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The homeodomain protein Cux1 interacts with Grg4 to repress p27^{kip1} expression during kidney development

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Abstract

The homeodomain protein Cux1 is highly expressed in the nephrogenic zone of the developing kidney where it functions to regulate cell proliferation. Here we show that Cux1 directly interacts with the co-repressor Grg4 (Groucho 4), a known effector of Notch signaling. Promoter reporter based luciferase assays revealed enhanced repression of *p27^{kip1}* promoter activity by Cux1 in the presence of Grg4. Chromatin immunoprecipitation (ChIP) assays demonstrated the direct interaction of Cux1 with *p27^{kip1}* in newborn kidney tissue *in vivo*. ChIP assays also identified interactions of Cux1, Grg4, HDAC1, and HDAC3 with *p27^{kip1}* at two separate sites in the *p27^{kip1}* promoter. DNAse1 footprinting experiments revealed that Cux1 binds to the *p27^{kip1}* promoter on the sequence containing two Sp1 sites and a CCAAT box ~500 bp from the transcriptional start site, and to an AT rich sequence ~1.5 KB from the transcriptional start site. Taken together, these results identify Grg4 as an interacting partner for Cux1 and suggest a mechanism of *p27^{kip1}* repression by Cux1 during kidney development.

Introduction

Cux1 is the murine homologue of the Drosophila gene cut. Cux1 contains four potential DNA binding domains: three 60 amino acid repeats, termed cut repeats, and the homeodomain (1–3). Mammalian cut proteins function as cell cycle-dependent transcription factors that can function as activators or repressors (4–12). Targets of repression by Cux1 include γ-globin (13), c-myc (14), myosin heavy chain (15), NCAM (16), CD8a (17), c-mos (18), MMTV long terminal repeats (19), gp91-phox (20), and the cyclin kinase inhibitors p21^{waf1} and p27^{kip1} (21–22). The binding of Cux1 protein to the promoters of target genes appears to be limited to tissues or developmental stages in which the target genes are not expressed. Upon terminal differentiation, Cux1 is downregulated or loses the ability to bind to the promoters, permitting transcription of the target genes. Cux1 represses transcription by two mechanisms: 1)

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Competition for CCAAT or SP1 binding site occupancy, preventing activation by the corresponding transcription factors, or 2) Active repression via carboxy-terminal repression domain following binding at distance from transcription start site (23). It has been suggested that the mechanism of active repression involves the direct recruitment of HDAC1 deacetylase (24). In addition, Cux1 was reported to recruit G9a histone lysine methyltransferase to repress transcription of the $p21^{waf1}$ promoter (7). During cell cycle progression, a nuclear isoform of the cysteine protease cathepsin L cleaves Cux1, forming the amino-truncated p110 Cux1 protein, which contains two cut repeats and the homeodomain (25). A shorter isoform, containing a single cut repeat and the homeodomain, is the result of transcription from an alternate promoter (26).

Mice carrying targeted deletions of Cux1 exhibit reduced growth, retarded differentiation of lung epithelia, hair follicle defects, reduced male fertility, and deficient T and B cell function (27–29). In contrast, transgenic mice ectopically expressing Cux1 exhibit multiorgan hyperplasia including an increase in the size of the kidneys, heart, liver, and testis, apparently resulting from the repression of $p27^{kip1}$ gene expression (22). These mice also develop glomerulosclerosis, renal interstitial fibrosis, and hepatic tumors (22,30–31). Cux1 transgenic mice have a similar kidney phenotype as $p27^{kip1}$ knockout mice (32–34), while $p21^{waf1}$ knockout mice do not exhibit renal hyperplasia (35), suggesting that $p27^{kip1}$, and not $p21^{waf1}$, may be the primary target of Cux-1 repression in the kidney. Another Cux1 transgenic mouse ectopically expressing p75 Cux1 exhibits a myeloid leukemia like myeloproliferative disease with reduced $p27^{kip1}$ expression in the spleen (36). Recently, p75 Cux1 transgenic mice have been reported to develop polycystic kidney disease, associated with reduced $p27^{kip1}$ expression and upregulation of c-myc (11).

The TLE/ Groucho (Grg) proteins are members of a family of co-repressor proteins that do not themselves bind to DNA but are recruited to DNA by interactions with DNA binding proteins (37). Once recruited to a promoter, the Grg proteins can recruit histone deacetylases or directly interact with histones or the basal transcriptional machinery, resulting in short-term or long-term repression (38–40). In yeast, the Groucho related protein, Tup1, can repress transcription by interacting with subunits of RNA polymerase II holoenzyme (41–42). TLE/Grg proteins are broadly expressed in developing organs and interact with multiple transcription factors involved in patterning and differentiation (43).

In Drosophila, Cut functions as a downstream effector of the Notch signaling pathway (1,44–46). The Notch pathway is highly conserved across most species. In mammals, there are four Notch receptors (Notch1–4) and five ligands (delta like 1, delta like 3, delta like 4, jagged 1, and jagged 2). Notch signaling is activated when jagged or delta ligands bind to Notch receptors, resulting in the proteolytic cleavage of the Notch receptor releasing the notch intracellular domain (NICD) (47–49). NICD translocates to the nucleus and associates with the RBP-jk transcription factor to activate the expression of Notch effector proteins, such as the HES or HERP family proteins (49). These proteins recruit Grg proteins as cofactors, and this complex functions to repress tissue specific genes such as Myo D and Mash during development (49–51). We have previously shown that Cux1 co-localizes with Notch pathway components in numerous tissues during embryogenesis and co-immunoprecipitates with Grg4 in a rat kidney epithelial cell line expressing a constitutively active Notch1, called RKE $Notch^{ic}$ cells (52–53). The RKE $Notch^{ic}$ cells exhibit significantly higher levels of Cux1 expression compared to vector only transfected cells, and show reduced $p27^{kip1}$ expression (53). Here we show a direct interaction between Cux1 and the co-repressor protein Grg4, and show that Cux1 is part of a complex bound to the $p27^{kip1}$ promoter in the native chromatin configuration in the developing kidney.

