Nucling mediates apoptosis by inhibiting expression of galectin-3 through interference with nuclear factor κB signalling

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Nucling is a novel apoptosis-associated molecule, which is involved with cytochrome *c*/Apaf-1/caspase-9 apoptosome induction following pro-apoptotic stress. In the present study, we show first that Nucling is able to interact with galectin-3. Galectin-3 is known to participate in many biological processes, including apoptotic cell death. Nucling was found to down-regulate the expression level of galectin-3 mRNA/protein. Nucling-deficient cells, in which galectin-3 expression is up-regulated, appeared to be resistant to some forms of pro-apoptotic stress as compared with wild-type cells. In addition, the preputial gland from Nucling-deficient mice expressed a significant level of galectin-3 and exhibited a high incidence of inflammatory lesions, indicating that Nucling plays a crucial role in the homoeostasis of this gland by interacting with the galectin-3 molecule and regulating the expression level of galectin-3. Up-regulation of galectin-3 was also observed in the heart, kidney, lung, testis and ovary of the Nucling-deficient mice. In order to confirm the functional interaction between Nucling and galectin-3, a well-documented candidate for the mediator of galectin-3 expression, NF-κB (nuclear factor κB), was investigated as well. Nucling was shown to interfere with NF-κB activation via the nuclear translocation process of NF-κB/p65, thus inhibiting the expression of galectin-3. Taken together, we propose that Nucling mediates apoptosis by interacting and inhibiting expression of galectin-3.

Key words: anoikis, apoptosis, galectin-3, Nucling, nuclear factor κB (NF-κB), preputial gland.

INTRODUCTION

Nucling is a novel protein isolated originally from murine embryonal carcinoma cells that is up-regulated during cardiac muscle differentiation [1]. cDNA for mouse Nucling encodes a polypeptide of 1411 amino acids, containing an ankyrin repeat, an aspartyl protease motif, a leucine zipper motif and two t-SNARE (target membrane soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) coiled-coil domains. Nucling mRNA is expressed in various adult tissues. Nucling protein localizes in the cytoplasm, especially around the nuclear membrane in mammalian cells. Moreover, the expression level of the Nucling gene transcript increases progressively during the early developmental stages in mice, and specifically at cardiomuscular differentiation *in vitro* and *in vivo* [1]. We have recently confirmed that several apoptotic signals can induce endogenous Nucling expression. Moreover, Nucling can induce cytochrome *c* release, followed by apoptosis in mammalian cells. It was also shown that Nucling recruits the Apaf-1–pro-caspase-9 complex for the induction of apoptosis following pro-apoptotic stress (T. Sakai, L. Liu, R. Mukai-Sakai, X. C. Teng, T. Mitani, M. Matsumoto, K. Toida, K. Ishimura, Y. Shishido, T. W. Mak and K. Fukui, unpublished work). βCAP73, a novel β-actin-specific binding protein, was isolated from a bovine endothelial cell library [2], and Nucling is regarded as the mouse homologue of βCAP73 on the basis of the sequence similarity.

Galectins are a family of carbohydrate-binding proteins defined by an affinity for β-galactoside and sequence identity with the carbohydrate-binding motif [3]. The family is composed of 14 members [4]. Among them, galectin-3, the only member of chimaeric type, contains a non-lectin part connected to a carbohydrate recognition domain [4,5]. Studies have previously demonstrated that galectin-3 has a broad distribution among different tissues and cell types [6–8]. Therefore galectin-3 may play a role in a number of biological activities, such as cell–cell and cell–matrix interactions [9,10], induction of pre-mRNA splicing [11], cell cycle regulation [12], angiogenesis [13], tumorigenesis [14,15] and, more importantly, cell growth and apoptosis [16,17]. It is reported that galectin-3 ectopically expressed in a human T-cell line protected the cell from apoptosis induced by pro-apoptotic stimuli [18]. In addition, studies on galectin-3-deficient mice support further the anti-apoptotic role of galectin-3 [19]. Much remains to be learned about how galectin-3 inhibits apoptosis, although early studies have suggested the involvement of Bcl-2 [20]. One recent report showed that galectin-3 translocates to the perinuclear membrane following exposure to a variety of apoptotic stimuli, and becomes abundant in the mitochondria, where it prevents mitochondrial damage and cytochrome *c* release [21].

Recent reports provide supporting evidence that NF-κB (nuclear factor κB) and Jun are involved in the regulation of galectin-3 expression [22]. Furthermore, galectin-3-deficient mice had fewer inflammatory cells after intraperitoneal challenge with reduced levels of NF-κB activation [19]. NF-κB is activated by various stimuli, including TNF-α (tumour necrosis factor-α), interleukin-1 and lipopolysaccharide [23], and regulates the expression of genes involved in the immune response, inflammatory processes and apoptosis [24,25]. Moreover, studies on mice deficient in NF-κB subunits revealed that NF-κB has an essential role in preventing TNF-α-induced cell death [26].

Abbreviations used: EMSA, electrophoretic mobility-shift assay; H/E, haematoxylin and eosin; JIA, juvenile idiopathic arthritis; MEF, murine embryonic fibroblasts; NF-κB, nuclear factor κB; NuclingΔN, a deletion mutant of Nucling lacking the N-terminal region; RT, room temperature; RT-PCR, reverse transcriptase-PCR; TNF-α, tumour necrosis factor-α; (t-)SNARE, (target membrane) soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling; WT, wild-type.

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In the present study, in order to investigate the signal transduction mechanism of Nucling we sought to identify Nucling-interacting proteins by screening a mouse embryo library using the yeast two-hybrid system. The result of the screening indicated positive interaction with Nucling by a galectin family member. Furthermore, we show that galectin-3 can interact with Nucling, with an up-regulated expression in several cell types or tissues of Nucling-deficient mice. We also found that NF-κB is involved in the functional interaction between Nucling and galectin-3 expression. Finally, we propose that Nucling may mediate apoptosis by interacting and inhibiting expression of galectin-3.

EXPERIMENTAL

Yeast two-hybrid system

A cDNA (1.5 kb) fragment encoding the C-terminus of Nucling was inserted into pGBKT7-GAL4 (ClonTech) to generate pGBKT7-GAL4–NuclingΔN (where 'NuclingΔN' represents a deletion mutant lacking the N-terminal region) as 'bait' (a tectonic schematic representation of this is shown in Figure 1A, together with that of Nucling [1]). As shown in Figure 1(A), full-length Nucling protein consists of several potential domains for protein–protein interaction, such as two t-SNARE coiled-coil domains, an ankyrin repeat and a leucine zipper motif. In order to circumvent this complexity of interacting domains, we first selected the C-terminal fragment of cDNA as bait, which contains only one t-SNARE coiled-coil domain and covers almost half of the full-length sequence. After verification of the sequence, AH109 yeast cells were sequentially transformed with pGBKT7-GAL4–NuclingΔN and a 17.5-day mouse embryo cDNA library (2.0×10^8 colony-forming units $\cdot$ ml^{-1}) in pACT (ClonTech). Selection was carried out by growth on SD/$-$ Trp, SD/$-$ Leu $-$ Trp and SD/α-X-gal $-$ Ade $-$ His $-$ Leu $-$ Trp plates (where X-gal represents 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside). Seventeen clones exhibiting activation of the *LacZ* reporter gene were identified by the β-galactosidase assay. These positive clones were isolated on Chroma Spin-1000 columns (ClonTech). Subsequent PCR amplification was performed using primers 5′-GTGAACTTGCGGGGTTTTTCAGTATCTACGT-3′ and 5′-CTATTCGATGATGAAGATACCCCACCAAACCC-3′ to amplify the cDNA inserts from all plasmids encoding candidate interacting proteins (the Advantage cDNA PCR kit). These proteins were sorted into groups on the basis of restriction digestion patterns. Since *Hae*III is a frequent-cutter restriction enzyme, it was used to digest the PCR product. Subsequently, a representative clone from each group was subjected to sequencing analysis. Lastly, the mini-prep DNA was transformed into *Escherichia coli* DH10B cells and purified.

