine kinases are differentially expressed. *Oncogene* **8**: 1807-1813

Srinivasan R, Mager GM, Ward RM, Mayer J, Svaren J (2006) NAB2 represses transcription by interacting with the CHD4 subunit of the nucleosome remodeling and deacetylase (NuRD) complex. *J Biol Chem* **281**: 15129-15137

Svaren J, Sevetson BR, Golda T, Stanton JJ, Swirnoff AH, Milbrandt J (1998) Novel mutants of NAB corepressors enhance activation by Egr transactivators. *Embo J* **17**: 6010-6019

Uchimura Y, Nakao M, Saitoh H (2004) Generation of SUMO-1 modified proteins in E. coli: towards understanding the biochemistry/structural biology of the SUMO-1 pathway. *FEBS Lett* **564**: 85-90

Table S1. Sequence of primers and siRNAs

Primer	*Sequence*
Nab2-F	AGGAAGAGGAGATCCGGAAG
Nab2-R	GTGTTGTCCCTCATGCAGAA
Krox20-F	CAGGAGTGACGAAAGGAAGC
Krox20-R	GACCAGAGGCTGAAGACTGG
GAPDH-F	AACTTTGGCATTGTGGAAGG
GAPDH-R	GGATGCAGGGATGATGTTCT
Id4prom-F	GCGCGGCTCTACAAATACTGC
Id4prom-R	AACCGCGCCTCCCAGCTCAAC
GFP-F	CAAGATCCGCCACAACATCG
GFP-R	GTCCATGCCGAGAGTGATCC
Oct4-F	CCAATCAGCTTGGGCTAGAG
Oct4-R	CTGGGAAAGGTGTCCCTGTA
siRNA Krox20#1	CGCCAAGGCCGUAGACAAA
siRNA Krox20#2	GCCCUUCCAGUGUCGGAUC
siRNA GFP	GGCACAAGCUGGAGUACAA