Materials and methods

Antibodies

Rabbit Anti-CDP (Cux1) (#sc-13024), goat anti-CDP (Cux1) (#sc-6327), rabbit anti-Grg4 (#sc-9125), rabbit anti-Grg1 (#sc-9121), and rabbit anti-p27 (#sc-528) antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-HDAC1 (#2062) and anti-HDAC3 (#2632) antibodies were purchased from Cell Signaling.

Immunohistochemistry

Kidneys were isolated from 3-day-old C57BL/6J mice and immersion fixed in 4% paraformaldehyde and embedded in paraffin. Five micron-thick tissue sections were de-paraffinized with xylene and hydrated with graded ethanols. Immunolabeling was performed as described previously (53).

Preparation of kidney lysates and western blot analysis

Kidneys isolated from 3-day-old mice were isolated and frozen immediately in dry ice and ethanol and stored at −80°C until used. Kidneys were thawed on ice and chopped with a sterile scalpel blade before use. The chopped kidneys were suspended in Ripa buffer (0.01M Sodium phosphate, pH 7.2; 125mM sodium chloride; 50mM sodium fluoride; 0.1% SDS; 1mM ethylenediaminetetra acetic acid; 1% sodium deoxycholate; 1%NP40; 4μg/ml aprotinin; 4μg/ml pepstatin; 4μg/ml leupeptin; 1mM PMSF (phenylmethylsulfonyl fluoride). The tissue was homogenized, using a dounce homogenizer, until no cellular clumps were visible, (~25 times up and down). This was incubated on ice for 20 minutes and centrifuged at 15000 rpm at 4°C for 30 minutes. The supernatant was collected and measured for protein concentration using BCA protein assay (Biorad). Western blotting was performed as described previously (53).

Co-immunoprecipitations

Coimmunoprecipitation assays were performed as previously described (53). Briefly, 60μl protein A-Sepharose beads were washed with RIPA buffer and were incubated with anti-Grg4, anti- Cux1, anti-HDAC1, or anti-HDAC3 antibodies. Immune complexes were washed and incubated with 500μg of protein (lysate) isolated from newborn kidney. Reactions were washed and analyzed by Western blot analysis.

Transient reporter assay

Transient luciferase assay was performed as described previously (22). Briefly, human embryonic kidney (293T) cells were cultured in Dulbecco's modified Eagle's medium (glucose concentration, 450mg/dl) supplemented with heat-inactivated 10% fetal bovine serum and 100U/ml penicillin and 100mg/ml streptomycin under humidified 5% CO2/95% air at 37°C. Cells were plated at a concentration of $6X10^5$ cells per well of twelve well plates 18 hrs prior to the transfection. Transient transfections were performed using Fugene transfection reagent (Roche Applied Science), using 0.5 μg of luciferase reporter plasmid containing p27 promoter sequence (−1609 to +178), along with 0.1 μg renilla-expressing plasmid (to correct for transfection efficiencies), pCMV/Cux1 and pKW/Grg4 at the concentrations indicated, and pcDNA3.1 (to control for non-specific vector effects). After 48 hours, cells were lysed, and luciferase and renilla activities were determined by enzyme assay kits. Luciferase activity was normalized to renilla activity as an internal transfection control. Comparisons between luciferase activity were made using one-way ANOVA. $P < 0.05$ was considered statistically significant.

GST pull down assay

Full length GST fusion protein (Grg4-GST) and full-length Cux1 were expressed in Escherichia coli BL21 (DE-3) cells by induction with isopropyl-B-thiogalactopyranoside for 2 hours at 30°C and 37°C respectively. Cell pellets were suspended in NETN buffer (20 mM Tris, pH 8.0; 100mM NaCl; 1mM EDTA; 0.5% Nonidet P-40) and native purification buffer (40 mM NaH_2 PO_4 pH 8.0 and .5 M NaCl) at 4°C supplemented with protease inhibitors (Roche), followed by lysis. Cleared lysates were suspended with washed Glutathione beads (Amersham) or probond resin (Invitrogen) and Grg4 or Cux1 was immobilized on glutathione beads or probond Ni agarose beads followed by extensive washings with the respective buffers listed above. The Cux1 protein was eluted by 250mM imidazole supplemented in the native buffer, pH 8.0. The GST pulldown assay was performed by incubating eluted Cux1 with glutathione-sepharose-bound GST, or GST fusion protein Grg4 in the binding buffer (10mM Tris, pH 7.6; 50mM NaCl; 5mM EDTA; 1%Triton-X 100; protease inhibitor) at 4°C for 1 hour. After incubation, the beads were washed three times with binding buffer and boiled in 6X sample buffer. The eluted binding proteins were electrophoresed in 4–6% gradient SDS-acrylamide gels (Biorad) and transferred to polyvinylidene fluoride (PVDF) membranes, followed by Western blot analysis.

Chromatin Immunoprecipitations

ChIP assays were performed using the EZ ChIP kit (Upstate Corp. Lake Placid, New York) according to manufacturer's directions, and as previously described (54). RKE cells or kidneys isolated from newborn mice were used for ChIP analysis. Briefly, equal aliquots of isolated chromatin were subjected to immunoprecipitation with an anti-Cux1, anti-Grg4, anti-HDAC1, anti- HDAC3, anti-RNA polymerase II, or IgG antibodies. DNA associated with immunoprecipitates was used as a template for PCR analysis with primers producing 249 bp (A), 317 bp (B), or 191 bp (C) fragments of the $p27^{kip1}$ promoter spanning −1609 to −1360 (A), −1272 to −955 (B), or −687 to −496 (C), relative to the transcription start site. Primers used were: **A)** 5'- AGCATTTTCGCCCTTCAAGAG-3' and 5'-CCAAGTGGGAAAGCCATTGC-3', **B)** 5'-TGAAGAGGCTTGAGAGCACTG-3' and 5'-TGGCTTGTTTGGAGCCTCAGTG-3', and **C)** 5'-AATGTCCTGGCGGCGGT-3' and 5'-GGAGGCTGACGAAGAAGAAGATG-3'. For newborn kidneys, $p27^{kip1}$ primers used were: 5'-CAGAGCAGGTTTGTTGGCAGTC-3' and 3'-GGCTGACGAAGAAGAAGATGATTG-5'. PCR conditions were determined to ensure that results were within linear range of the PCR. Results obtained from unrelated antibody controls combined with enrichment when specific antibodies were used confirmed they were in the linear range of product amplification and not a consequence of non-specifically immunoprecipitating chromatin.