Co-immunoprecipitation assay and immunoblotting

Cos7 cells were transiently co-transfected with plasmids using Effectene® Transfection Reagent (Qiagen). Cells were harvested and lysed in a lysis buffer containing 20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % (v/v) Triton X-100, 0.5 % (w/v) deoxycholate, 0.1 % (w/v) SDS and Complete™ protease inhibitor cocktail (Roche) at 24 h post-transfection. Cell extracts were clarified by centrifugation at 12 000 ***g*** for 30 min at 4 °C, and the supernatant was immunoprecipitated with anti-Flag M2 affinity gel (Sigma) by incubating overnight at 4 °C. The beads were washed five times with the lysis buffer and suspended in SDS loading buffer. Subsequently, the co-immunoprecipitated and lysate samples were subjected to an immunoblot assay with anti-Flag M2 monoclonal antibody–horseradish-peroxidase conjugate (Sigma) or c-Myc 9E10 antibody–horseradish-peroxidase conjugate (Santa Cruz Biotechnology).

Immunofluorescence staining

Cos7 cells were plated on to Lab-Tek Chamber Slides (Nalge Nunc International, Naperville, IL, U.S.A.), transiently transfected with FLAG–Nucling and/or c-Myc–galectin-3, and cultured overnight. After a brief wash at room temperature (RT; $\approx$ 25 °C) in PBS, the cells were fixed in 3.7 % (w/v) paraformaldehyde in PBS for 15 min at RT. After several washes in PBS, the cells were immersed in PBS containing 0.2 % Triton X-100 for 5 min at RT, and washed three more times with PBS. Samples were incubated for 30 min at RT with 10 % goat serum. After three further washes, cells were incubated with FITC–anti-Flag M2 antibody (Sigma) and anti-Myc (Ab-1) antibody for 2 h at RT. After a further three washes with PBS, the coverslips were incubated with Texas Red™ (TXRD)-conjugated secondary antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL, U.S.A.) or FITC–anti-mouse IgG (Zymed Laboratories, Inc., San Francisco, CA, U.S.A.) for 1 h. Lastly, coverslips were analysed and viewed under an Olympus FluoView microscope.

Northern blot analysis

Total RNA of MEFs (murine embryonic fibroblasts) or preputial glands from Nucling$^{+/+}$ and Nucling$^{-/-}$ mice was isolated with the Isogen reagent (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. After electrophoresis through formaldehyde-containing agarose gels, RNAs were transferred on to Hybond N$^+$ nylon membranes (Amersham Biosciences). Probes were labelled with [α-^{32}P]dCTP (NEN Life Science Products, Inc.) using Ready-To-Go DNA Labelling Beads ($-$ dCTP) (Amersham Biosciences). After hybridization overnight at 65 °C, blots were washed three times in 2 $\times$ SSC (where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.1 % SDS for 15 min (25 °C) and four times in 0.2 $\times$ SSC/0.1 % SDS for 30 min (65 °C). The blots were exposed to Kodak™ BioMax™ MS film at $-$ 70 °C for 0.8–3 h.

EMSA (electrophoretic mobility shift assay)

The NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega) was labelled with [γ-^{32}P]ATP (NEN Life Science Products, Inc.) using T4 polynucleotide kinase. MEF nuclear extracts (1 μg/μl) were added to 5 μl of gel shift binding buffer [20 % (v/v) glycerol/5 mM $MgCl_2$/2.5 mM EDTA/2.5 mM dithiothreitol/250 mM NaCl/50 mM Tris/HCl (pH 7.5)/0.25 mg/ml poly(dI-dC)]. For competition or gel-shift experiments, the binding was performed in the presence of 1 μl (1.75 pmol/μl) of unlabelled NF-κB oligonucleotide or 5 μg of NF-κB–p65 antibody (Santa Cruz Biotechnology Inc.) respectively. The mixture was incubated for 20 min on ice before addition of 1 μl of ^{32}P-labelled NF-κB oligonucleotide probe. The resulting complexes were resolved by electrophoresis on 4 % polyacrylamide gels. Finally, the gels were dried and analysed by autoradiography.

RT-PCR (reverse transcriptase-PCR)

Total RNA from preputial glands was extracted from Nucling$^{+/+}$ and Nucling$^{-/-}$ mice using Isogen. cDNA synthesis was performed using a Superscript Preamplification System for First

Strand cDNA Synthesis kit (Invitrogen), according to the manufacturer's instructions. Synthetic oligonucleotide PCR primers for mouse Nucling were 5′-TGATCACCCAGGACCCGGAAGTTACC-3′ (sense) and 5′-GGTGCTCTTTGAGGGCGAGGAAGTG-3′ (antisense). A 25 μl reaction mixture containing 1 μl of cDNA sample as the template, 200 nM PCR primers, 0.2 mM dNTPs, 1 mM $MgCl_2$, and 2.5 units of AmpliTaq Gold® enzyme (Applied Biosystems, Foster City, CA, U.S.A.) in AmpliTaq Gold® buffer was subjected to amplification in a DNA thermal cycler (GeneAmp PCR system 9700, Applied Biosystems). PCR was performed for 34 cycles (95 °C, 1 min; 60 °C, 2 min; 72 °C, 2.5 min), with an initial incubation at 96 °C for 10 min, and final extension at 72 °C for 5 min. Samples (10 μl) of the amplified product were resolved by 1.2 % (w/v) agarose gel electrophoresis, and visualized by ethidium bromide staining. RT-PCR of glyceraldehyde-3-phosphate dehydrogenase served as a control.

Immunohistochemistry

Frozen sections (10 μm thick) containing the maximum diameter of the tissue under study were subjected to immunohistochemistry. Sections were fixed with acetone, and endogenous peroxidase was quenched with 0.3 % hydrogen peroxide in methanol. Sections were subsequently incubated for 30 min with normal serum in the VECTASTAIN® ABC kit (Vector Laboratories, Burlingame, CA, U.S.A.) to block non-specific antibody binding, and exposed to biotin-conjugated anti-mouse Mac-2 monoclonal antibody (dilution 1:50) at 37 °C for 2 h. Finally, the sections were subjected to a peroxidase reaction with 3,3′-diaminobenzidine for 5 min, resulting in a brown-coloured precipitate at the antigen site. Between each step, the slides were washed four times with PBS buffer.

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) assay

The TUNEL assay was performed on frozen sections (10 μm thick) using the TACS™ 2 TdT-Blue Label in Situ Apoptosis Detection Kit (Trevigen).

RESULTS

Nucling can interact with galectin-3

In search of proteins that interact with Nucling, we chose the N-terminal-region-lacking mutant NuclingΔN as 'bait' (Figure 1A) to screen a 17.5-day mouse embryo cDNA library using the yeast two-hybrid system. Out of 2.0×10^8 diploid colony-forming units screened, 17 positive colonies were detected. Nine of the seventeen candidates, including galectin-4, interacted with Nucling in immunoprecipitation assays of expressed proteins in mammalian cells. Since the galectin family, including galectin-1 and -3, are reported to be involved in apoptosis [27], we examined the possibility that galectin family members interact with Nucling. To confirm any direct interaction between Nucling and galectin-1, -3, and -4, we performed co-immunoprecipitation analysis. As shown in Figure 1(B), we observed that full-length Nucling could interact with galectin-3 and galectin-4, but not with galectin-1 (results not shown). In contrast, NuclingΔN could interact with galectin-4, but not with galectin-3.

