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REGULAR ARTICLE

Chicken GRIFIN: binding partners, developmental course of localization and activation of its lens-specific gene expression by L-Maf/Pax6

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Abstract

Tissue lectins appear to be involved in a broad range of physiological processes, as reflected for the members of the family of galectins by referring to them as adhesion/growth-regulatory effectors. In order to clarify the significance of galectin presence, key challenges are to define their binding partners and the profile of localization. Having identified the chicken *g*alectin-*r*elated *i*nter*f*iber prote*in* (C-GRIFIN) as lens-specific protein present in the main body of adult lens, we here report its interaction with lens proteins in ligand blotting. The assumption for pairing with α-, β- and δ-crystallins was ascertained by mass spectrometric detection of their presence in eluted fractions obtained by affinity chromatography. Biochemical and immunohistochemical monitoring revealed protein presence from about 3-day-old embryos onwards, mostly in the cytoplasm of elongated posterior cells, later in secondary lens fiber cells. On the level of gene expression, its promoter was activated by transcription factor L-Maf alone and together with Pax6 like a crystallin gene, substantiating C-GRIFIN's status as lens-specific galectin. Using this combined strategy for counterreceptor and expression profiling by bio- and histochemical methods including light, electron and fluorescence microscopy, respective monitoring in lens development can now be taken to the level of the complete galectin family.

Keywords Crystallin · Galectin · Lectin · Lens · Promoter

Introduction

The eye lens is a tissue entirely composed of a single cell type. Thus, the study of the lens offers the attractive opportunity to relate a certain cell's phenotypic fate (starting from its origin in the epithelial ectoderm) to expression of proteins without the added complexity of presence of other cell populations. The following course of development covers the route from the

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germinative zone with mitotically active, undifferentiated cells up to spatially highly organized, elongated lens fiber cells. Of note, they have lost all organelles to preclude scattering of light. In this report, we focus on a protein recently detected in adult chicken lens that belongs to a family of effectors known to be active in intra- and intercellular communication.

On the biochemical level, the special function of the lens calls for the need to accomplish the unique and exceptionally high-concentration and high-order packing of long-lived and highly stable intracellular proteins. In addition, they must have the required transparency and diffracting properties, as realized by crystallins most abundantly found in the lens (Wistow and Piatigorsky 1988; Bloemendal et al. 2004; Graw 2009; Bassnett et al. 2011; Clark et al. 2012). This term is purely technical. After all, crystallins are most often already known cytoplasmic constituents that have activities *sui generis*. For example, they are chaperones such as two members of the small heat shock protein family (αA- and αB-crystallins) in all vertebrates or enzymes additionally fulfilling this special task such as argininosuccinate lyase (δ-crystallin) in most birds and in reptiles (Piatigorsky and Wistow 1989; Wistow 1993; Slingsby et al. 2013). During development, expression patterns of crystallins do not seem to be simply co-regulated. Instead, even homologous crystallins can be differentially expressed and accumulation rates differ, as monitoring regulation of α-, β- and δ-crystallins in chicken revealed (Parker et al. 1988; Kondoh et al. 1991; Inoue et al. 1992).

In addition to the crystallins, further proteins are known to be present in the lens at substantial amounts (for complete chicken lens proteome, please see Chen et al. 2016). Among them is the *g*alectin-*r*elated *i*nter-*f*iber prote*in* (GRIFIN), a member of the family of adhesion/growth-regulatory galectins (Cooper 2002; Kaltner et al. 2017, 2018a; García Caballero et al. 2018). The multifunctional character of galectins, especially their capacity for *cis*/*trans*-bridging of counterreceptors with glycan and peptide motifs as binding partners in- and outside of cells (Harrison and Chesterton 1980; Gabius 1997; Liu et al. 2002; Kopitz et al. 2017; Kasai 2018; Xiao et al. 2018), provides incentive to explore the physiological relevance of this protein. Such a study will be a step to elucidate the functionality of a tissue lectin; this class of proteins is often considered as a tool, e.g., wheat germ agglutinin (WGA) as a marker for eye lens membrane (Kistler et al. 1986; Lim et al. 2005).