DNase 1 footprint analysis

The $p27^{kip1}$ −1609/-1360 (fragment A) and −687/-496 (fragment C) amplification products from the ChIP analysis were used for DNA footprinting. DNA fragments obtained from the ChIP analysis were cloned into PCR Topo TA cloning vector (Invitrogen). DNase1 footprinting was performed using a core footprinting System from Promega. Briefly, double-stranded, single ^{32}P-end-labeled probe was obtained by digesting the plasmid with Spe1 and Not1 to obtain the double stranded 249bp and 191bp fragments. The 5' ends were then dephosphorylated and radiolabeled, followed by restriction digestion with Pme1 and Pst1 for $p27^{kip1}$ −1609/-1360 (fragment A) and $p27^{kip1}$ −687/-496 (fragment C) respectively, so that only the 3' end remained labeled. These probes were incubated with in vitro synthesized Cux1, which was generated using the Tnt quick coupled transcription/translation kit and transcend tRNA kit (Promega) according to manufacturer's directions. Various concentrations of DNase 1 were added and samples were incubated for one minute, followed by the addition of stop

solution. Following purification, the DNA samples were electrophoresed through a 6% polyacrylamide, 7M urea sequencing gel at 1500V for 2 hours. Gels were dried and visualized by autoradiography.

Results

Cux1 is the murine homologue of the Drosophila gene Cut. In Drosophila, Cut is regulated by the Notch signaling pathway. Previously we showed that Cux1 expression is upregulated by an activated Notch1 in rat kidney epithelial (RKE) cells (Notch^{ic} cells), while p27^{kip1}, a target of repression by Cux1, is downregulated (53). Moreover, we identified Grg4, a mammalian homologue of the co-repressor groucho, as a potential interacting partner of Cux1 in RKE cells. Grg4 was significantly upregulated in Notch^{ic} cells and co-immunoprecipitated with Cux1. In addition, Cux1 and Grg4 are co-expressed in the nephrogenic zone of the developing kidney, with highest expression in the presumptive podocytes of capillary loop staged glomeruli. Taken together, our previous results raised the possibility that Cux1 interacts with Grg4 to regulate gene expression during kidney development.

Cux1 interacts with Grg4 *in Vivo* and *in Vitro*

To determine whether Cux1 associates with Grg4 in the developing kidney, reciprocal co-immunoprecipitation assays were performed. Cux1 was immunoprecipitated from newborn kidney lysates from 3-day old wild type (WT) and Cux1 transgenic (TG) mice, followed by Western blotting for the presence of Grg4. Grg4 protein was coimmunoprecipitated with Cux1 from the kidney lysates, but not in control precipitations (Figure 1A). Similar immunoprecipitation experiments were performed showing that Cux1 protein was coimmunoprecipitated with Grg4 from newborn kidney lysates (Figure 1A). The presence of Groucho binding sites on Cux1 raised the possibility that Cux1 can physically interact with Grg4. To test this directly, we performed pulldown assays using bacterially expressed GST fused full-length Grg4 and full-length Cux1 protein. GST fused Grg4 protein was immobilized on glutathione agarose beads and incubated with bacterially expressed Cux1. This showed that Cux1 interacts directly with GST-Grg4 (Figure 1B). In contrast, the GST protein alone incubated with Cux1 showed no interaction. These results indicate a direct interaction between Grg4 and Cux1, and suggest that Cux1 and Grg4 form a complex during kidney development. The weaker interaction between Cux1 and Grg4 detected by the pulldown assay, compared to the co-immunoprecipitation results, suggests that direct interaction of Cux1 with Grg4 may be stabilized by other proteins present in a complex.

Grg4 enhances the ability of Cux1 to repress p27^{kip1} promoter activity

We previously showed that Cux1 represses p27^{kip1} promoter activity in a reporter assay. In addition, transgenic mice ectopically expressing Cux1 exhibit multiorgan hyperplasia, similar to p27^{kip1} knockout mice (22). Moreover, rat kidney epithelial cells stably transfected with the intracellular active form of Notch 1 (Notch^{ic}) showed increased Cux1 and Grg4 expression and decreased p27^{kip1} expression (53). To evaluate the functional importance of Grg4 on Cux1 mediated p27^{kip1} repression, we compared the ability of Cux1 to repress p27^{kip1} promoter activity with or without Grg4 (Fig 2). Unsynchronized 293T cells were cotransfected with a p27^{kip1}/luciferase reporter construct and either the empty CMV vector, or the CMV/Cux1 expression construct, with or without a CMV/Grg4 expression construct. In three separate experiments, the luciferase activity was significantly reduced in the presence of Cux1 and Grg4, compared with Cux1 alone, suggesting that Cux1 repression of p27^{kip1} involves Grg4.