In order to examine the subcellular localization of Nucling and galectin-3, we performed an immunofluorescence analysis of the transfected cells. As shown in Figures 1(C) and 1(D), we found that galectin-3 existed homogeneously in both the nucleus and the cytoplasm of cells, as was reported previously [8]. On the other hand, Nucling was found to be localized around the nuclear membrane, and also diffusely in cytoplasm. However, when Nucling and galectin-3 were co-transfected into COS7 cells, the immunofluorescence staining revealed that the expression profiles of Nucling and galectin-3 were mutually exclusive in cytoplasm; that is, the area where galectin-3 staining was negative corresponded to the area in the cytoplasm that was Nucling-positive (Figure 1D).

Nucling is a negative regulator of galectin-3 by inhibiting NF-κB activity

On the basis of the observation shown in Figures 1(C) and 1(D), we surmised that Nucling might down-regulate the expression of galectin-3. To confirm this hypothesis, we first investigated whether Nucling overexpression results in altered expression of endogenous galectin-3 at the cellular level (Figure 2A). We overexpressed full-length or mutant Nucling (NuclingΔN) in NIH-3T3 cells. Cell lysates subsequently showed that endogenous galectin-3 expression levels were obviously decreased in the Nucling-positive cell line (Figure 2A, lane 1), but did not change in the Nucling-null or mutant-transfected cell lines (Figure 2A, lanes 2–4).

On the other hand, in order to elucidate the physiological function of Nucling *in vivo*, we have produced Nucling-deficient mice generated by targeted disruption of the Nucling gene (T. Sakai, L. Liu, R. Mukai-Sakai, X. C. Teng, T. Mitani, M. Matsumoto, K. Toida, K. Ishimura, Y. Shishido, T. W. Mak and K. Fukui, unpublished work). We then evaluated the expression level of galectin-3 in Nucling-deficient cells. Primary MEFs were prepared from embryos of wild-type (WT) and mutant ($Nucling^{-/-}$) mice. Immunoblot analyses were then performed to reveal that galectin-3 expression was markedly up-regulated in the $Nucling^{-/-}$ cells (Figure 2B, lane 2) compared with WT cells (Figure 2B, lane 1). We therefore concluded that Nucling was able to inhibit the expression of galectin-3.

Anoikis is the name given to a special form of cell death, namely detachment-induced cell death or suspension-induced apoptosis. Galectin-3 is reported to be able to protect human breast epithelial cells against apoptosis induced by anoikis [9]. There is also evidence that galectin-3 prevents mitochondrial damage and cytochrome *c* release [21,28]. Nucling is shown to induce cytochrome *c* release following pro-apoptotic stress (T. Sakai, L. Liu, R. Mukai-Sakai, X. C. Teng, T. Mitani, M. Matsumoto, K. Toida, K. Ishimura, Y. Shishido, T. W. Mak and K. Fukui, unpublished work). To determine whether galectin-3 is regulated by Nucling during the stress of anoikis or H_2O_2-induced apoptosis, we compared the expression levels of galectin-3 in two genotypes, WT ($Nucling^{+/+}$) and $Nucling^{-/-}$ cells. The protein expression level of galectin-3 was significantly higher in the Nucling-deficient group than in WT, both in the absence and presence of pro-apoptotic stress (Figure 2B, lanes 3–6). The RNA expression of galectin-3 was also up-regulated in $Nucling^{-/-}$ MEFs relative to that in $Nucling^{+/+}$ cells, and further enhancement of the up-regulation was observed in both cell lines under conditions of anoikis and H_2O_2 exposure (Figure 2C, land 3–6). Nucling is therefore considered to be a negative regulator of galectin-3 expression both at the RNA and the protein level.

Although it is possible that Nucling negatively regulates galectin-3 by interacting with this molecule at the protein level, it is still unclear how Nucling leads to transcriptional down-regulation of galectin-3. In order to clarify the functional interaction between Nucling and galectin-3, we postulated the possibility that additional factor(s) is (are) involved in the

