RBP-Jκ/SHARP Recruits CtIP/CtBP Corepressors To Silence Notch Target Genes

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Notch is a transmembrane receptor that determines cell fates and pattern formation in all animal species. After ligand binding, proteolytic cleavage steps occur and the intracellular part of Notch translocates to the nucleus, where it targets the DNA-binding protein RBP-Jκ/CBF1. In the absence of Notch, RBP-Jκ represses Notch target genes through the recruitment of a corepressor complex. We and others have identified SHARP as a component of this complex. Here, we functionally demonstrate that the SHARP repression domain is necessary and sufficient to repress transcription and that the absence of this domain causes a dominant negative Notch-like phenotype. We identify the CtIP and CtBP corepressors as novel components of the human RBP-Jκ/SHARP-corepressor complex and show that CtIP binds directly to the SHARP repression domain. Functionally, CtIP and CtBP augment SHARP-mediated repression. Transcriptional repression of the Notch target gene *Hey1* is abolished in CtBP-deficient cells or after the functional knockout of CtBP. Furthermore, the endogenous *Hey1* promoter is derepressed in CtBP-deficient cells. We propose that a corepressor complex containing CtIP/CtBP facilitates RBP-Jκ/SHARP-mediated repression of Notch target genes.

The Notch signaling pathway plays a critical role in the cell fate determination of various lineages (for a review, see reference 13). Notch is typically involved in binary cell fate decisions in *Drosophila melanogaster*, including neurogenesis and myogenesis. In mammals, Notch regulates numerous cell fate decisions during hematopoiesis, neurogenesis, and many other tissue-developing processes. Notch signaling has two opposing functions; depending on developmental stage, it either suppresses or facilitates differentiation. For example, Notch signaling promotes T-cell differentiation while inhibiting B-cell development (for a review, see reference 22). Ligand binding to the Notch receptor leads to proteolytic processing within the transmembrane domain, resulting in the release of the Notch intracellular domain, Notch IC (5, 31). Notch IC translocates to the nucleus and acts as a transcriptional coactivator in association with the DNA-binding protein RBP-Jκ (25, 30). RBP-Jκ was originally classified as a transcriptional repressor in vertebrates and as a transcriptional activator in *Drosophila melanogaster*. This paradox was resolved with the realization that repression and activation via RBP-Jκ involved the recruitment of distinct corepressors and coactivator complexes, as reviewed in reference 13. Notch IC binding to RBP-Jκ is crucial for the switch from the repressed state to the activated state. Notch IC first displaces corepressors from RBP-Jκ, resulting in the derepression of promoters containing RBP-Jκ-binding sites. Then Notch IC recruits several coactivators, in-

cluding Mastermind-like protein MAML and p300 (21, 37, 39). The focus of this paper is to further characterize the corepressor that shuts down the transcription of Notch-regulated genes.

Recent evidence indicates that corepressors use redundant mechanisms to direct repression. These mechanisms involve effects on the sequestration and function of the basal transcription machinery as well as targeted modifications of chromatin structure, as reviewed in reference 35. Corepressors often contain histone deacetylases (HDACs) in order to establish locally a tightly packed chromatin configuration. Examples of such corepressors are Groucho-, Sin3-, and CtBP-containing corepressor complexes. Several of these corepressor complexes can act in either an HDAC-dependent or an HDAC-independent manner, depending on the promoter context. One good example of such different usage of repression mechanisms is the transcriptional repression at the Ikaros and E2F-Rb target genes mediated by the CtIP (CtBP-interacting protein) and CtBP corepressors (10, 16). A comprehensive review of the roles of the CtBP protein in transcriptional repression has been recently described (4).

CtBP was originally identified through its ability to interact with a five-residue motif, PLDLS, in the carboxy terminus of the E1A adenoviral transforming protein (4, 23). The involvement of CtBP has since been identified in a number of signaling pathways via interaction with key regulators. In the Wnt pathway, CtBP interacts with TCF-4, leading to the silencing of certain Wnt target genes in the absence of a Wnt signal (36). An inhibitory SMAD involved in transforming growth factor-β signaling, SMAD6, can recruit CtBP, thereby repressing bone-morphogenetic protein-induced transcription of Id1 (14).

CtIP was originally isolated by its ability to interact with CtBP (24). Transcriptional repression by CtIP is mediated not only through recruitment of CtBP but also via direct contact with the general transcription factors TFIID and TFIIB (11).

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CtIP is a ubiquitously expressed 125-kDa nuclear protein that is capable of forming homodimers via the conserved N-terminal coiled-coil domain (6). CtIP also binds LMO4, a transcription factor overexpressed in over 50% of primary breast cancers, and Ikaros, a master regulator in lymphocyte development (10). The LXCXE motif of CtIP is essential for the transcriptional repression activity regulated through binding to the retinoblastoma (Rb) protein and Rb-related protein p130 (16). CtIP also plays a role in DNA repair by interacting with BRCA-1. Mutations abrogating BRCA1-CtIP binding have been shown to result in the deregulation of the cell cycle, leading to oncogenesis (41).

In higher eukaryotes, SHARP (*S*MRT/*H*DAC-1-*a*ssociated *r*epressor *p*rotein) physically interacts with both RBP-Jκ and the SMRT corepressor (20, 27). In *Drosophila*, Hairless (H) is the link between the RBP-Jκ homolog Suppressor of Hairless (SuH) and the corepressors dCtBP (17) and Groucho (2). However, *Drosophila* Hairless and mammalian SHARP share no sequence homology. SHARP is a large protein of approximately 450 kDa containing four RNA recognition motifs (RRMs) at its N terminus and a highly conserved SPOC-domain at its C terminus (1). Here, we further explored the mammalian RBP-Jκ/SHARP corepressor complex by identifying CtBP and CtIP as new components. We show that the SHARP C-terminal repression domain is necessary and sufficient to repress transcription mediated by CtIP and CtBP in both a *trans*-repression assay and the Notch target gene *Hey1*. Transcriptional repression is enhanced by increasing amounts of CtIP and CtBP. Furthermore, we demonstrate that the *Hey1* Notch target gene is strongly derepressed in CtBP-deficient mouse embryonic fibroblasts. Finally, we can purify an endogenous RBP-Jκ complex that contains CtIP and CtBP. Therefore, we propose that CtIP and CtBP are novel components of the RBP-Jκ corepressor complex that is required for the transcriptional repression of Notch target genes.

MATERIALS AND METHODS

Plasmids. The bait vector for two-hybrid screening, pGBT-SHARP(3291-3664), was constructed by inserting the blunted 1,355-bp NcoI fragment from pcDNA3-FLAG3-SHARP(2002-3664) into the blunted BamHI site from pGBT9 (Clontech). Expression vectors for the Gal4 fusion proteins, G4-VP16, G4-SHARP-RD-VP16, and G4-VP16-SHARP-RD, used in the transcriptional repression assay were made using PCR-assisted cloning. Details on the construction of the pFA-CMV (Stratagene)-based expression plasmids are available on request. The pGa981/6 luciferase reporter plasmid as well as the pCMV-RBP-VP16, pcDNA-3-mNotch-1ΔE, pcDNA3-FLAG2-SHARP, and pcDNA3-FLAG3-SHARP(2002-3664) expression plasmids was described previously (20, 21). The SHARP-specific expression plasmid pcDNA3-FLAG2-SHARP-RD (C-terminal repression domain only) as well as pcDNA3-FLAG2-SHARPΔC and pcDNA3-FLAG3-SHARP(2002-3411) lacking the repression domain was made using PCR-assisted cloning (construction details available on request). For the bacterial expression plasmid pGEX-2TK-SHARP-RD, the SHARP repression domain was amplified by PCR (5′-CGGGATCCGAATTCCAGCCAGCCCC-3′ and 5′-ATCCCGGGTCACACGGAGGCAATGACAATCATG-3′), digested with BamHI and XmaI, and inserted into the corresponding sites of the pGEX-2TK vector (Amersham). The vectors for the expression of glutathione *S*-transferase (GST)-SHARP deletion mutants (amino acids [aa] 3477 to 3628, 3477 to 3604, and 3477 to 3545) were constructed as follows. Starting from pcDNA3-FLAG2-SHARP-RD, PCR products were amplified using the upstream primer 5′-ATTAATACGACTCACTATAGGGAGACC-3′ and the following downstream primers: 5′-GCCTCGAGTTAGATCTGCAGCACGTAGGCAG-3′, 5′-TACTCGAGTTACTGCAGGTAAGTGATGAAGGC-3′, and 5′-TACTCGAGTTAGGGCCCTCCTTCAGAAAGGG-3′. The PCR products were then digested with EcoRI and XhoI and inserted into the corresponding sites of pGEX6P1 (Amersham). The eukaryotic expression vector for T7-tagged human CtBP1 and

the prokaryotic expression vector for GST-CtBP1 were provided by G. Chinnadurai. The CtIP expression vector (pSP6-CtIP) was a gift from R. Baer. Myc-tagged CtIP constructs (wild type, Δ LXCXE, and ΔPLDLS) were supplied by J. R. Nevins, and CtIP constructs pcDNA3.1HA-CtIP [59 to 320] and pcDNA3.1HA-CtIP (281 to 620) were provided by J. E. Visvader. A 1.6-kb fragment was isolated after XbaI digestion of pEFrHAPGKpuropA-CtIP (aa 371 to 897), which was supplied by J. E. Visvader, and inserted into the XbaI site of pcDNA3-FLAG1, resulting in pcDNA3-FLAG1-CtIP (aa 371 to 897). After EcoRI/XbaI digestion of pGEXCtIP (aa 620 to 897), which was supplied by R. Baer, a 1.1-kbp fragment was isolated and inserted into the corresponding sites of pcDNA3-FLAG3, resulting in pcDNA3-FLAG3-CtIP (aa 620 to 897). The luciferase reporter plasmid for the *Hey1* promoter (−95/+87) was a gift from M. Gessler, and the E1A expression plasmids E1A-Exon2 (pc-dl1119) and E1A-Exon2ΔCID (pc-dl1135) were supplied by C. Svensson.