Co-expression and Co-immunoprecipitation of HDACs with Cux1

One mechanism of transcriptional repression by Cux1 involves the recruitment of HDAC1 (24). Groucho proteins can also act as cofactors either alone or by recruiting HDACs (55–

57). To begin to determine whether Cux1 interacts with HDACs in the developing kidney, we evaluated the expression of HDAC1 and HDAC3. HDAC1 was expressed throughout the nephrogenic zone, with highest levels in the ureteric buds, and lower levels in the condensing mesenchyme and early nephric structures, including S-shaped bodies and capillary loop staged glomeruli (Fig. 3A). Similarly, HDAC3 was expressed in the nephrogenic zone, including the ureteric bud and S-shaped bodies. In capillary loop stage glomeruli, HDAC3 expression was observed in the presumptive podocytes (Fig. 3B). The expression of Cux1 (not shown) is broader and overlaps with the expression of both HDAC1 and HDAC3 (53). Grg4 was expressed at low levels in the nephrogenic zone, but was upregulated in the presumptive podocytes of capillary loop staged glomeruli (Fig. 3C), where it overlapped with the expression of HDAC3 (Fig. 3D–F). To begin to determine whether Cux1 repression involves the recruitment of HDACs, we asked whether an interaction between Cux1 and HDAC proteins could be detected in vivo. HDAC1 was immunoprecipitated from kidneys isolated from 3-day-old mice, followed by Western blotting for the presence of Cux1. Cux1 protein was coimmunoprecipitated with HDAC1 from the kidney lysates, but not in control preparations (Fig. 3G). Similar immunoprecipitation experiments were performed showing that Grg4 was coimmunoprecipitated with HDAC3 from the newborn kidney lysates (Fig. 3G).

Cux1, Grg4, HDAC1, and HDAC3 are present in complexes bound to the native promoter of *p27^{kip1}*

Our previous studies evaluating Cux1 transgenic mice revealed p27^{kip1} as a potential direct target of Cux1 during nephrogenesis. To directly determine whether Cux1 interacts with the native p27^{kip1} promoter, we performed chromatin immunoprecipitation (ChIP) analysis using chromatin isolated from Notch^{ic} cells or from newborn mouse kidneys. The ChIP assays were carried out using IgG (negative control), anti-Cux1, anti-Grg4, anti-HDAC1, anti-HDAC3, and anti-RNA polymerase II. Multiple primer sets were designed to amplify fragments of the p27^{kip1} promoter from 150–350 bp in length. Together, these primer sets spanned the entire length of the p27^{kip1} promoter. We found that two of the primer sets amplified the p27^{kip1} promoter following ChIP (panel A and C). The first positive primer set amplified a 191 bp fragment that contained two Sp1 sites and the CCAAT box at −543 to −522 relative to the transcription start site and is shown in panel C of Fig 4. The second primer set amplified a 249 bp fragment located at ~ −1.5 kb from the transcription start site and is shown in panel A of Fig 4. Amplification of sequences between regions A and C produced no Cux1 bound products. These results clearly show that Cux1 is part of a complex bound to two different regions of the p27^{kip1} promoter in its native chromatin configuration. Therefore, p27^{kip1} is a direct in vivo transcriptional target of Cux1, providing a mechanism for the regulation of cell cycle progression by Cux1 during kidney development. Moreover, our results show that Grg4, HDAC1 and HDAC3 are part of a complex bound to the same regions of the p27^{kip1} promoter in its native chromatin configuration.

Identification of a novel region on the p27^{kip1} promoter bound by Cux1

To identify the DNA sequence in the p27^{kip1} promoter bound by Cux1 in vivo, we performed DNAse 1 footprinting analysis using the amplification products from the ChIP assays. The ChIP positive amplification products (p27/191) and (p27/229) were end-labeled and incubated with in vitro synthesized Cux1. We observed protection of a 38 bp region overlapping two SP1 sites and a CCAAT box within region B (Figure 5, panel B). A second protected region was identified in the p27^{kip1} promoter in region C (Figure 5, panel C). This novel site of interaction between Cux1 and the p27^{kip1} promoter is in a region located ~1.5 kb from the transcription start site and contains two ATTTG repeats, sequences previously shown to be bound by Cux1 (58).

DISCUSSION

During kidney development, Cux1 is highly expressed in the nephrogenic zone, where it is associated with cell proliferation. Cux1 is down regulated at later stages of nephrogenesis, when cell proliferation is decreased (59). Transgenic mice ectopically expressing Cux1 develop renal hyperplasia resulting from the down regulation of $p27^{kip1}$ (22). In addition, promoter reporter assays demonstrated that Cux1 repressed $p27^{kip1}$ promoter activity in a concentration dependent manner. Cux1 can be regulated by the Notch signaling pathway suggesting that cell proliferation induced by Notch signaling may occur through the down regulation of $p27^{kip1}$. To begin to determine the mechanism of $p27^{kip1}$ repression by Cux1, we evaluated whether Cux1 interacts with the Notch effector protein groucho homologue Grg4 during kidney development

Two mechanisms of repression have been described for Cux1 protein: 1) Passive repression via competition for CCAAT or Sp1 binding site occupancy, preventing activation by the corresponding transcription factors, or 2) active repression via a carboxy terminal repression domain following binding at a distance from the transcription start site (23). The first mechanism is thought to involve rapid and unstable DNA binding activity and involves the first two cut repeats, while the second mechanism is thought to involve stable DNA binding activity and involves the third cut repeat and the homeodomain (2). Our results suggest that Cux1 can bind to the Sp1 and CCAAT sequences within the $p27^{kip1}$ promoter. This site has previously been shown to be required for $p27^{kip1}$ promoter activity, suggesting that Cux1 may engage in passive repression (60). However, we also identified a novel DNA binding site within the $p27^{kip1}$ promoter that is bound by Cux1. The interaction between Cux1 and Grg4, both by co-immunoprecipitation and pull down assays, suggests that Cux1 repression of $p27^{kip1}$ involves the recruitment of Grg4 to the promoter. This is supported by the observation that Grg4 significantly enhanced the repression of $p27^{kip1}$ promoter activity by Cux1 in a reporter assay. Grg proteins cannot bind DNA by themselves, but interact with other transcription factors. One mechanism of repression by Grg proteins is the recruitment of HDACs to form a repression complex (37,41–43). The chromatin immunoprecipitation of Cux1, in addition to Grg4 and HDAC1/3, on the native $p27^{kip1}$ promoter, raises the possibility that $p27^{kip1}$ repression by Cux1 involves the formation of a repression complex.