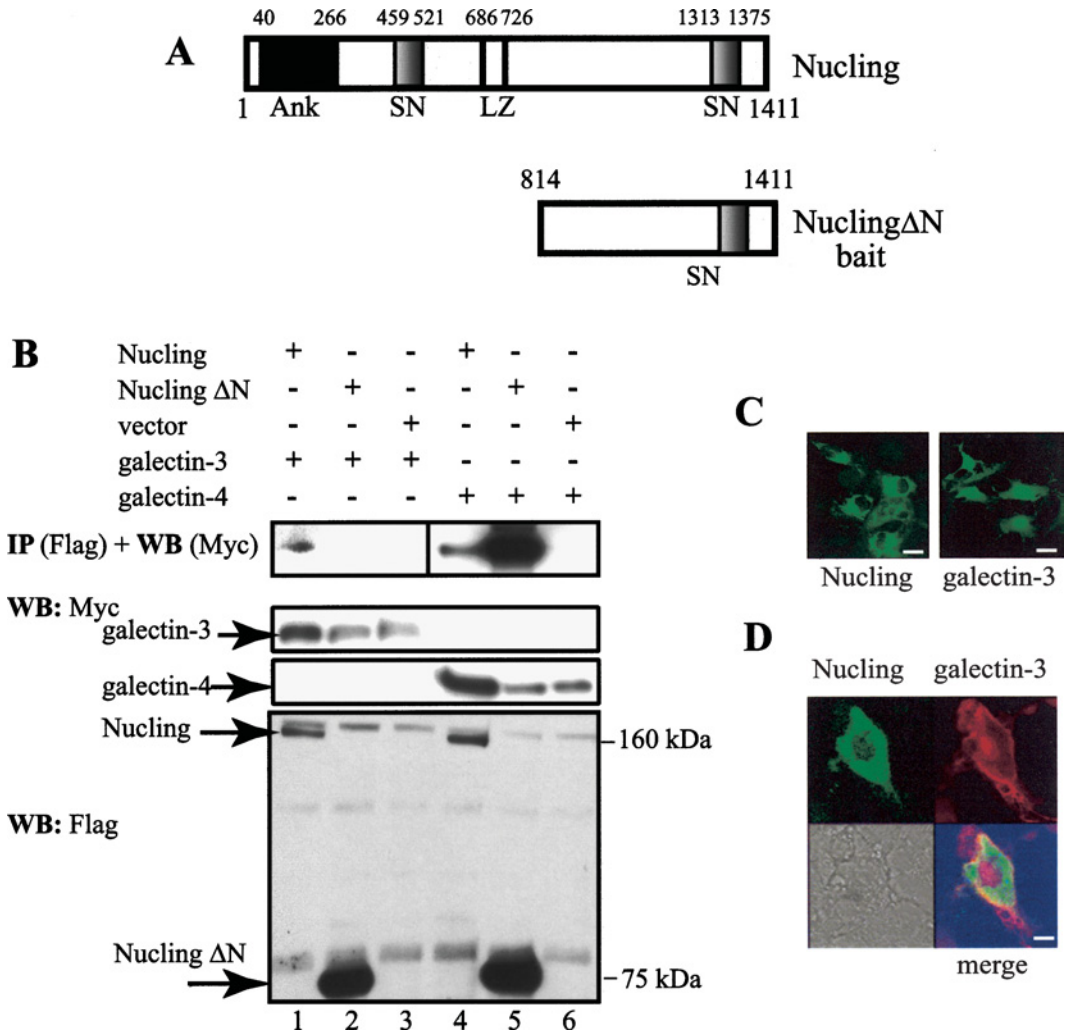


Figure 1 Interaction of Nucling with galectin-3

(**A**) Primary structures of the full-length Nucling and its deletion mutant (NuclingΔN; amino acids 814–1411) used as bait in a yeast two-hybrid assay are illustrated with typical motifs. Ank, SN and LZ represent ankyrin repeat region, t-SNARE coiled-coil domain and leucine zipper motif respectively. (**B**) COS-7 cells were transiently co-transfected with FLAG–Nucling/FLAG–NuclingΔN/vector and Myc–galectin-3/Myc–galectin-4. Lysates were subjected to co-immunoprecipitation assay (IP) with anti-Flag antibody. Immunoblot analysis (WB) was performed with anti-Myc antibody. The presence of galectin-3 or galectin-4 and Nucling or NuclingΔN in the same lysates were verified by immunoblotting with anti-Myc antibody and anti-FLAG antibody respectively. (**C** and **D**) Subcellular localization of transfected galectin-3 (**C**, left panel) or Nucling (**C**, right panel) alone, and co-transfected with galectin-3 and Nucling together (**D**) in COS-7 cells were analysed by subjecting cells to immunofluorescence staining. The bar represents 10 μm.

inhibition of galectin-3 expression by Nucling. We found an NF-κB binding sequence (5′-GGGAGATCCC-3′) in the genomic sequence of Mac-2 (990–999 bp) [29], which matches completely with the consensus κB recognition sequence (5′-GGG-RNNYYCC-3′).

This observation led us to examine and compare the level of NF-κB between WT and Nucling-deficient MEFs. Immunoblot analysis of nuclear and cytoplasmic proteins revealed that the NF-κB–p65 protein level was markedly higher in the nuclear fraction of Nucling$^{-/-}$ MEFs (Figure 2D, lane 1) than in that of Nucling$^{+/+}$ MEFs (lane 2), but in the cytosolic fraction no obvious difference was observed (lanes 3 and 4).

Furthermore, in order to investigate the capacity of proteins from nuclear extracts of Nucling$^{+/+}$ and Nucling$^{-/-}$ MEFs to interact with oligonucleotides containing a consensus binding sequence for NF-κB, EMSA was performed (Figure 2E). The results from the gel-shift study showed that the DNA-binding capacity of one of the NF-κB complexes from Nucling$^{-/-}$ MEF nuclear extracts was increased compared with that from Nucling$^{+/+}$ MEFs (Figure 2E, lanes 2 and 3, arrow A). Addition of an antibody specific for the p65 subunit of NF-κB clearly attenuated this DNA-binding activity (Figure 2E, lanes 4 and 5, arrow A). To confirm the specificity of NF-κB binding in this assay, a non-labelled NF-κB oligonucleotides probe was used as competitor. As shown in Figure 2(E), lanes 6 and 7, non-labelled probe was able to compete for the binding of two complexes (shown by arrows A and B), indicating the formation of p65–p50 (arrow A) and p50–p50 (arrow B) complexes in this assay. Taken together, these results indicate that Nucling might inhibit NF-κB activity by interfering with the nuclear translocation of NF-κB–p65 from the cytoplasm.

The preputial gland of Nucling$^{-/-}$ mice exhibited inflammatory lesions

In order to examine the pathophysiological significance of Nucling at the whole animal level, we then analysed the WT

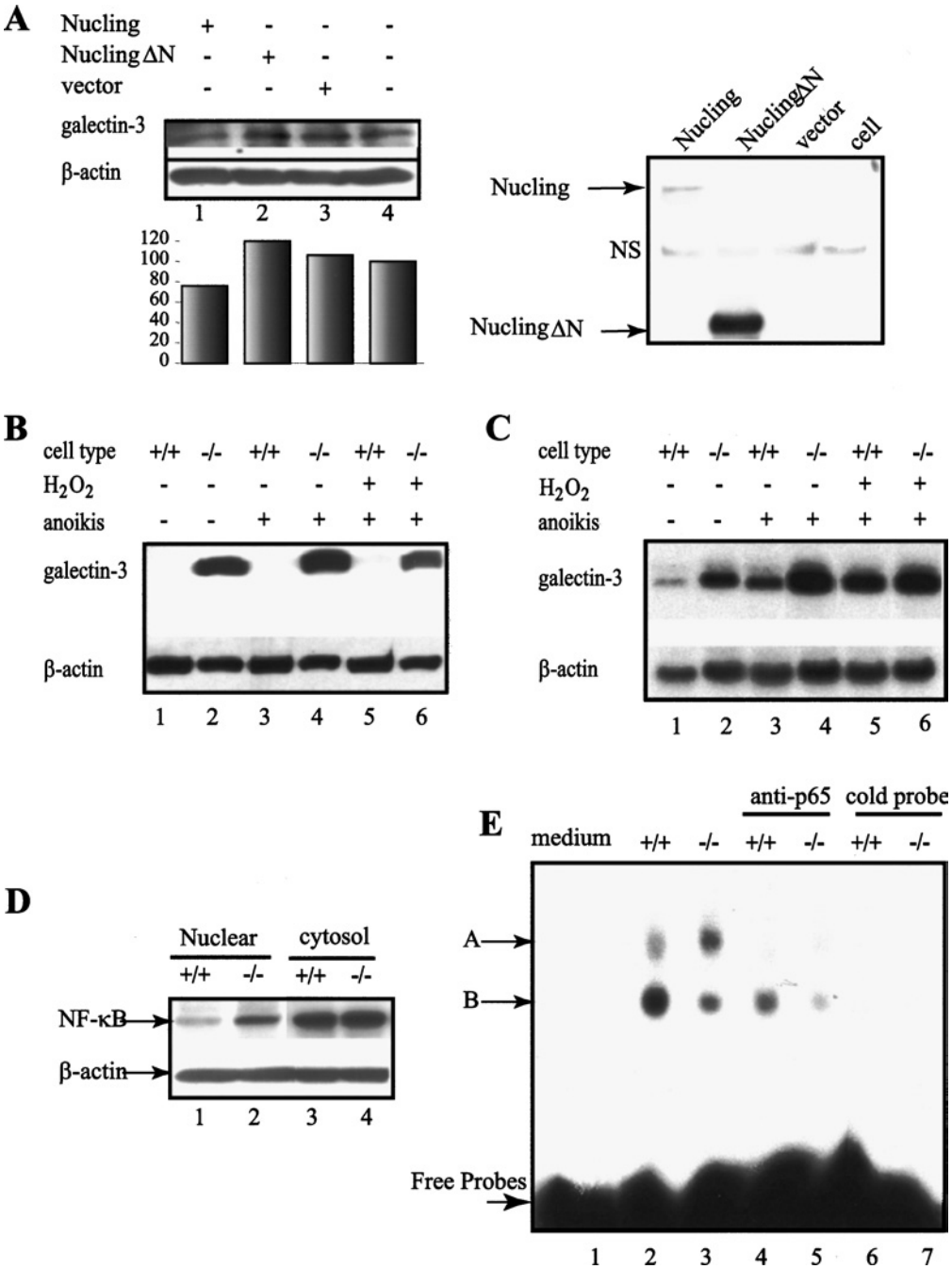


Figure 2 Up-regulation in the level of protein and mRNA of galectin-3 in MEFs of Nucling-null mice