Cell lines. The HEK-293 (ATCC CRL 1573) and HeLa (ATCC CCL 2) cell lines as well as mouse embryonic fibroblasts (as described in reference 8), which were kindly provided by J. D. Hildebrand, were grown at 37°C under 5% CO_2 in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal calf serum.

Yeast two-hybrid screening. Yeast (*Saccharomyces cerevisiae*) two-hybrid screening was performed using Gal4 fusion proteins as previously described (38). Briefly, *Saccharomyces cerevisiae* strain Y153 was transformed with the pGBT-SHARP(3291-3664) bait plasmid using the lithium acetate method and stably maintained in the absence of tryptophan. Yeasts were subsequently transformed with a pACT-based cDNA library derived from Epstein-Barr virus-transformed human peripheral lymphocytes and grown (2.8 million primary transformants) on His/Leu/Trp dropout plates containing 20 mM 3-aminotriazole. His⁺ colonies were analyzed for beta-galactosidase activity using the X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) filter assay as previously described (38). Prey plasmids from clones with positive results in both assays were isolated and propagated in *Escherichia coli* strain DH10B and sequenced using the gal843 primer (5′-GCGTTTGGAATCACTACAGGG-3′).

Preparation of cell extracts. Whole-cell lysates were prepared as follows. Cells were washed three times in phosphate-buffered saline (PBS) and pelleted by centrifugation at 300 × *g*. The pellet was resuspended in 5 volumes of ice-cold CHAPS lysis buffer consisting of 10 mM 3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 50 mM Tris-HCl (pH 7.9), 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride and incubated on ice for 40 min. The lysate was cleared by centrifugation at 80,000 × *g* for 30 min. Protein concentrations were determined using the Bradford assay method (Bio-Rad). Extracts were used for immunoprecipitation, in vitro interaction assays, and Western blotting.

In vitro protein translation. Proteins were translated in vitro in the presence of [³⁵S]methionine using the reticulocyte lysate-coupled transcription/translation system according to the manufacturer's instructions (Promega). Translation and labeling quality were monitored by SDS-PAGE.

GST pull-down assay. GST fusion proteins were expressed in *E. coli* strain BL21-CodonPlus-RIL (Stratagene) and stored as whole bacterial lysates. GST protein and GST fusion proteins (approximately 1 μg) were immobilized on Sepharose beads (Amersham) and incubated at 4°C for 1 h together with in vitro-translated proteins under rotation. The reaction mixtures were washed three times with 600 μl buffer A (40 mM HEPES [pH 7.5], 5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 1% Nonidet P40 [NP-40], and 100 mM KCl) and three times with buffer B (equivalent to buffer A, but containing 300 mM KCl). After these washing steps, the reaction mixtures were boiled and proteins were separated by SDS-polyacrylamide gel electrophoresis. Gels were dried and exposed to X-ray films. In addition to cell-free synthesized and radiolabeled proteins, extracts from transfected HEK-293 cells were also used in pull-down assays. In these cases, proteins interacting with GST fusion proteins were detected by Western blotting.

Cell sorting and quantitative RNA analysis. A total of 10^6 mouse embryonic fibroblasts was transfected either with an expression vector for enhanced green fluorescent protein (EGFP) (pEGFPC1; Clontech) or together with expression vectors for T7-CtBP1 or FLAG-CtBP1. Forty-eight hours after transfection, cells were harvested and GFP-positive cells (2×10^5) were sorted on a FACS Star Plus cell sorter (Becton Dickinson). Total RNA was isolated, and mRNA expression levels were quantified using real-time PCR (TaqMan; PE Applied Biosystems). For PCR, cDNAs were reverse transcribed from 2 μg of total RNA. The PCR (denaturation 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min) was performed using SYBR green PCR core reagents (PE Applied Biosystems) and primer combinations listed below. Endogenous cyclophilin mRNA levels were used as internal controls. The following primers were used: mCyclophilin-F, 5′-ATGGTCAACCCCACCGTGT-3′; mCyclophilin-R,

5′-TTCTTGCTGTCTTTGGAACTTTGTC-3′; mHey1F, 5′-CACTGCAGG AGGGAAAGGTTATT-3′; mHey1R, 5′-GCCAGGCATTCCCGAAAC-3′; mNotch-1F, 5′-ACATCCGTGGCTCCATTGTCTA-3′; and mNotch-1R, 5′-TCTTGTAAGGAATATTGAGGCTGCC-3′.

DNA transfection and luciferase assay. A total of 10^6 HEK-293 cells was transfected in 90-mm culture dishes with 5 to 10 μg of expression plasmid DNA using calcium phosphate coprecipitation as previously described (21). Proteins were extracted 24 h after transfection and assayed for protein expression or used in pull-down assays. HeLa cells were transfected (2×10^5) in 35-mm culture dishes with 2 μg of reporter plasmid DNA together with various amounts of expression plasmid using the FuGENE transfection reagent (Roche). Mouse embryonic fibroblasts were transfected with Lipofectamine (Invitrogen). Luciferase activity was determined from at least four independent transfections with 20 μl of cleared lysate in an LB 9501 luminometer (Berthold) using the luciferase assay system from Promega. All transfections were normalized using total cellular protein concentrations (Bradford assay; Bio-Rad).

Immunofluorescence assay. HEK-293 cells were cultured on glass coverslips in a 25-well plate (Bibby Sterilin, Ltd.) at a density of 10^5 cells per cm². After 16 h, cells were transfected with 500 ng of expression plasmids. Cells were rinsed with PBS 24 h after transfection and fixed and permeabilized with 0.1% Triton X-100. Nonspecific immunostaining was blocked by incubating the cells in 3% bovine serum albumin in PBS with 0.1% Tween 20. An antibody directed against the FLAG epitope (M5; Sigma) was used for detection of SHARP, and an antibody directed against the Myc epitope (9B11; Cell Signaling) was used for detection of the CtIP proteins. Staining was performed using an Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG; Molecular Probes), or a Cy3-conjugated goat anti-mouse IgG (Jackson Immuno Research) secondary antibody. After washing and mounting, the cells were analyzed using a fluorescence microscope (DMIRB; Leica).

Western blotting. Western blotting was performed as previously described (21). For the detection of the endogenous proteins, the following antibodies were used: anti-RBP-Jκ (rat monoclonal IgG2a, T6709 [Institute of Immunology Co., Ltd.] secondary antibody; peroxidase-conjugated goat anti-rat IgG [Dianova]), anti-CtIP (rabbit polyclonal IgG, 612L, raised against residues 58 through 369 [gift from R. Baer and described in reference 40] secondary antibody; peroxidase-conjugated donkey anti-rabbit IgG [Amersham]), anti-CtBP (rabbit polyclonal IgG, H-440 [Santa Cruz]), and anti-p65/RelA (goat polyclonal IgG, C-20 [Santa Cruz] secondary antibody; peroxidase-conjugated rabbit anti-goat IgG [Dianova]). To analyze coimmunoprecipitated CtBP1 proteins, membranes were incubated with the primary antibody directed against the T7 epitope (Bethyl). Coimmunoprecipitated CtIP proteins were detected with an antibody directed against the Myc epitope (9B11; Cell Signaling). As a secondary antibody, a 1:7,000 dilution of peroxidase-conjugated sheep anti-mouse IgG (Amersham) or a 1:5,000 dilution of peroxidase-conjugated donkey anti-rabbit antibody (Amersham) was used.

Coimmunoprecipitation. Immunoprecipitation was carried out using HEK-293 cell extracts 24 h after cotransfection with FLAG2-SHARP-RD (aa 3411 to 3663) and expression plasmids for human CtBP1 or CtIP. Cells were lysed in 900 μl CHAPS lysis buffer. Extracts were incubated with 40 μl of an agarose-conjugated anti-FLAG antibody (M2; Sigma) at 4°C overnight. Beads were washed three times with CHAPS lysis buffer containing 300 mM LiCl. After a further wash with CHAPS lysis buffer containing 150 mM LiCl, the beads were resuspended in SDS-PAGE loading buffer.