There are four domains in Cux1 that could potentially be involved in an interaction with Groucho proteins. The N-terminus of Cux1 protein contains the sequence FALNSLL that is similar to the Eh1 (Engrailed like homeodomain1 sequence) motif designated by the consensus sequence FXIXXIL (43). This sequence was originally identified in the Drosophila homeodomain proteins Engrailed (En) and Goosecoid and shown to mediate the interaction of these homeodomain proteins with groucho (61–62). In addition, the mammalian homeodomain proteins PRH and Six3 interact with groucho family members through the Eh1 domain (63–64). Three additional groucho interaction motifs exist in Cux1. These PKPW motifs are found in each of the cut repeats (CR) (65). This sequence is similar to the consensus sequence (W/F/Y)(K/R)P(WFY) used to recruit Groucho proteins to transcription factors (43). Further studies will be required to identify the specific sites of interaction between Cux1 and Grg4.

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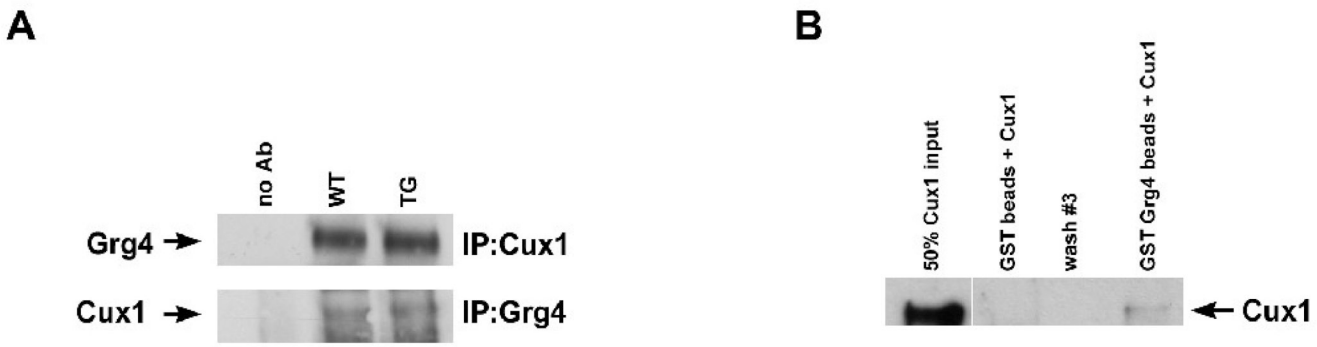

Figure 1. In vivo and in vitro interactions between Cux1 and Grg4

A: Kidney lysates from 3-day-old wild type (WT) and Cux1 transgenic (TG) mice were immunoprecipitated with rabbit polyclonal anti-Cux1 (top panel) or rabbit polyclonal anti-Grg4 (bottom panel) as indicated. The immunoprecipitates were then blotted for Grg4 (top panel) or Cux1 (bottom panel) as indicated. Grg4 can be seen in WT and TG kidneys, after immunoprecipitation with Cux1 (right lanes, top panel). Cux1 can be seen in WT and TG kidneys, after immunoprecipitation with Grg4 (right lanes, bottom panel). No bands are observed when protein G beads alone are precipitated (no Ab). **B:** Bacterially expressed Cux1 protein was incubated with GST- Grg4 fusion protein immobilized on Glutathione agarose beads. Following washing, GST beads alone with Cux1, wash #3, and GST-Grg4 immobilized beads with Cux1 were transferred to a membrane and blotted with antibody directed against Cux1 protein. Only GST-Grg4 with Cux1 protein and the Cux1 input showed a positive band, indicating a direct physical interaction between Cux1 and Grg4.Lane 1 shows 50% of the total purified recombinant Cux1 protein used for the assay.