(**A**) NIH3T3 cells were transiently overexpressed with Nucling, NuclingΔN or vector. Lysates were subjected to immunoblot analysis with anti-Mac2 antibody to check the level of endogenous galectin-3 (upper-left panel). The signals were scanned and calculated using NIH Image Analysis Software (lower-left panel). The presence of Nucling or NuclingΔN was revealed with anti-Flag M2 antibody (right panel). (**B**) MEFs of WT and Nucling$^{-/-}$ mice were treated with or without 0.25 % trypsin/EDTA (anoikis) and 0.8 mM H_2O_2 for 16 h. Then, the level of galectin-3 protein in MEFs was examined with galectin-3 antibody (Mac-2). To confirm the equal loading of the protein in each lane, the same blot was probed with β-actin antibody (Sigma). (**C**) Total RNA of MEF was isolated with Isogen from WT and Nucling$^{-/-}$ embryos, and then Northern blotting was performed with a ^{32}P-labelled cDNA fragment of galectin-3. The same blot was probed with β-actin (ClonTech). (**D**) Translocation of NF-κB–p65 from the cytoplasm to the nucleus in Nucling$^{-/-}$ and Nucling$^{+/+}$ MEFs was examined by immunoblot analysis. Nuclear and cytosolic fraction proteins were used for determination of the NF-κB–p65 level. A monoclonal anti-NF-κB–p-65 antibody was used at a dilution of 1:500. The equal loading of the protein in each lane was confirmed by β-actin antibody. (**E**) Nuclear extracts from Nucling$^{+/+}$ and Nucling$^{-/-}$ MEFs were isolated and analysed by EMSA as described in the Experimental section.

and knock-out mice. No obvious phenotypic differences were observed initially between Nucling$^{+/+}$ and Nucling$^{-/-}$ mice. However, after the age of 4 months, apparent swellings of preputial glands were frequently observed in Nucling$^{-/-}$ mice. The preputial gland is a part of the male reproductive system, appearing as a paired structure on either side of the penis in the male mouse. This gland is reported to play an important role in the production of olfactory substances, which attract the opposite sex [30]. When mutant mice reached the age of 12 months, more than 50 % of Nucling$^{-/-}$ and 30 % of Nucling$^{+/-}$ males displayed the same symptoms, i.e. swelling of the preputial gland, under specific pathogen-free conditions (Table 1 and

Table 1 Occurrence of swelling in male Nucling$^{-/-}$ mice

Data show the percentage of mice with preputial gland swelling over a range of ages (15–48 weeks) for the various genotypes. The numbers in parentheses show the number of mice examined for each experiment.

		Proportion of mice with preputial swelling (%)						
Genotype	Age (weeks) . . .	15	21	24	34	38	44	48
+/+		0 (10)	0 (10)	0 (10)	0 (5)	0 (5)	0 (5)	0 (5)
+/−		0 (16)	0 (16)	0 (16)	7.1 (14)	12.5 (14)	25 (10)	37.5 (10)
−/−		3.4 (29)	6.9 (29)	10.3 (29)	17.2 (29)	29.2 (24)	41.7 (24)	58.3 (24)

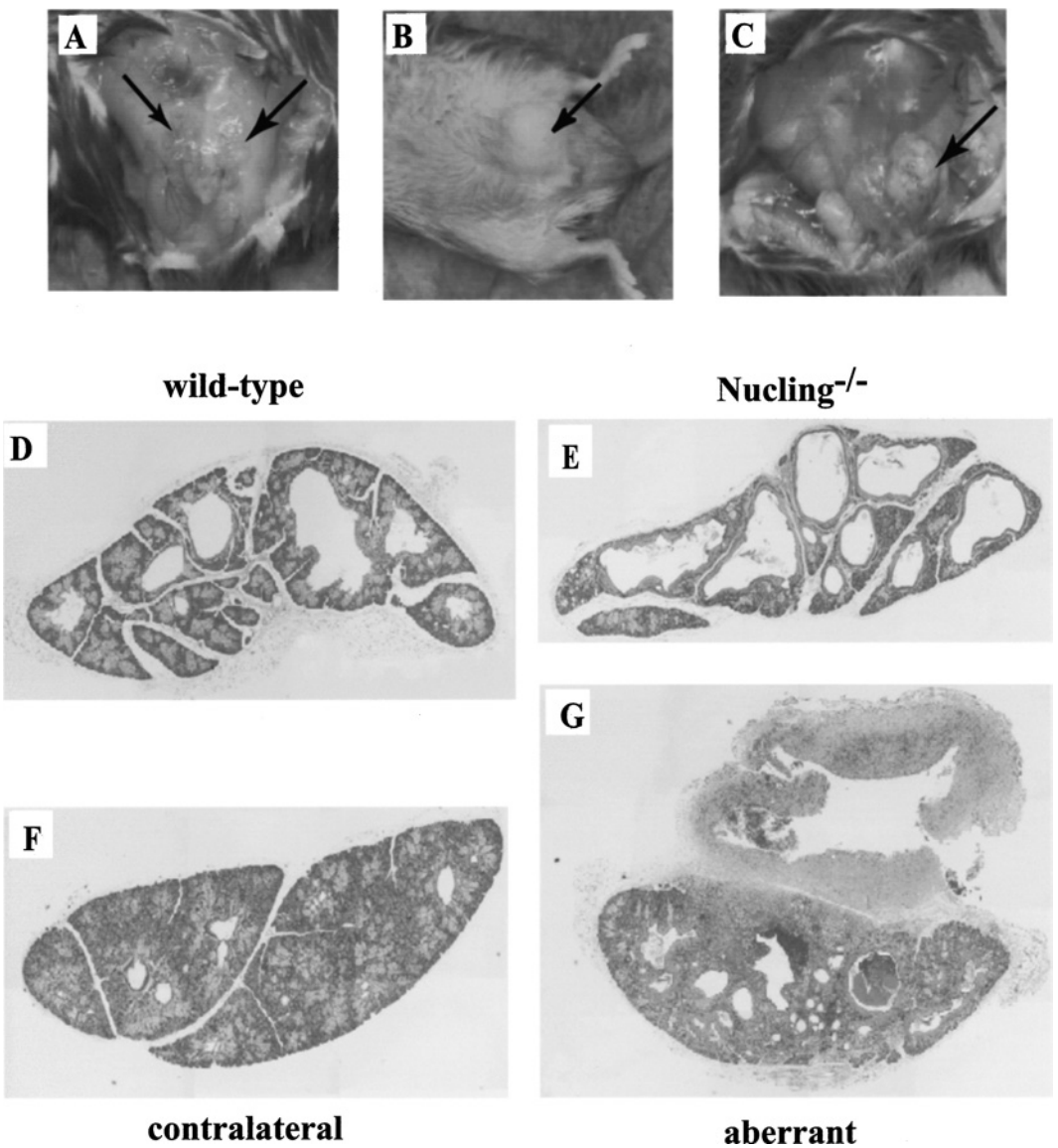


Figure 3 Inflammatory lesions of the preputial gland of male Nucling$^{-/-}$ mice

(**A**) Preputial gland of a 5-month-old WT male mouse after dissection. (**B**) Enlarged preputial gland (arrow) of a 5-month-old mutant male. (**C**) Aberrant preputial gland (arrow) after dissection. (**D**–**G**) H/E staining of paraffin-embedded sections (7 μm thick) of preputial glands from WT (**D**) and Nucling$^{-/-}$ (**E**–**G**) 5-month-old mice. Samples were obtained from the preputial glands of Nucling$^{-/-}$ mice without swelling (**E**), from those with swelling (**G**) and from the contralateral side (**F**).