GRIFIN is present throughout vertebrates, commonly expressed in lens fiber cells of rat, zebrafish and chicken irrespective of the loss of carbohydrate-binding activity in mammals (Ogden et al. 1998; Ahmed and Vasta 2008; García Caballero et al. 2016a). Chicken GRIFIN (C-GRIFIN) is a compact and highly stable homodimer in crystals and in solution (Ruiz et al. 2018). In adult tissues, its expression is confined to the ocular lens, at about 0.016% of total protein among 1934 proteins at 15 days post-hatch compared to about 0.5% in adult rat lens (Ogden et al. 1998; Chen et al. 2016; García Caballero et al. 2016a; Manning et al. 2017a, 2018a). This current status of knowledge poses the questions on the nature of potential binding partners for C-GRIFIN (to be addressed biochemically and galectin histochemically), on its developmental course of gene expression (to be monitored biochemically and immunohistochemically) as well as on promoter sequence motifs and transcription factors, especially Maf recognition elements (MAREs) and L-Maf, which exert control on gene expression.

In order to address the given issues, we performed blotting of lens extract using C-GRIFIN as a sensor to detect binding partners and affinity chromatography on resin-immobilized C-GRIFIN together with mass spectrometric (MS) identification of eluted proteins. Monitoring of C-GRIFIN expression in the course of development was done by RT-PCR and Western blotting as well as immunohistochemistry by light, electron and fluorescence microscopy (here also performing double labeling using labeled C-GRIFIN and adding the study of a second chicken galectin (CG), i.e., CG-2, as internal control). The influence of distinct sequence motifs on the transcriptional activity was assessed by luciferase-based reporter assays using the natural C-GRIFIN promoter sequence and a panel of engineered (by deletions and mutations) variants together with expression vectors for transcription factors. They were selected due to known impacts of the produced protein on vertebrate lens induction and differentiation (Grainger 1992; Kondoh 1999; Chow and Lang 2001). The presented results provide evidence for interactions involving crystallins and two other galectins as well as define the expression profile from the earliest positive stage to adult tissue together with evidence for positive control of C-GRIFIN gene transcription by promoter sequence motifs binding L-Maf and Pax6.

Materials and methods

Antibodies and galectins

C-GRIFIN and CG-2 were purified after recombinant production by affinity chromatography, rigorously controlled for purity and activity and labeled under activity-preserving conditions with the N-hydroxysuccinimide ester derivative of biotin (Sigma-Aldrich, Munich, Germany), as described in detail previously (Kaltner et al. 2016; García Caballero et al. 2016a). Moreover, they were used as antigens for immunizing rabbits to obtain polyclonal immunoglobulin G (IgG) preparations, which were processed by Western blotting and ELISA to detect any cross-reactivity with the complete set of purified CGs, which was then completely removed by affinity chromatography, as described (Kaltner et al. 2002; García Caballero et al. 2016a).

Ligand blotting

Extract of a lens from a 19-week-old chicken was prepared in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% (*w*/*v*) NP-40) and processed, as described previously (Gabius et al. 1991). In detail, proteins of extract samples (with protein quantities of 0.25–6 μg) were separated in a SDS polyacrylamide gel (4% stacking gel, 15% running gel) at 200 V for 45 min; proteins were then transferred from the gel onto a nitrocellulose membrane (0.2 μm pore size; Schleicher & Schuell, Dassel, Germany) by tank blotting. Non-specific sites on the matrix for binding to the lectin/antibody were saturated by incubation with a solution of Tris-buffered saline (TBS, pH 7.5) containing 0.1% Tween 20 (TBS-T) and 3% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at room temperature. Incubation with biotinylated or label-free C-GRIFIN (1 μg/ml) was performed for 2 h at room temperature. After extensive washing with TBS-T solution, membranes incubated with biotinylated protein were exposed to avidin-horseradish peroxidase conjugate (0.5 μg/ml; Sigma-Aldrich) for 1 h at room temperature, while membranes incubated with unlabeled C-GRIFIN were exposed to a solution containing anti-C-GRIFIN IgG fraction (2 μg/ml, overnight at 4 °C) followed by incubation with anti-rabbit IgG-horseradish peroxidase conjugate (0.5 μg/ml, 1 h at room temperature; Sigma-Aldrich). Effect of the cognate sugar was tested by a pre- and co-incubation step of C-GRIFIN in blocking buffer containing 75 mM lactose, a mixture of 75 mM lactose and 1 mg/ml of the glycoprotein asialofetuin, 200 mM lactose or 75 mM non-cognate sugar mannose. Binding by ionic interactions was impaired by presence of 0.5 M NaCl. Detection of probe-dependent chemiluminescence was performed using a substrate mixture with 1.25 mM sodium salt of luminol in 3 ml 0.1 M Tris-HCl (pH 8.6), 0.3 ml of a 6.7 mM *p*-coumaric acid solution in dimethylsulfoxide, and 0.9 μl of a 30% H_2O_2 solution at room temperature. Imaging was carried out with a ChemiDoc Touch system and its analysis software Image Lab 5.2.1 (Bio-Rad, Munich, Germany).