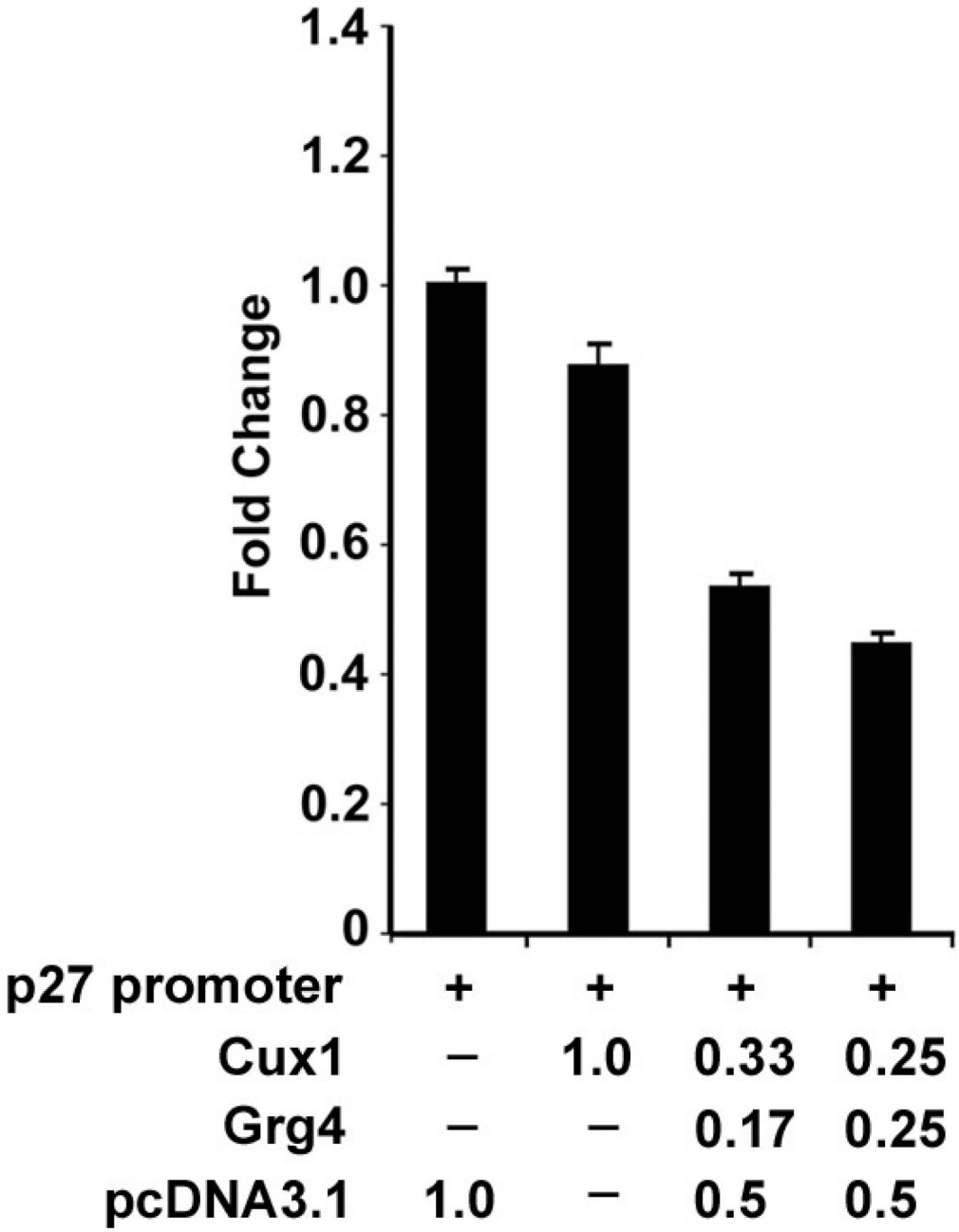

Figure 2. Repression of p27^{kip1} promoter activity by Cux1 and Grg4

293T cells were transiently transfected with 1 μg of reporter construct containing p27 upstream sequences from −1609 to +178 fused to the luciferase reporter gene (+), together with different concentrations of the Cux1 and Grg4 expression vectors (amounts shown are in μg) or with empty pcDNA3.1 expression vector (amounts shown are in μg). In the presence of Grg4, significantly less Cux1 is required to repress p27 promoter activity (3rd and 4th bar). Promoter activity was plotted as fold change, normalized to the expression of a co-transfected renilla expression construct. Activity is expressed as the mean of three separate experiments performed in triplicate. Error bars indicate standard deviation. ANOVA showed a significant

reduction in luciferase activity following cotransfection with Grg4 in a dose dependent manner ($P < 0.002$).

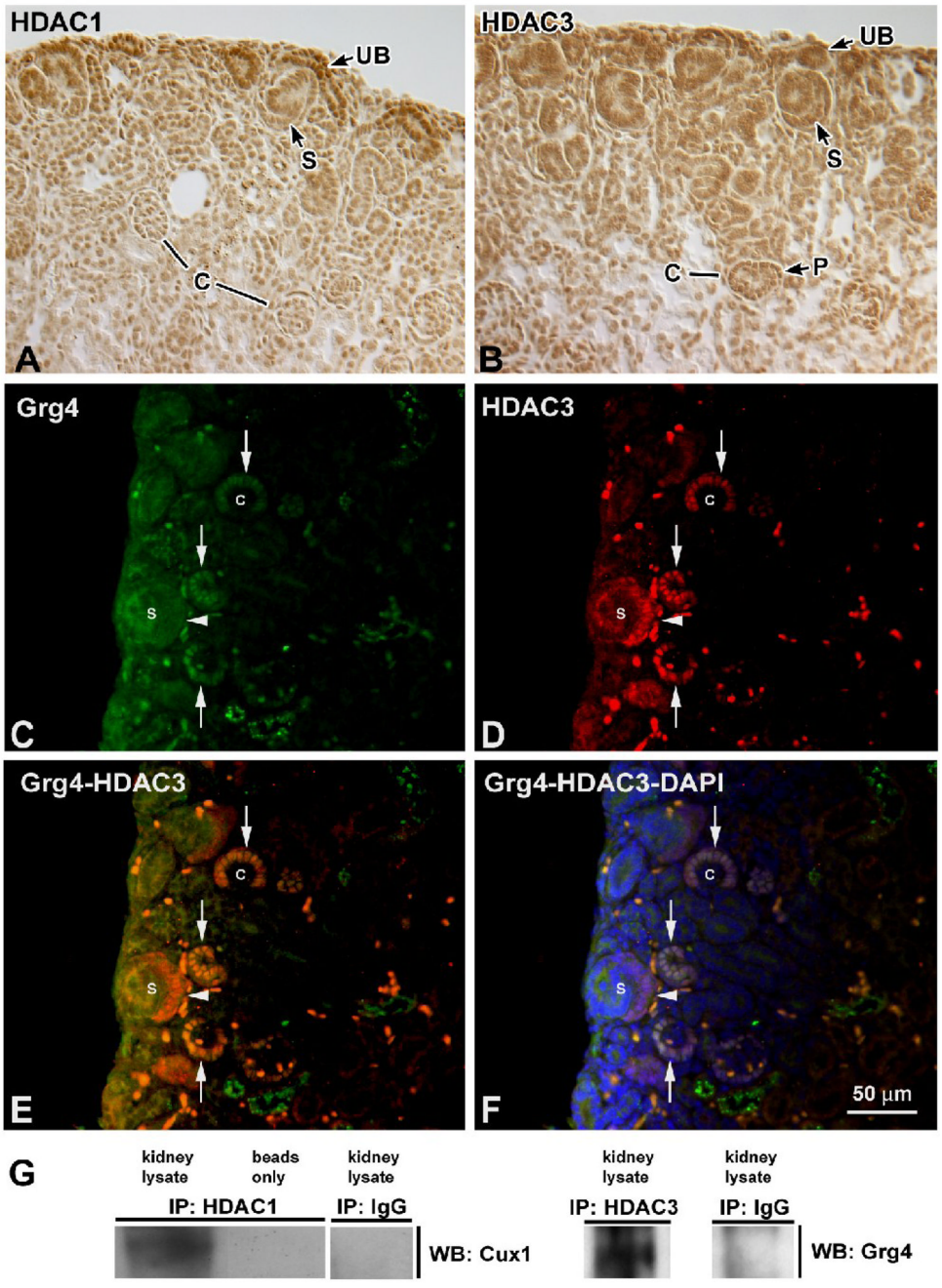

Figure 3. Co-localization and interaction of Cux1, Grg4, and HDACs

A: HDAC1 was expressed at highest levels in the nephrogenic zone, where it was localized to mesenchyme, ureteric buds (UB), and early nephric structures, including S-shaped bodies (S), but down regulated in capillary loop staged glomeruli (C). **B:** HDAC3 was also expressed in the nephrogenic zone, including the ureteric bud (UB) and S shaped bodies (S). In capillary loop stage glomeruli (C), HDAC3 expression was observed in the presumptive podocytes (P). **C**: Grg4 was expressed at low levels in the nephrogenic zone, including S-shaped bodies (arrowhead), and upregulated in the presumptive podocytes of capillary loop staged glomeruli (arrows). **D:** HDAC3 was similarly expressed in the nephrogenic zone, with highest levels in the presumptive podocytes (arrows) and in S-shaped bodies (arrowheads). **E, F**: Co-