Figures 3A–3C). The occurrence of pathological lesions was extremely high in mice with mutant allele(s) compared with their WT counterparts. Dissection of these abnormal preputial glands revealed the presence of viscous fluid in the swelling. In all the mice with abnormal glands, except one mouse with bilateral lesions, only one side of the gland was positive for swelling. Histopathological changes in the preputial gland were then analysed by H/E (haematoxylin and eosin) staining (Figures 3D–3G). On the aberrant side with the swollen preputial gland, we observed keratinization, inflammation, granulomatous

lesions, duct contraction and duct blockage (Figure 3G). In contrast, on the contralateral side of the preputial gland, we found that the number of luminal ducts was decreased, and cells were undergoing extreme hyperplasia (Figure 3F). In contrast, no marked difference could be observed between the normal-sized gland in Nucling$^{-/-}$ and that in WT mice, except for the abundant presence of luminal duct dilatation in Nucling$^{-/-}$ mice (Figures 3D and 3E). Therefore we consider that Nucling may also play a crucial role in the maintenance of the physiological function of the mouse preputial gland.

Galectin-3 is involved in inflammatory lesions of the Nucling$^{-/-}$ preputial gland

In galectin-3-null mutant mice, galectin-3 was shown to be involved in the control of an acute inflammatory process [31]. Galectin-3 expression has also been shown to be strongly up-regulated during inflammation in acute renal failure of the rat [32], and in the presence of certain pathological conditions, such as atherosclerotic lesions, ischaemic brain lesions, JIA (juvenile idiopathic arthritis) and diabetes [33–36]. It is possible that galectin-3 is also involved in inflammatory lesions of the preputial gland. Therefore we examined first whether Nucling is expressed in the male preputial gland of WT mice by RT-PCR analysis. As shown in Figure 4(A) (lanes 1 and 3), we could detect the expression of Nucling in the preputial gland as well as in the heart. The expression level of galectin-3 in the preputial gland was then investigated in WT mice, Nucling$^{-/-}$ mice without swelling and Nucling$^{-/-}$ mice with swelling, including both the aberrant and contralateral sides, by Northern blot analysis. Galectin-3 mRNA was markedly increased in the Nucling$^{-/-}$ preputial gland as compared with the WT. Moreover, the expression of galectin-3 was significantly up-regulated on both the aberrant side and the contralateral side of Nucling$^{-/-}$ mice with swelling (Figure 4B). In contrast, we could not detect the expression of galectin-4 in the preputial gland by Northern blot analysis (Figure 4C).

Expression of galectin-3 is up-regulated in the preputial gland of Nucling-null mice

To examine the localization of galectin-3 expression in the preputial gland, we then performed an immunohistochemical analysis of the gland (Figures 5A–5D). Galectin-3 was found to be negative for staining in the preputial gland of WT mice (Figure 5A). In contrast, in the preputial gland of Nucling$^{-/-}$ mice with or without swelling, galectin-3 was detected with a remarkably high level of staining around luminal ducts (Figures 5B–5D). Moreover, the expression of galectin-3 on the aberrant side of the preputial gland with swelling was much stronger, as shown in Figure 5(C). The immunostaining pattern of galectin-3 expression was in good accord with that of RNA expression (Figure 4B).

Apoptosis is attenuated in the preputial gland of Nucling$^{-/-}$ mice

It is reported that the accumulation of inflammatory cells was mainly due to down-regulated apoptosis [35]. In order to investigate whether Nucling-induced apoptosis is involved in the inflammatory lesions in the preputial gland, we performed the TUNEL assay (Figures 6A–6D). Around the luminal ducts (Figure 6A) and in acinous tissue (results not shown) of the WT preputial gland, many TUNEL-positive cells were observed. In contrast, few TUNEL-positive cells were observed around luminal ducts in the Nucling$^{-/-}$ preputial gland (Figures 6B–6D); furthermore, TUNEL-positive cells were hardly detected in acinous tissue on the aberrant side of the preputial gland of Nucling$^{-/-}$ mice either. On the basis of this observation, we speculated that Nucling is involved in apoptosis around luminal ducts of the preputial gland. The pattern of the presence of TUNEL-positive cells is in sharp contrast with that of galectin-3 expression in Figures 5(A)–5(D). We hypothesize that down-regulation of the expression of galectin-3, through the interaction of Nucling with galectin-3, induces apoptosis around the luminal ducts.

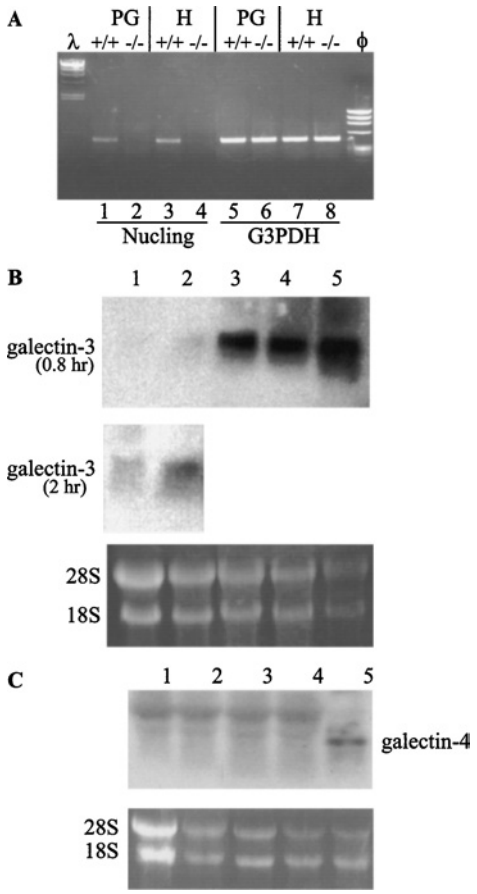


Figure 4 Up-regulation in expression of galectin-3, but not of galectin-4, in inflammatory lesions of the preputial gland

(**A**) Nucling expression was detected in mouse preputial gland (PG) by RT-PCR. Heart tissue (H) was used as a positive control. (**B**) Northern blot analysis of preputial gland was performed using 20 μg of total RNA from the WT preputial gland (lane 1), the Nucling$^{-/-}$ preputial gland without swelling (lane 2), the aberrant side with swelling (lane 3) and the contralateral side (lane 4). Intestine was used as a positive control (lane 5). Hybridization was performed with ^{32}P-labelled mouse galectin-3 probe. (**C**) Northern blot analysis for galectin-4 was also performed with ^{32}P-labelled mouse galectin-4 probe. The bottom panels in (**B**) and (**C**) show ethidium bromide staining of 28 S and 18 S RNA as a control for RNA loading.

Expression of galectin-3 is generally up-regulated in tissues of Nucling-null mice

Besides the preputial gland, we also investigated the expression of galectin-3 in other tissues. Proteins were harvested from tissues of both male and female WT and Nucling$^{-/-}$ mice. The expression of galectin-3, as compared with that observed in the preputial gland, was up-regulated in the heart (Figure 7, lanes 1–4), lung (lanes 5–8), kidney (lanes 9–12), ovary (lanes 13 and 14) and testis (lanes 15 and 16) of Nucling$^{-/-}$ mice.