Injection of mRNA into *Xenopus laevis* embryos and whole-mount in situ hybridization. Microinjections of capped mRNA of mNotch-1ΔE, SHARP, and SHARPΔC and whole-mount in situ hybridization using a digoxigenin-labeled antisense RNA probe for *N-tubulin* were performed as previously described (20). Stained embryos were postfixed overnight in MEMFA (0.1 M morpholinepropanesulfonic acid [MOPS], 2 mM EGTA [pH 8.0], 1 mM MgSO$_4$, 3.7% formaldehyde) and depigmented using 10% H$_2$O$_2$ in methanol. The uninjected side was used as a control for normal primary neurogenesis.

Purification of RBP-Jκ DNA-binding complexes. For the preparation of the DNA affinity column, a 340-bp fragment containing 12 RBP-Jκ-binding sites was isolated by NsiI/HindIII digestion of pGa981/6. Approximately 0.15 nmol of the fragment was biotinylated and immobilized on streptavidin Sepharose (Amersham). Whole-cell extract from 10^9 Jurkat T cells in a total volume of 10 ml was first precleared by incubating with streptavidin Sepharose alone at 4°C for 1 h. The precleared lysate was then incubated with the affinity column at 4°C for 2 h. The supernatant was collected, and, after three washing steps with 7 ml of CHAPS lysis buffer, DNA-binding complexes were each eluted with 1 ml of CHAPS lysis buffer containing increasing NaCl concentrations (elution E1, 180 mM; E2, 200 mM; E3, 300 mM; E4, 400 mM; E5, 500 mM; E6, 700 mM; and E7,

1,000 mM) and analyzed by electrophoretic mobility shift assay (EMSA) and Western blotting.

EMSA. Cell extract, flowthrough, washing steps, and eluted fractions (2 μl) were used for electromobility gel shift assays in a binding buffer consisting of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 0.5 mM DTT, and 4% glycerol. For binding reaction, 2 μg poly(dI-dC) (Amersham) and approximately 0.5 ng of ^{32}P-labeled oligonucleotides were added. The sequence of the double-stranded oligonucleotide FO233 (5′-CCTGGAACTATTTTCCCACGGTGC CCTTCCGCCCATTTTCCCACGAGTCG-3′) corresponds to the two RBP-Jκ-binding sites within the EBVTP-1 promoter. The reaction products were separated using 5% polyacrylamide gels with 1× Tris-glycine-EDTA at room temperature. Gels were dried and exposed to X-ray films.

RESULTS

Functional characterization of the SHARP repression domain. Previous studies have shown that the evolutionarily conserved repression domain of SHARP (SHARP-RD), also called the SPOC (*Spen paralog and ortholog C*-terminal) domain, resides at the C terminus (1, 20, 27). Here, we show that the SHARP-RD is necessary and sufficient to repress transcription in transient cotransfection experiments. In addition, we demonstrate that SHARPΔC, lacking the repression domain, acts in a dominant negative fashion in a Notch-dependent neurogenesis assay using *Xenopus laevis* embryos. SHARP-RD was fused to the Gal4 DNA-binding domain and the VP16 transactivation domain as shown in Fig. 1A. The expression plasmids G4-VP16, G4-SHARP-RD-VP16, and G4-VP16-SHARP-RD were transiently cotransfected into HeLa cells together with the pFR-LUC reporter construct containing five Gal4-binding sites. The Gal4-VP16 control exhibited a dose-dependent increase of luciferase activity, whereas this effect was abrogated by either the Gal4-SHARP-RD-VP16 or the Gal4-VP16-SHARP-RD expression plasmid (Fig. 1B). This suggests that SHARP-RD alone is sufficient to recruit additional corepressors capable of overriding the strong effect of the VP16 transcriptional activator. Subsequently, we investigated whether full-length SHARP requires its C-terminal repression domain for RBP-Jκ-mediated transcriptional regulation. The expression plasmids for RBP-VP16, full-length SHARP, or a truncated SHARP lacking the C-terminal repression domain were transiently transfected into HeLa cells together with an RBP-Jκ-responsive reporter construct, pGa981/6 (Fig. 1C). This luciferase reporter plasmid contains six repeats of the EBNA-2-responsive element within the Epstein-Barr virus TP-1 promoter upstream to a minimal β-globin promoter. Cotransfection of the RBP-VP16 expression plasmid resulted in approximately 100-fold transcriptional activation. Transactivation mediated by RBP-VP16 was gradually reduced after cotransfection of the full-length corepressor, SHARP. In contrast, SHARP lacking its C-terminal repression domain (SHARPΔC) had a dominant negative effect, further stimulating the reporter gene instead of repressing it. Therefore, the SHARP-RD is necessary for transcriptional repression in transient transfection assays.

We have shown previously that SHARP acts as a Notch antagonist during *Xenopus laevis* neurogenesis (20). To test the role of the SHARP repression domain in this context, we overexpressed SHARPΔC in one half of the embryo. Whole-mount in situ hybridization for *N-tubulin* allowed for the identification of primary neurons in *Xenopus laevis* neurulae (Fig. 1D). As shown previously, embryos injected with Notch-1ΔE mRNA had no lateral and, in some cases, no intermediate primary neurons in the injected side (Fig. 1D, panel b). In
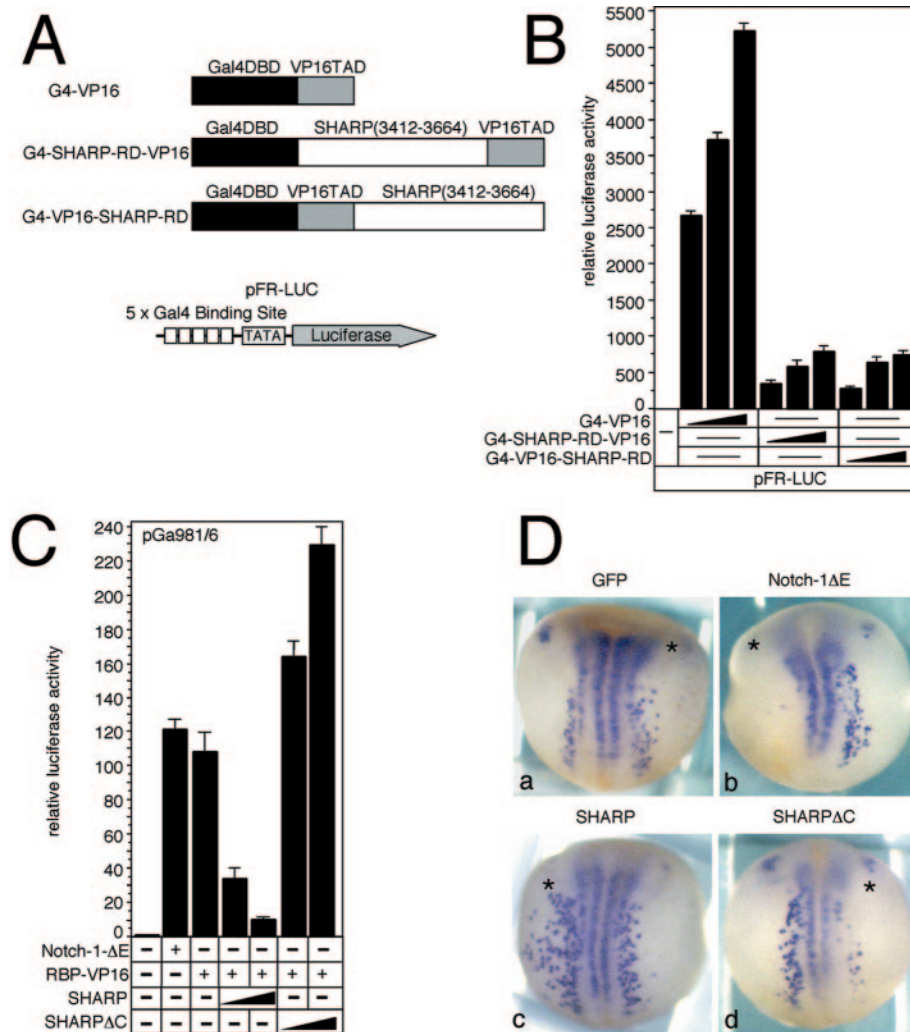
FIG. 1. Functional characterization of SHARP-RD. (A) Schematic representation of the Gal4-VP16 expression and reporter constructs for SHARP-RD used in the transcriptional repression assay. (B) SHARP-RD is necessary and sufficient to repress Gal4-VP16-mediated transcription. The pFR-LUC reporter construct (2 μg) was transfected alone or together with increasing amounts (5 ng, 10 ng, and 20 ng) of the indicated Gal4-VP16 expression constructs into HeLa cells. (C) Dominant negative effects of SHARP lacking its C-terminal repression domain. In contrast to SHARP, SHARPΔC stimulates RBP-VP16-mediated transcription. The pGA981/6 reporter construct (2 μg) was transfected alone or together with plasmids expressing either Notch-1ΔE (20 ng) or RBP-VP16 (20 ng) as well as with increasing amounts (100 ng and 250 ng) of either SHARP or SHARPΔC expression plasmids into HeLa cells. Luciferase activity was determined in 100-μg portions of total-cell extracts and normalized to the basal promoter activity of the reporter construct. Mean values and standard deviations from four independent experiments are shown. +, with the indicated construct; −, without the indicated construct. (D) Dominant negative effects of SHARP lacking its C-terminal repression domain. SHARPΔC induces a neurogenic phenotype in *Xenopus laevis* embryos. Embryos were injected with 100 pg GFP expression plasmid alone (panel a) or together with 100 pg mNotch-1ΔE mRNA (panel b), 2.4 ng full-length SHARP mRNA (panel c), or 2.4 ng of SHARPΔC mRNA (panel d) in one cell at the two-cell stage. Whole-mount in situ hybridization for *N-tubulin* shows primary neurons. The injected sides are marked with an asterisk.