localization of HDAC3 and Grg4 without (E) or with (F) DAPI in capillary loop staged glomeruli (arrows). **G:** Newborn kidney lysates were immunoprecipitated (IP) with protein G beads alone (beads only), rabbit IgG (IgG), rabbit polyclonal anti-HDAC1 (left panel), or rabbit polyclonal anti-HDAC3 (right panel) as indicated. The immunoprecipitates were then blotted for Cux1 (left panel) or Grg4 (right panel) as indicated. Cux1 can be seen in newborn kidneys after immunoprecipitation with HDAC1 (left lane, left panel), and Grg4 can be seen in newborn kidneys after immunoprecipitation with HDAC3 (left lane, right panel).

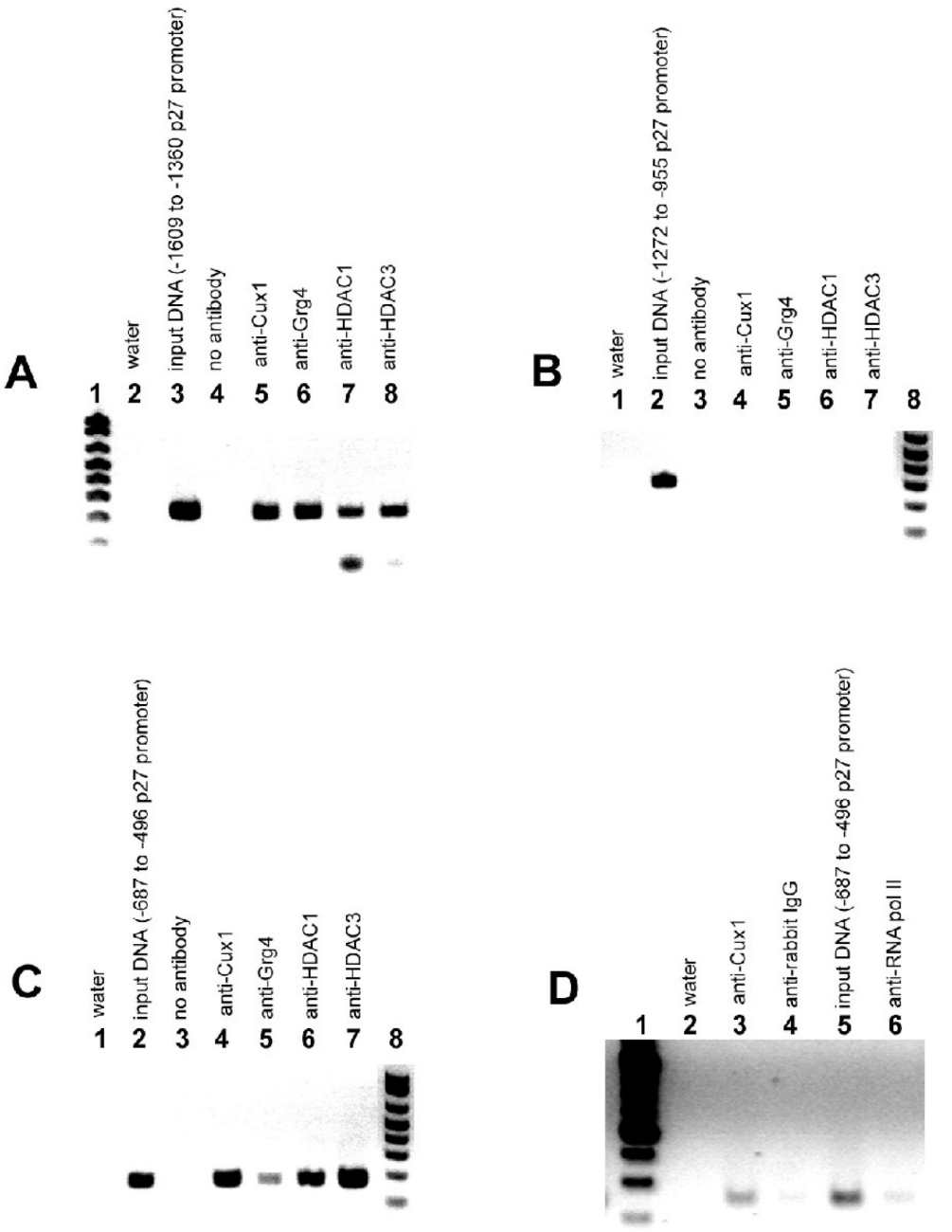

Figure 4. *p27^Kip1* is a direct Cux1 target in the developing kidney

Complexes isolated from Notch^{ic} cells (A–C) or from newborn kidneys (D) were immunoprecipitated with anti-Cux1, anti-Grg4, anti-HDAC1, anti-HDAC3, or anti-RNA polymerase II antibodies followed by reverse cross-linking of protein and DNA, and PCR amplification of a 249 bp fragment of the p27kip1 promoter spanning −1609 to −1360 (A), or a 191 bp fragment of the p27kip1 promoter spanning −687 to −496 (C, D), relative to the transcription start site. **A:** Inverse image of ethidium-stained agarose gel showing PCR amplification of a 249 bp p27kip1 product from input DNA (lane 3), following ChIP with antibody directed against Cux1 (lane 5), Grg4 (lane 6), HDAC1 (lane 7), and HDAC3 (lane 8), indicating that Cux1, Grg4, HDAC1, and HDAC3 interact with a site within the 5’ region