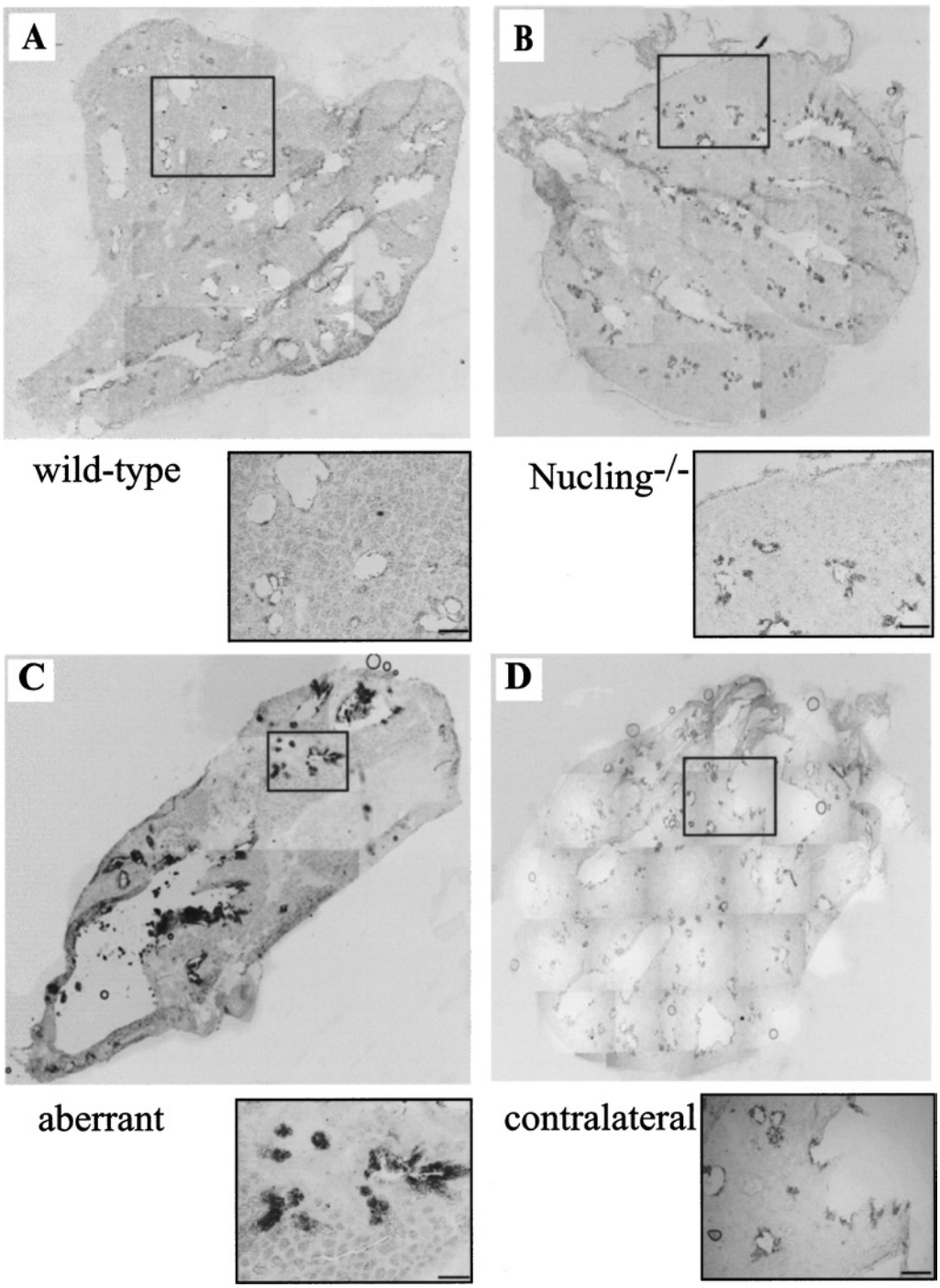


Figure 5 Immunohistochemical analysis of up-regulation of galectin-3 in preputial gland of Nucling-null mice

(**A**–**D**) Immunohistochemical localization of galectin-3 in preputial gland sections. Samples were obtained from 20-month-old WT mice (**A**), Nucling$^{-/-}$ mice (**B**) and abnormal Nucling$^{-/-}$ mice, including the aberrant side (**C**) and the contralateral side (**D**). Frozen sections were probed with Mac-2 antibody. Original magnification $\times$ 50.

DISCUSSION

In the present study, two major targets have been achieved: that is, the identification of galectin-3 as a protein interacting with Nucling and a pathophysiological analysis of Nucling-deficient mice. βCAP73 was shown to interact with β-actin [2]. Since we used NuclingΔN as 'bait' to screen for the protein interacting with Nucling, β-actin may not be detected as positive clones by the yeast two-hybrid system in our study. However, galectin-4 was found to interact strongly with this bait. The interaction was confirmed further by immunoprecipitation assay (Figure 1B). In this assay, the binding capacity of NuclingΔN for galectin-4 was greater than that of full-length Nucling, supporting further the validity of using NuclingΔN as the bait (Figure 1B). In addition, several clones were identified which interact with Nucling, and some of them have been reported to be involved in apoptosis

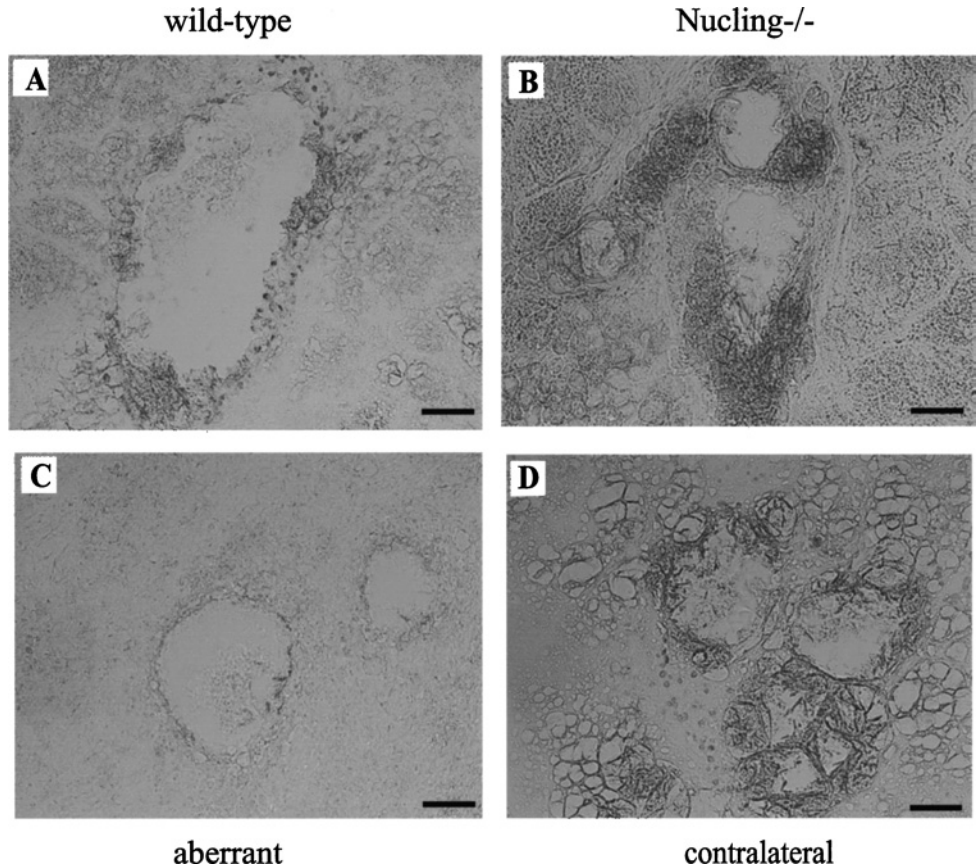


Figure 6 Detection of apoptotic cells around luminal ducts of the preputial gland

(**A**) Luminal ducts of the preputial gland in WT mice. (**B**) Luminal ducts of the preputial gland in Nucling$^{-/-}$ mice. (**C**) Aberrant side of the abnormal Nucling$^{-/-}$ preputial gland. (**D**) Contralateral side of the abnormal Nucling$^{-/-}$ preputial gland. Original magnification × 200.