contrast, overexpression of SHARP resulted in more primary neurons and a broader zone of lateral primary neurons in the injected sides (Fig. 1D, panel c). Interestingly, overexpression of SHARPΔC induced, like Notch-1ΔE, a neurogenic phenotype (Fig. 1D, panel d), resulting in a loss of primary neurons (75 out of 138 neurons [54%]). SHARPΔC showed a dominant negative effect on primary neurogenesis, which was analogous to our experiments assessing gene activation.

SHARP associates with the CtBP corepressor. During *Drosophila melanogaster* Notch signaling, H can interact with the RBP-Jκ homolog SuH as well as corepressors such as dCtBP

(2). In mammalian Notch signaling, SHARP interacts with both RBP-Jκ and corepressors such as SMRT (20, 27). Here, we show that endogenous RBP-Jκ also interacts strongly with GST-SHARP (Fig. 2A). So far, no sequence similarities could be detected between *Drosophila* H and mammalian SHARP. Assuming that SHARP may still have functional similarities to *Drosophila* H, the interaction of SHARP and the CtBP corepressor was tested in vivo. HEK-293 cells were transfected with an expression plasmid for FLAG-tagged SHARP-RD alone or together with T7-tagged human CtBP1. SHARP was immunoprecipitated using an anti-FLAG antibody coupled to beads.
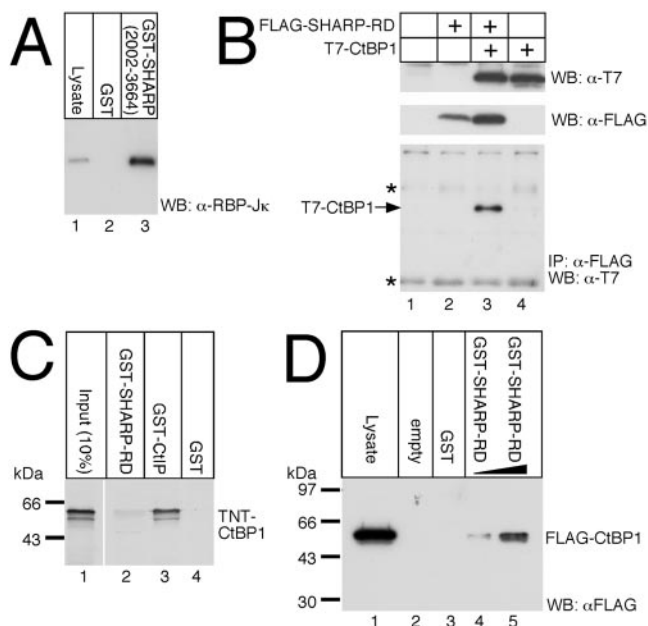
FIG. 2. (A) Endogenous RBP-Jκ proteins bind strongly to GST-SHARP(2002-3664) immobilized on glutathione Sepharose beads. (B through D) Association of CtBP1 with the repression domain of SHARP. (B) Expression plasmids for T7-tagged CtBP1 and FLAG-tagged SHARP-RD were transfected into HEK-293 cells. Expression of transfected SHARP-RD and CtBP1 was detected via Western blotting using antibodies against the T7 (upper blot) or FLAG tags (middle blot). CtBP1 coimmunoprecipitated with SHARP-RD with the antibody against the FLAG epitope from only lysates of cells transfected with both expression constructs (lower blot, lane 3). Coimmunoprecipitated CtBP1 proteins were detected on Western blots by using the anti-T7 antibody. The asterisks indicate antibody heavy and light chains. +, with the indicated construct. IP, immunoprecipitation. (C) Radiolabeled CtBP1 translated in vitro in reticulocyte lysate binds only weakly, if at all, to GST-SHARP-RD (lane 2) but strongly to GST-CtIP immobilized on Sepharose beads (lane 3). (D) CtBP1 proteins from transfected HEK-293 lysates bind GST-SHARP-RD (lanes 4 and 5). Binding proteins in the pull-down assay were separated by SDS-PAGE; RBP-Jκ and FLAG-CtBP1 were detected by Western blotting. WB, Western blot.

Subsequently, the coimmunoprecipitated T7-CtBP protein was detected on Western blots using an antibody against the T7 tag (Fig. 2B).

In an attempt to verify these results in an in vitro system, a GST pull-down assay was conducted using a glutathione *S*-transferase fusion protein for the SHARP repression domain (GST-SHARP-RD). Glutathione-Sepharose beads were coated with bacterially expressed GST or GST-SHARP-RD and used as bait for cell-free synthesized and radiolabeled CtBP1. No binding or extremely weak binding of in vitro-translated CtBP1 to GST-SHARP-RD was detected (Fig. 2C, lane 2) compared to that of our positive control, in vitro-translated CtIP (Fig. 2C, lane 3, see also Fig. 3). However, GST-SHARP-RD bound FLAG-tagged CtBP in cell lysates from FLAG-CtBP1-transfected HEK-293 cells (Fig. 2D). We concluded either that posttranslational modifications were necessary for SHARP-CtBP binding or that binding was indirect and required a bridging factor present in HEK-293 lysates.

SHARP interacts with the CtIP corepressor directly. A yeast two-hybrid screen was performed using the SHARP repression
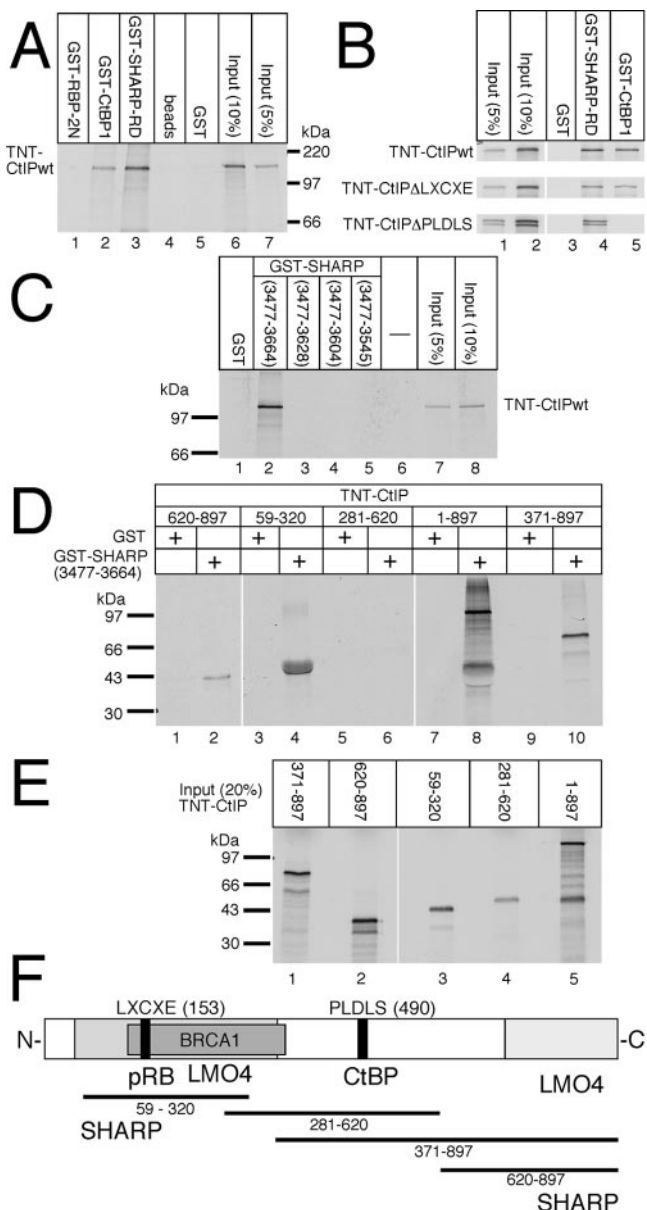