of the p27kip1 promoter in vivo. **B:** To control for non-specific chromatin immunoprecipitation, a 317 bp product from −1272 to −955 of the p27kip1 promoter was amplified from input DNA (lane 2), following ChIP with antibody directed against Cux1 (lane 4), Grg4 (lane 5), HDAC1 (lane 6), and HDAC3 (lane 7). **C:** PCR amplification of a 191 bp p27kip1 product from input DNA (lane 2), following ChIP with antibody directed against Cux1 (lane 4), Grg4 (lane 5), HDAC1 (lane 6), and HDAC3 (lane 7), indicating that Cux1, Grg4, HDAC1, and HDAC3 interact with a site within the 3' region of the p27kip1 promoter in vivo. **D:** PCR amplification of a 191 bp p27kip1 product from input DNA (lane 5), following ChIP with antibody directed against Cux1 (lane 3), and RNA polymerase II (lane 6), indicating that Cux1 interact with a site within the 3' region of the p27kip1 promoter in newborn kidneys in vivo. In A controls were water (lane 2) and no antibody (lane 4). In both B and C, controls were water (lane 1) and no antibody (lane 3). In D controls were water (lane 2) and normal rabbit IgG (lane 4).

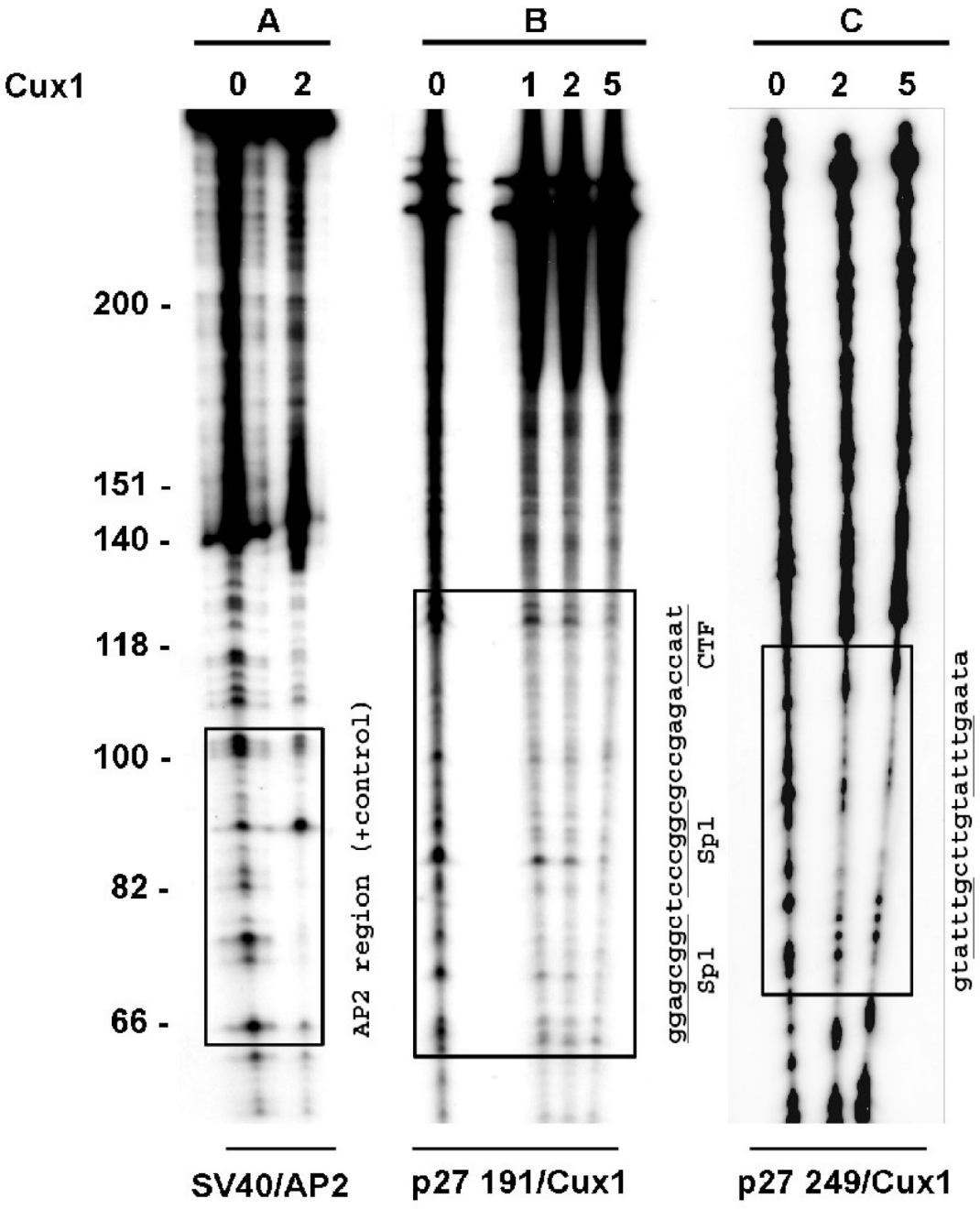

Figure 5. Two separate Cux1 binding sites on the p27^{kip1} promoter

DNAse I footprint analysis of the p27^{kip1} promoter regions bound by Cux1 in vivo. −687 to −496 (panel B) and −1609 to −1360 (panel C) PCR amplification products from ChIP assays were radiolabeled and incubated with purified in vitro synthesized Cux1 protein (amounts shown are in μl) and then digested with DNase1. Regions of DNA protected from DNase1 digestion are indicated by boxed regions. The corresponding sequence for each protected region is shown, with the previously identified Cux1 target sequences underlined. Panel A shows radiolabeled SV40 DNA following incubation with AP2 protein and DNase1 digestion as a positive control. Boxed region shows region of DNA protected from DNase1 digestion.