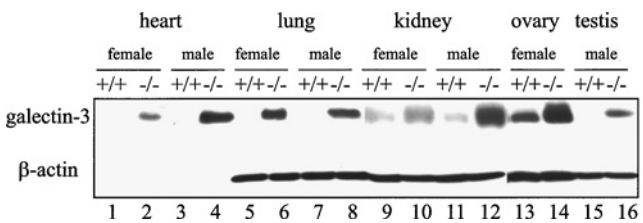


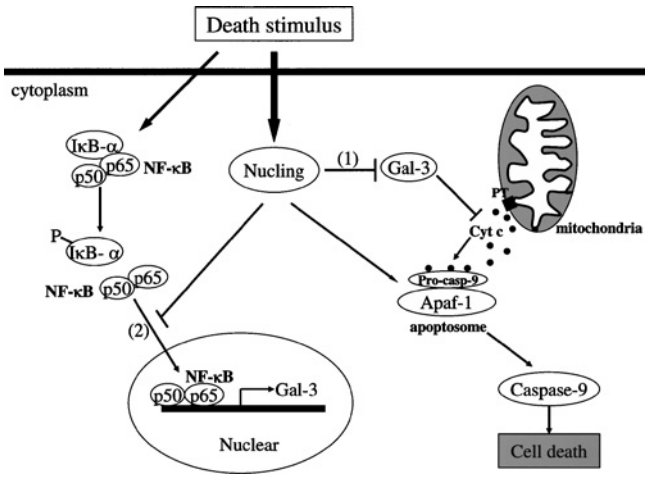
Figure 7 Expression of galectin-3 is generally up-regulated in tissues of Nucling-null mice

Extracted proteins from the heart (lanes 1–4), lung (lanes 5–8), kidney (lanes 9–12), ovary (lanes 13 and 14) and testis (lanes 15 and 16) were analysed by immunoblotting. Galectin-3 level in these tissues was examined with Mac-2 antibody. The same blot was probed with β-actin antibody (Sigma). This antibody does not stain adult cardiac and skeletal muscles.

(results not shown). Nucling was found to induce cytochrome *c* release, up-regulate Apaf-1 expression and recruit the Apaf-1–pro-caspase-9 complex for the induction of apoptosis following pro-apoptotic stress (T. Sakai, L. Liu, R. Mukai-Sakai, X. C. Teng, T. Mitani, M. Matsumoto, K. Toida, K. Ishimura, Y. Shishido, T. W. Mak and K. Fukui, unpublished work). In the present study, we have shown that Nucling-deficient cells are resistant to apoptosis (Figure 6B). These findings support further the pro-apoptotic role of Nucling. In addition, galectin-3 was demonstrated to interact with Nucling (Figure 1B). It is now well documented that galectin-3 has a critical role in the inhibition of apoptosis [9,10,17–20]. However, the signal transduction mechanism(s) of galectin-3 in relation to apoptosis is poorly understood. In the present study, we have shown that galectin-3 may negatively regulate apoptosome-dependent apoptosis by interacting with Nucling, a novel pro-apoptotic molecule.

It has been reported that NF-κB is involved in the regulation of galectin-3 expression [22]. We also found an NF-κB binding sequence (5′-GGGAGATCCC-3′) in the genomic sequence of Mac-2. We then showed that Nucling may inhibit NF-κB activation by interfering with the nuclear translocation of NF-κB–p65 from the cytoplasm (Figures 2D and 2E). The molecular mechanism underlying this regulatory role of Nucling in NF-κB activation remains to be investigated; however, the presence of the ankyrin repeat domain in both NF-κB [37] and Nucling may indicate the interaction of these molecules. In contrast with NF-κB-p65, the DNA-binding capacity of the NF-κB–p50 complex was lower in Nucling-deficient MEF nuclear extracts than that in Nucling$^{+/+}$ MEFs (Figure 2E, lanes 2 and 3, arrow B). Further detailed studies are necessary to analyse the precise mechanism of how Nucling regulates the NF-κB complexes containing p50 and p65 subunits. Taken all together, we postulate a distinct functional role of Nucling in the signalling pathway to apoptosis, as shown in Scheme 1.

The histopathological examination of Nucling-deficient mice revealed that preputial gland inflammatory lesions were found in more than 50 % of the Nucling$^{-/-}$ mice from our production colonies under specific pathogen-free conditions (Table 1).



Scheme 1 A possible pathway for the control of apoptosis involving Nucling, galectin-3 and NF-κB

The Figure shows that: (1) Nucling directly interacts with galectin-3; and (2) Nucling interferes with NF-κB activity through preventing the nuclear translocation of the NF-κB–p65 complex from the cytoplasm.

Interestingly, galectin-3 expression was up-regulated in swollen preputial glands from Nucling$^{-/-}$ mice (Figures 4B, 5C and 5D). It has been reported that galectin-3 may be secreted through a classical or non-classical secretory route under inflammatory conditions [38–40], and may function in activating various cells regulating cell adhesion, attracting inflammatory cells to the site of inflammation [41]. NF-κB is also reported to be involved in inflammatory responses. We considered that NF-κB may be a key mediator in the regulation of galectin-3 and inflammatory responses in Nucling-deficient mice, and increased galectin-3 expression is tightly correlated with a longer duration of inflammatory lesions.

In addition, galectin-3 was localized around the luminal ducts in the preputial gland of Nucling$^{-/-}$ mice (Figures 5B–5D), in which impaired apoptosis was detected (Figures 6B–6D). Our results are in agreement with those of Harjacek et al. [35], who showed that increased galectin-3 expression is correlated with defective mononuclear cell apoptosis in patients with JIA. Therefore we conclude that galectin-3-induced defective apoptosis is one of the key processes leading to inflammation of the preputial gland in Nucling$^{-/-}$ mice.

On the other hand, there are reports suggesting that apoptosis is an important mechanism in ductal morphogenesis [42], and that p53-independent apoptosis is primarily involved in the formation of ducts [43]. In addition, the formation of vascular-like structures requires apoptotic cell death through the activation of a caspase-dependent mechanism and mitochondrial cytochrome *c* release [44]. However, it is not yet fully explained which apoptotic pathway regulates the formation of the ducts. In our study, apoptosis-positive and galectin-3-positive cells accumulated around the luminal ducts in the preputial gland (Figures 5B–5D, and Figure 6A), and the number of ducts in the gland was also decreased, as shown in Figures 3(F) and 3(G). These findings seem to imply that Nucling-induced apoptosis plays an important role in the formation of luminal ducts to maintain the function of the preputial gland.

Regarding the phenotype of Nucling$^{-/-}$ mice other than the inflammatory lesions in the preputial gland, mutant mice displayed no defects that were similar to those of Apaf-1$^{-/-}$ [45] or caspase-9$^{-/-}$ mice [46], including forebrain hyperplasia and embryonic lethality. Although this phenotypic discrepancy might come from the existence of unknown redundant molecules or pathways, it is not clear at present whether Nucling is an essential molecule or not for apoptosis pathways during development. These studies are currently under investigation.

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