FIG. 3. CtIP interacts with the SHARP repression domain. (A) Cell-free synthesized CtIP binds specifically to GST-CtBP1 (lane 2) and GST-SHARP-RD (lane 3) but not to GST-RBP-2N (lane 1). (B) The LXCXE motif (lane 4, middle) and the PLDLS motif (lane 4, lower) within CtIP are dispensable for binding of CtIP to SHARP-RD. (C) CtIP binds to the C terminus of SHARP-RD. Interaction is lost already after the deletion of 36 aa (lane 3). (D and E) SHARP-RD binds to N-terminal (aa 59 to 320) and C-terminal (aa 620 to 897) regions of CtIP. GST proteins were immobilized on Sepharose beads and incubated with in vitro-translated, radiolabeled CtIP proteins. After extensive washing, proteins were eluted and separated on SDS-PAGE. +, with the indicated construct. (F) Schematic representation of CtIP and its interaction domains. SHARP-RD binds, like LMO4, to an N-terminal and a C-terminal domain of CtIP.

domain fused to the Gal4 DNA-binding domain and a human B-cell library (38). We identified SMRT and NCor, which had been previously described to interact with SHARP (27). CtIP was among the newly identified interacting proteins and fit the
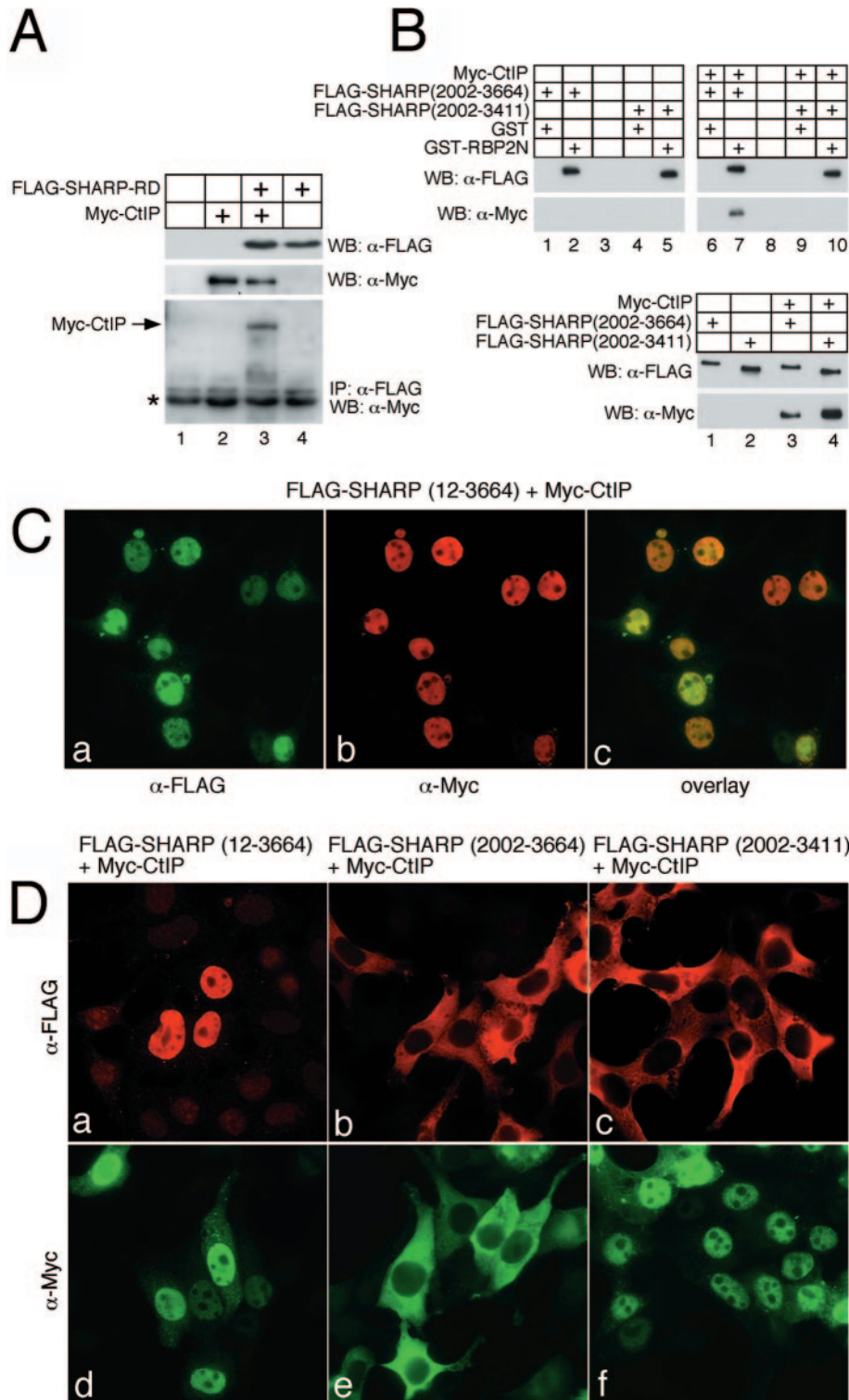
FIG. 4. SHARP interacts with CtIP in vivo and links CtIP with RBP-Jκ. (A) Expression of transfected SHARP-RD and CtIP was detected by Western blotting using antibodies against the FLAG (upper panel) or the Myc tags (middle panel). CtIP was coimmunoprecipitated together with SHARP-RD using an antibody directed against the FLAG-epitope exclusively from lysates where both proteins were expressed (lower panel, lane 3). Coimmunoprecipitated CtIP proteins were detected by Western blotting using an anti-Myc antibody. The asterisk indicates the heavy chain of the anti-FLAG antibody. (B, top) Either GST protein or GST-RBP-2N was immobilized on Sepharose beads and incubated with HEK-293 lysates expressing the SHARP(2002-3664) (lanes 1 and 2) or SHARP(2002-3411) (lanes 4 and 5) alone or together with Myc-CtIP protein (lanes 6, 7, 9, and 10). Only when both SHARP(2002-3664) and Myc-CtIP were expressed was a ternary complex formed with GST-RBP-2N (lane 7). This complex was not formed when a C-terminally truncated form of SHARP was expressed together with Myc-CtIP (lane 10). (B, bottom) Expression of the SHARP and CtIP proteins was verified by Western blotting. (C) HEK-293 cells were transiently transfected with an expression plasmid for

requirements of a possible bridging factor for SHARP and CtBP1 interaction.

The interaction of SHARP and CtIP was verified in vitro using the pull-down assays with GST-SHARP-RD (Fig. 3A and B). Full-length CtIP strongly bound to GST-SHARP-RD (Fig. 3A, lane 3) as well as to CtBP (Fig. 3A, lane 2) but not to GST alone (Fig. 3A, lane 5) or GST-RBP-2N (Fig. 3A, lane 1). The CtIP protein contains a binding site for the Rb protein LXCXE in addition to the binding site for CtBP, PLDLS. Neither of these sequences are necessary for the SHARP/CtIP interaction (Fig. 3B, lane 4), but, as previously shown, the PLDLS motif is required for the CtIP/CtBP interaction (24). Pull-down assays were also performed with in vitro-translated CtIP and various GST-SHARP-RD deletion constructs. Deletion of the C-terminal 36 aa of SHARP-RD completely abolished binding to CtIP (Fig. 3C). While this 36-aa segment is necessary for CtIP interaction, it is not sufficient for binding (data not shown). Pull-down assays using GST-SHARP-RD and various in vitro-translated and radiolabeled CtIP proteins identified two regions of CtIP as binding to GST-SHARP-RD, the N terminus (aa 59 to 320) and the C terminus (aa 620 to 897) (Fig. 3D to F). The same interaction domains were previously identified for transcription factor LMO4 (32).

The SHARP/CtIP interaction was further examined in vivo using coimmunoprecipitation experiments. FLAG-tagged SHARP-RD was expressed either alone or together with Myc-tagged CtIP in HEK-293 cells. After the precipitation of SHARP-RD from cell lysates using a monoclonal anti-FLAG antibody, CtIP was detected on Western blots using an antibody against the Myc-tag. CtIP was detected in the coimmunoprecipitate only when both proteins were expressed (Fig. 4A, lane 3). Furthermore, SHARP colocalized with CtIP in the cell nucleus (Fig. 4C). In fact, we demonstrated that CtIP colocalizes with wild-type SHARP(12-3664) in the nucleus and SHARP lacking a nuclear localization signal (aa 2002 to 3664) in the cytoplasm (Fig. 4D, panels a and d and panels b and e). CtIP did not colocalize in the cytoplasm with SHARP(2002-3411) lacking C-terminal RD (Fig. 4D, panels c and f).

Importantly, we show that GST-RBP-2N, a splice variant of RBP-Jκ, FLAG-SHARP, and Myc-CtIP form a complex (Fig. 4B). FLAG-SHARP(2002-3664) or C-terminally truncated SHARP(2002-3411) lacking the RD was coexpressed with Myc-CtIP in HEK-293 cells. GST-RBP-2N specifically bound FLAG-SHARP proteins and Myc-CtIP. However, this ternary RBP-SHARP/CtIP complex did not form when cell lysate expressing the truncated FLAG-SHARP protein was used (Fig. 4B, lanes 7 and 10).

SHARP, CtIP, and CtBP act together to repress transcription. The significance of our biochemical findings that CtIP and CtBP are novel constituents of the RBP-SHARP corepressor complex was investigated using a transrepression assay for CtIP and CtBP function on SHARP-mediated transcriptional repression. Expression plasmids for either Gal4-VP16 or Gal4-VP16-SHARP-RD in combination with expression plasmids for CtBP1 or CtIP were cotransfected into HeLa cells together with a Gal4-responsive reporter construct. Neither CtBP1 nor CtIP on its own could disrupt Gal4-VP16-mediated transactivation (Fig. 5A). However, when G4-VP16-SHARP-RD was used in the transrepression assay, the expression of either CtBP1 or CtIP increased the repressive effect of SHARP (Fig. 5A). In addition, a CtIP construct lacking the CtBP interaction domain (CtIP ΔPLDLS) failed to repress transcription (Fig. 5A). SHARP-dependent transcription requires, in part, the presence of CtBP (Fig. 5B). Mouse embryonic fibroblasts (MEFs) were transfected with the Gal4-dependent reporter construct pFR-LUC and the SHARP-RD specific expression plasmid G4-VP16-SHARP-RD. In CtBP-deficient MEFs, SHARP-mediated repression is less effective than in the heterozygous MEFs. Importantly, we could demonstrate that the transcriptional repression of CtIP, when it functions as a corepressor for SHARP, is dependent on CtBP (Fig. 5C). MEFs were transfected with the reporter construct and the SHARP-RD-specific expression plasmid G4-VP16-SHARP-RD, together with increasing amounts of a CtIP expression plasmid. A dose-dependent repressive activity for CtIP was detected only in heterozygote MEFs, not in CtBP1/CtBP2-deficient MEFs (Fig. 5C). These results strongly suggest that CtIP corepressor function with SHARP-RD requires CtBP.

SHARP-mediated repression requires the CtBP corepressor. *Hey1* was identified as a Notch target gene in mammals (15). To test the effect of CtBP- and CtIP-mediated transcriptional repression on a naturally existing Notch-responsive promoter, we performed cotransfection experiments with the human *Hey1* promoter fused to the luciferase gene. Cotransfection of the *Hey1* promoter construct together with the RBP-VP16 activator into HeLa cells strongly activated transcription, and increasing amounts of full-length SHARP resulted in a clear repression of RBP-VP16-mediated transactivation (Fig. 6A). In order to investigate the functional role of CtBP in transcriptional repression, the ability of the E1A adenoviral protein to derepress *Hey1* transcriptional control was explored. It was previously shown that the portion of the E1A protein encoded by exon 2 strongly binds CtBP, thereby relieving CtBP-mediated transcriptional repression (33). Cotransfection of increasing amounts of E1A exon 2 lessened CtBP-mediated repression of the *Hey1* promoter in HeLa cells, thereby increasing promoter activity up to sixfold (Fig. 6B). A mutant E1A exon 2 that no longer binds to CtBP, however, had no effect on transcriptional activity of the *Hey1* promoter. Similar experiments conducted in CtBP-deficient mouse embryonic fibro-

SHARP(12-3664) and Myc-tagged CtIP. Cells were fixed 24 h after transfection, permeabilized, and immunostained using anti-FLAG and anti-Myc antibodies. The subcellular localization of SHARP (green, panel a) and CtIP (red, panel b) was assayed by fluorescence microscopy. (D) HEK-293 cells were transfected with various SHARP expression constructs together with Myc-CtIP as indicated. Subcellular protein localization was visualized using immunofluorescence staining, as described above. Both, wild-type SHARP (panel a) and CtIP (panel b) proteins are localized predominantly in the nucleus. Transfection of SHARP lacking the nuclear localization signal (aa 2002 to 3664, panel b) resulted in the cytoplasmic localization of CtIP (panel e). Transfection of SHARP(2002-3411) lacking both the nuclear localization signal and the RD (panel c) resulted in a restoration of the nuclear localization of CtIP (panel f).
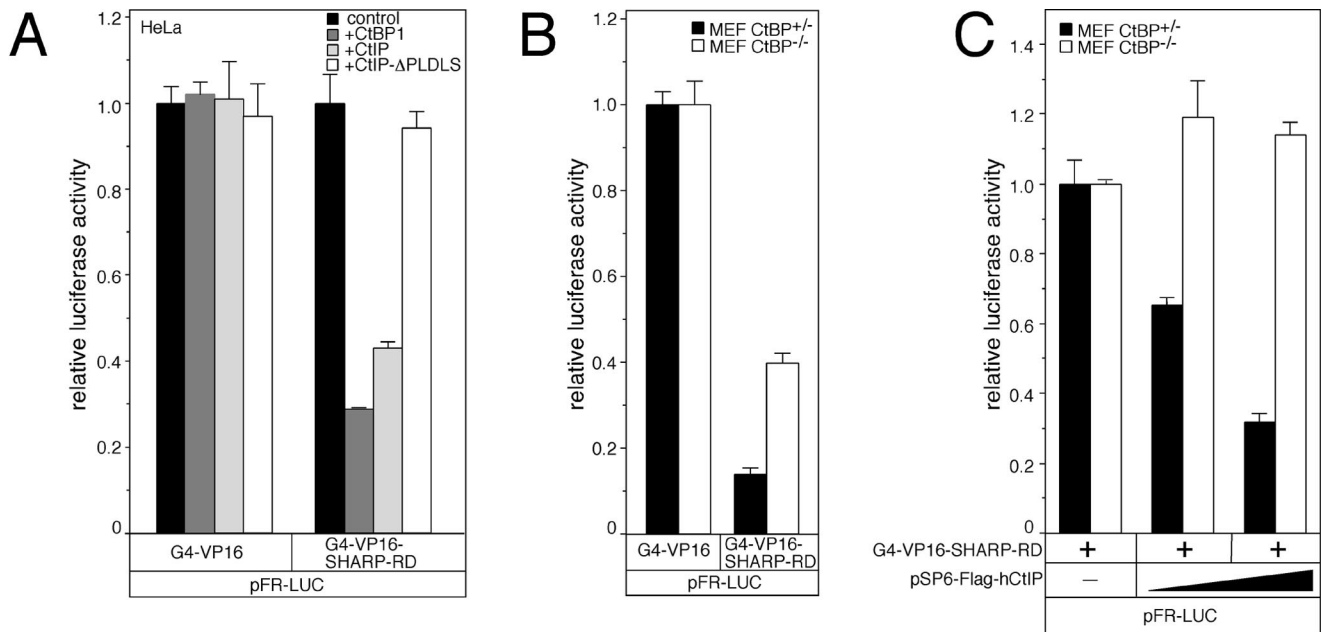
FIG. 5. CtIP and CtBP act as SHARP corepressors in the SHARP transcriptional repression assay. (A) The pFR-LUC reporter construct (2 μg) was transfected into HeLa cells together with either Gal4-VP16 or Gal4-VP16-SHARP-RD plasmids (20 ng) and expression constructs for CtBP1, CtIP, or CtIP-ΔPLDLS (100 ng). Cotransfection of CtBP1 or CtIP represses SHARP-RD-mediated transcription (right) but not VP16-mediated transcription (left). (B) SHARP-RD-mediated repression is less effective in CtBP-deficient MEFs. The pFR-LUC reporter construct (2 μg) was transfected into MEFs heterozygous (black bars) or CtBP-deficient (white bars) together with G4-VP16 (20 ng) or the G4-VP16-SHARP-RD plasmid (20 ng). Luciferase activity was normalized to the transcriptional activity of Gal4-VP16. Mean values and standard deviations from 12 independent experiments are shown. (C) CtIP corepressor function depends on CtBP in the SHARP repression assay. The pFR-LUC reporter construct (2 μg) was transfected into MEFs heterozygous (black bars) or CtBP-deficient (white bars) together with the G4-VP16-SHARP-RD plasmid (20 ng) and increasing amounts (100 and 200 ng) of CtIP expression plasmids. Luciferase activity was determined from 100-μg portions of total-cell extracts and normalized to the transcriptional activity of the Gal4-VP16 and Gal4-VP16-SHARP-RD constructs alone. Mean values and standard deviations from four independent experiments are shown.

blasts showed that transfection of increasing amounts of CtBP1 rescued transcriptional repression of the *Hey1* promoter (Fig. 6C). Additionally, CtBP1-induced repression was relieved by the cotransfection of wild-type E1A exon 2 but not by mutant E1A exon 2. Neither wild-type nor mutant E1A exon 2 has an effect on *Hey1* promoter activity in the absence of CtBP1. Finally, we utilized quantitative reverse transcription-PCR of either heterozygous or CtBP-deficient MEFs to demonstrate that in the absence of CtBP, the endogenous *Hey1* message is strongly derepressed or upregulated (Fig. 6D). When CtBP was reintroduced into CtBP-deficient MEFs, repression at the endogenous *Hey1* promoter was partially restored (Fig. 6E).

Formation of the endogenous corepressor complex. Both biochemical and functional data suggest the presence of an endogenous RBP-Jκ/SHARP-CtIP/CtBP corepressor complex. We set out to isolate also the endogenous complex taking advantage of the high affinity of RBP-Jκ to its DNA-binding site 5′-CGTGGG AA-3′. RBP-Jκ itself binds tightly to a biotinylated multimerized RBP-Jκ-oligonucleotide column as seen in EMSA and Western blotting with anti-RBP-Jκ antibodies (peak fractions 8 to 10, elution fractions E3 to E5; Fig. 7). As monitored by silver staining and Western blotting, our DNA affinity purification approach yielded a defined spectrum of bands for the putative RBP-Jκ-containing complex(es) (data not shown); the achieved purification was from 60-fold (E2) to 800-fold (E6). Importantly, CtIP coelutes with RBP-Jκ in elution fractions E4 and E5. CtBP can

still be found to bind to the RBP-Jκ oligonucleotide column. However, most of it comes off in elution E1 (180 mM NaCl).

We propose that both CtIP and CtBP are new constituents of the mammalian RBP-Jκ (Notch) corepressor complex. Because SHARP is capable of interacting with both RBP-Jκ and CtIP directly, it is conceivable the SHARP plays a functionally similar role to *Drosophila* Hairless.

DISCUSSION

RBP-Jκ is the central player in the transcriptional regulation of Notch target genes and functions in both transcriptional repression and activation. Notch IC enters the nucleus, binds to RBP-Jκ, and activates target genes. In the absence of Notch IC, RBP-Jκ recruits a corepressor complex that keeps Notch target genes inactive. Previously, we described SHARP as an RBP-Jκ-interacting corepressor (20). SHARP is able to repress transcription of Notch target genes, inhibits Notch-mediated transactivation, and rescues Notch-induced inhibition of primary neurogenesis in *Xenopus laevis* embryos. Here, we further investigated the mechanism of RBP-Jκ/SHARP-mediated repression by identifying the CtIP/CtBP corepressors as novel components of the corepressor complex. We show that the RBP-Jκ/SHARP-CtIP/CtBP complex physically interacts in vitro and in vivo. We can purify an endogenous RBP-Jκ corepressor complex that contains CtIP and CtBP. In addition,
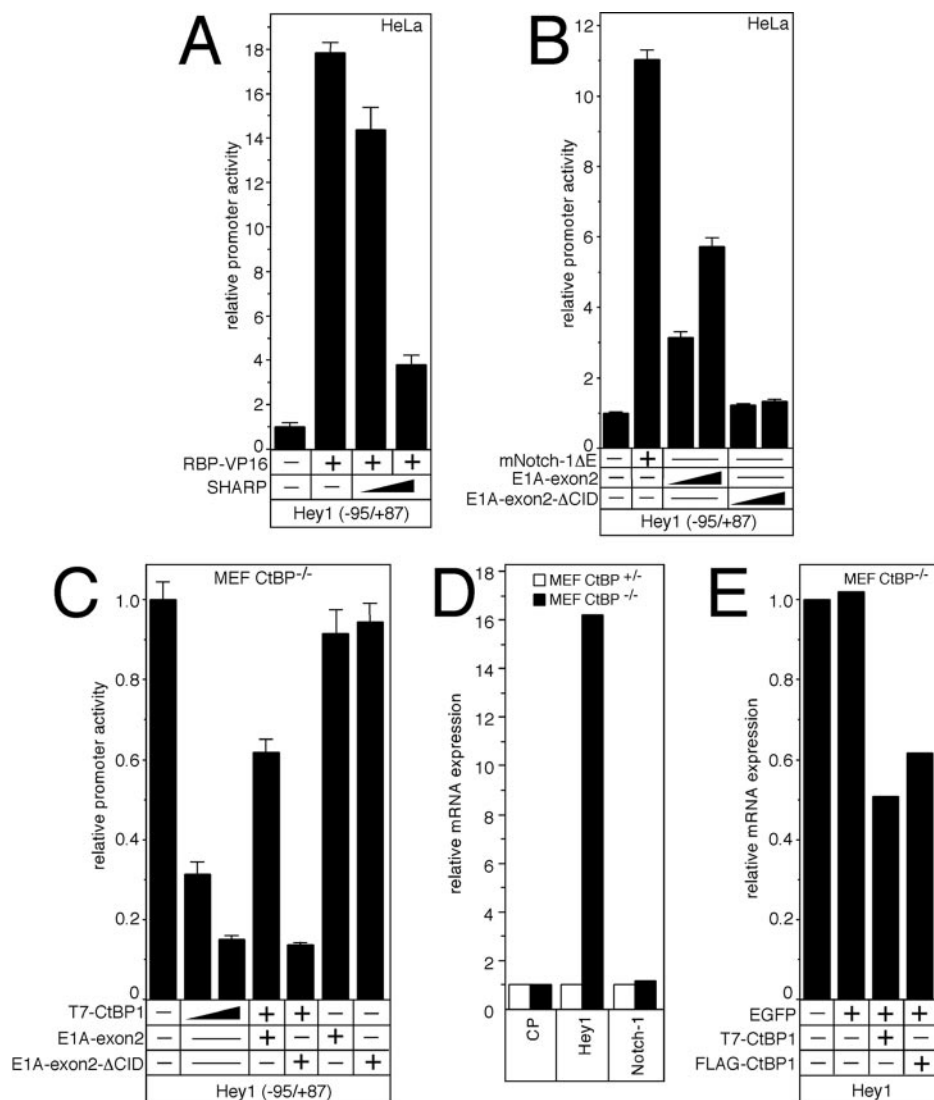
FIG. 6. SHARP and CtBP act as corepressors for *Hey1* transcription. (A) The *Hey1* reporter construct (2 μg) was transfected into HeLa cells alone or together with RBP-VP16 expression plasmids (60 ng) and increasing amounts of SHARP expression construct (200 and 400 ng). SHARP repressed RBP-VP16-mediated transcription of the human *Hey1* promoter. (B) Cotransfections were performed with 40 ng of mNotch-DE expression plasmids or increasing amounts (100 and 200 ng) of E1A-Exon2 expression constructs. Expression of the E1A CtBP-binding motif resulted in the derepression of the *Hey1* promoter. (C) CtBP-deficient MEFs were cotransfected with CtBP1 expression plasmids (100 and 200 ng) alone or together with either the E1A-Exon2 expression plasmid (100 and 200 ng) or a construct lacking the CtBP-binding motif (E1Aexon2-ΔCID). Only coexpression of the E1A CtBP-binding motif, together with T7-CtBP1, relieved CtBP1-mediated repression in CtBP-deficient MEFs. Luciferase activity was determined from 100-μg portions of total-cell extracts and normalized to the basal promoter activity of the reporter construct. Mean values and standard deviations from four independent experiments are shown. (D) *Hey1* transcription is upregulated in CtBP-deficient MEFs. CP, cyclophilin. *Hey1* and Notch-1 mRNA levels were examined using real-time PCR. The mRNA levels were normalized to the endogenous cyclophilin mRNA levels for each cell type. One representative experiment of six is shown. (E) Expression of CtBP1 proteins in CtBP-deficient MEFs represses *Hey1* transcription. Cells were transfected with T7-CtBP1 or Flag-CtBP1 together with pEGFPC1. GFP-positive cells were sorted, and *Hey1* mRNA levels were examined. The mRNA levels were normalized to the endogenous cyclophilin mRNA levels. One representative experiment of four is shown. +, with the indicated construct; −, without the indicated construct.

we demonstrate that the CtBP corepressor is required for SHARP-mediated transcriptional repression using CtBP-deficient MEFs and the functional knockdown of CtBP by adenoviral E1A exon 2, that CtBP is required for the repression of the Notch target gene *Hey1* (Fig. 5 and 6), and that endogenous *Hey1* is derepressed in CtBP-deficient MEFs. Taken together, three independent lines of evidence are presented

here, showing CtBP to be essential for silencing the Notch target gene *Hey1*.

CtIP and CtBP as novel components of RBP/SHARP-corepressor complex. Both HDAC-independent and -dependent mechanisms have been proposed for RBP-Jκ-mediated transcriptional repression and are not necessarily mutually exclusive. It was shown that RBP-Jκ is able to recruit an HDAC/
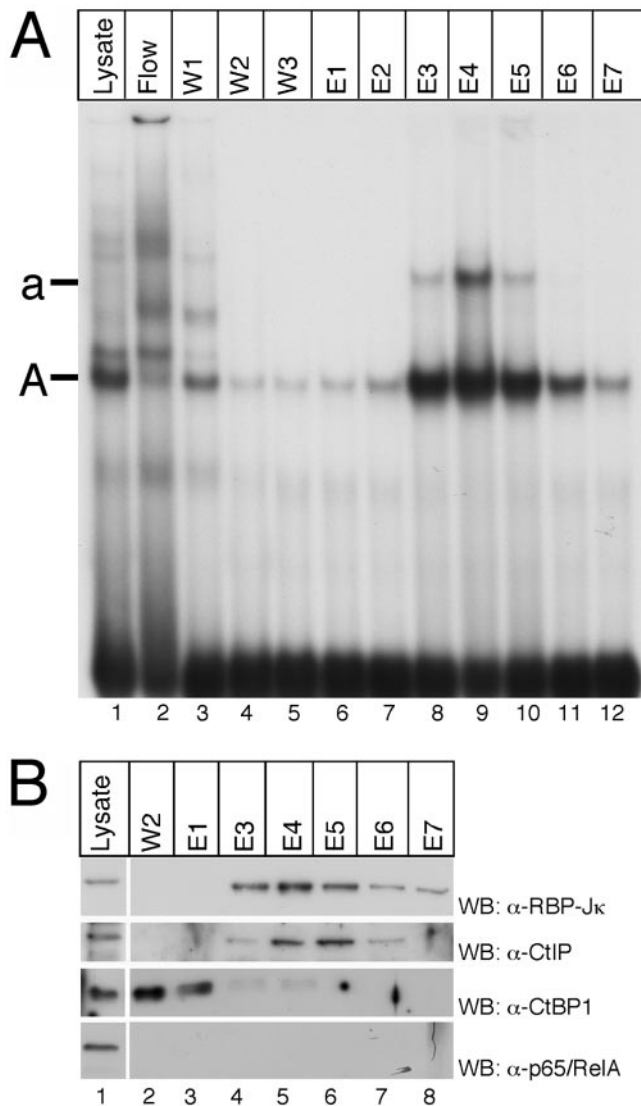
FIG. 7. Purification of endogenous RBP-Jκ complexes by DNA affinity chromatography. (A) A DNA fragment containing 12 RBP-Jκ-binding sites was biotinylated and immobilized with streptavidin Sepharose. The column was incubated with cellular extract from Jurkat T cells. After washing, the DNA-binding activity was eluted with increasing NaCl concentrations as indicated in Materials and Methods. RBP-Jκ-specific DNA-binding activity (complexes A [single occupancy] and a [double occupancy] were eluted in fractions E3 to E6 (lanes 8 to 11). (B) Western analysis of lysate (lane 1), washing step 2 (lane 2), and eluted fractions (lanes 3 to 8) using the indicated antibodies. WB, Western blot.

SMRT/SKIP-containing complex (9, 42). In contrast, Honjo and coworkers showed recently that the activity of the *Hes1* and *Hes5* promoters is not increased in RBP-Jκ-deficient T cells (34). One interpretation is that neither RBP-Jκ nor an associated RBP-Jκ corepressor complex is needed for transcriptional repression. In our case, looking at the endogenous Notch target gene *Hey1* (Fig. 6D and E), the absence of corepressor CtBP does lead to strong derepression. This discrepancy might be explained by different regulation at different Notch target genes.

Our previous findings describing SHARP-mediated repression as being sensitive to trichostatin agree with findings that HDAC activity is required for optimal RBP-Jκ corepressor function (20). Here, we further elucidated the mechanism of RBP-Jκ-mediated transcriptional repression by identifying the CtIP and CtBP corepressors as novel constituents of the RBP-Jκ/SHARP corepressor complex. Transcriptional repression via the joint action of CtIP and CtBP has previously been demonstrated for Rb/p130- and Ikaros-mediated repression (10, 16). In both cases, Ikaros and Rb utilize HDAC-dependent and -independent mechanisms of transcriptional repression. CtBP is a broadly expressed corepressor protein that binds Pro-X-Asp-Leu-Ser (PXDLS) motifs present in diverse transcriptional regulators. CtBP also binds to HDACs and Polycomb group proteins (26). The ability of CtBP to form homodimers may allow it to serve as a link between these transcriptional silencing complexes and regulators bearing PXDLS motifs. Previously, Shi and colleagues described a CtBP repressor complex that exhibited a number of enzymatic activities, including HDAC and histone methyltransferases (29). Most recently, the same group showed that lysine-specific demethylase 1, another component of the CtBP complex, is capable of demethylating histone H3 at the Lys 4 position (28). Combining the data we presented here with these findings allows speculation that histone demethylation plays a role in Notch target gene repression. Alternatively, the recruitment of Polycomb-containing repressor complexes via CtIP/CtBP may be involved in maintaining Notch target gene silencing.

Is SHARP the functional analog of *Drosophila* Hairless? SHARP belongs to the split ends (Spen) protein family, bearing characteristic N-terminal RRMs and a conserved SPOC domain (1). Spen-like proteins have been identified in vertebrates but also in *Caenorhabditis elegans* and *Drosophila*. However, it is unlikely that *Drosophila* Spen (dSpen) is the functional SHARP homolog. (i) Despite the homologies between dSpen and SHARP within the N-terminal RRMs and the C-terminal SPOC domain, the intervening region shows only poor homology and differs greatly in size. (ii) We have shown that SHARP physically interacts with RBP-Jκ via a well-defined interaction domain and antagonizes Notch-mediated transcriptional activation (20). The RBP-Jκ-interaction domain of SHARP is highly conserved from human to *Xenopus* but is not present in dSpen. Furthermore, no physical interaction between dSpen and SuH, the RBP-Jκ ortholog in *Drosophila*, has yet been reported.

SuH was originally identified on the basis of its dominant suppression of the H phenotype, and genetic evidence indicates that H negatively regulates the activity of SuH and the Notch pathway (17). H is a potent antagonist of Notch pathway activity and binds not only to SuH but also to dCtBP and Groucho corepressors (2, 17). However, to date, no mammalian homolog of H has been identified. Functionally analogous to *Drosophila* H, the vertebrate protein SHARP plays the bridging role between RBP-Jκ and corepressors. Since *Drosophila* H is able to recruit the dCtBP corepressor, we investigated whether SHARP had a similar function and recruits the mammalian CtBP protein. The initial requirement is that SHARP is capable of interacting with CtBP, and, indeed, we show here that this protein coimmunoprecipitates. However, the

SHARP-CtBP interaction is not direct but requires an additional bridging factor, CtIP.

The C-terminal SPOC domain of SHARP in transcriptional repression. We and others have previously reported SHARP to be important for transcriptional repression. The murine homolog of SHARP, MINT, is required for the repression of the homeodomain transcriptional repressor, Msx2 (18). The SHARP SPOC domain interacts with the corepressors SMRT and NCor (27). X-ray crystallography of the SHARP SPOC domain shows conserved residues that are important for the interaction with SMRT or NCor corepressors (1). SMRT and NCor, in turn, recruit larger multiprotein complexes containing HDAC activity.

Here we show that the SPOC domain alone is able to repress transcription. Furthermore, the C-terminal 36 amino acids are required to interact with the coiled-coil domains of the CtIP corepressor. Therefore, we suggest that the SHARP SPOC domain binds not only SMRT and NCor but also corepressors CtIP and CtBP. RBP/SHARP-mediated repression possibly recruits redundant corepressors that work at different Notch target genes in combination or alone and that function not only in an HDAC-dependent but also in an HDAC-independent manner.

The role of CtIP/CtBP in Notch signaling in vivo. It is well established that Notch signaling is important for several differentiation decisions during embryonic development. It would follow that deficiency of key components such as RBP-Jκ, SHARP, CtBP, or Notch target genes should cause severe developmental defects. As expected, RBP-Jκ deficiency in mice causes gross developmental anomalies, resulting in embryonic death before day 10.5 of gestation (19). MINT-deficient mice die at embryonic day 12.5 to 13.5 and exhibit defects in cardiac development (12). Inactivation of CtIP leads to early embryonic lethality mediated by a G_1 restraint (3). Here, we show that the Notch target gene *Hey1* is negatively regulated by corepressors SHARP, CtIP, and CtBP. Deficiency in *Hey1*/*Hey2* results in death after embryonic day 9.5 (E9.5) with vascular defects. Similar defects are observed in Jagged1 and Notch1 knockout mice (7). Deficiency in the CtBP corepressor leads to embryonic death at embryonic day 10.5 due to defects in extraembryonic vascularization (8). The different knockout mouse models clearly demonstrate important roles for RBP-Jκ, SHARP, CtIP, and CtBP in early embryonic development, as expected for mice with aberrant Notch signaling.

In summary, our results indicate that corepressors CtIP and CtBP are novel components of the RBP/SHARP corepressor complex and that CtBP is required for the repression of the Notch target gene *Hey1*. It will be interesting to investigate the impact of CtIP and CtBP on Notch signaling and nuclear hormone signaling in vertebrate systems. Conditional knockout mice will be key in supplying many answers that describe the control mechanisms in these signaling pathways.

ACKNOWLEDGMENTS

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