Affinity chromatography of lens extracts

Protein extracts of lenses from 3-day-old chickens were prepared in PBS (20 mM phosphate buffer, 150 mM NaCl, pH 7.2) containing 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonylfluoride, 10 μM leupeptin and 0.4 μM aprotinin. Activated resin for protein conjugation (Affi-Gel10; Bio-Rad) was loaded with C-GRIFIN up to 11.4 mg/ml of slurry thoroughly washed and equilibrated. 0.5 ml of the suspension was incubated overnight at 4 °C with 1 ml of clear extract solution (protein content adjusted to 20 mg/ml) under gentle shaking. Following this incubation step, resin was carefully washed in a column with PBS to remove any unbound material. Maintained binding activity of resin-attached C-GRIFIN was ascertained by a binding assay using the glycoprotein asialofetuin as a ligand, as described (Gabius et al. 1991). Proteins associated with the C-GRIFIN-presenting beads after the washing procedure were released from the resin with 20 mM phosphate buffer containing either 0.5 M NaCl or 75 mM lactose. The eluted samples were re-equilibrated in 5 mM PBS using PD10® columns (GE HealthCare, Munich, Germany), then concentrated in an Amicon® ultrafiltration unit with an YM5 membrane (Merck, Darmstadt, Germany) and lyophilized.

Tryptic digestion

Protein samples (5 μg each) were dissolved in 40 mM ammonium bicarbonate (ABC). Processing followed an optimized protocol (Michalak et al. 2017) starting with reduction of disulfide bonds by supplementing each sample with 2 μl of a solution of 10 mM DTT and then keeping the mixtures in 40 mM ABC for 60 min at 45 °C on a thermomixer (600 rpm). In order to alkylate thiol groups of cysteine residues, 1 μl of a solution of 55 mM iodoacetamide (IAA) in 40 mM ABC was added, the resulting mixtures were then incubated for 30 min at 25 °C on a thermomixer (600 rpm) in the dark. Excess of IAA was inactivated by adding 2.5 μl of a solution of 10 mM DTT and incubating this mixture for 15 min at 37 °C on a thermomixer (600 rpm). Subsequently, 2 μl of a solution of 40 mM ABC was pipetted containing a total trypsin amount of 0.1 μg (Promega, Mannheim, Germany) establishing a protein to protease ratio of 50:1. Samples were incubated at 37 °C overnight. The solutions were evaporated to dryness and redissolved in 0.1% TFA/2.5% hexafluoroisopropanol and subsequently analyzed by nanoLC-ESI-MS/MS.

ESI-MS/MS analysis

Tryptic peptide mixtures were separated using a nanoAcquity UPLC system (Waters GmbH, Eschborn, Germany). Peptides were trapped on a nanoAcquity C18 column, 180 μm × 20 mm, particle size 5 μm (Waters). Separation was performed on a C18 column (BEH 130 C18 100 μm × 100 mm, particle size 1.7 μm; Waters) with a flow rate of 400 nl/min. Chromatography was carried out using a 2-h stepped linear gradient of solvent A (96.9% water, 3% DMSO, 0.1% formic acid) and solvent B (99.9% acetonitrile and 0.1% formic acid) in the following sequence from 0 to 4% B in 1 min, from 4 to 30% B in 80 min, from 30 to 45% B in 10 min, from 45 to 90% B in 10 min, 10 min at 90% B, from 90 to 0% B in 0.1 min and 9.9 min at 0% B. NanoUPLC was coupled online to a nano-electrospray source of a linear ion trap quadrupole (LTQ) Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Dreieich, Germany). A Pico-Tip Emitter tip (type 360 μm OD × 20 μm ID, 10 μm; New

Objective, Woburn, MA, USA) was used for sample ionization and introduction into the mass spectrometer. It was operated in the sensitive mode with the following parameters: ESI voltage was set to 2400 V, the capillary temperature was 200 °C and the normalized collision energy was 35 V. Full-scan spectra were acquired in a mass-to-charge ratio (*m*/*z*) range from 350 to 2000 in the profile mode with a mass resolution of 60,000 at *m*/*z* 400. Simultaneously, six most abundant precursor ions from the full-scan spectrum were selected for MS/MS fragmentation in the LTQ. Data were acquired in centroid mode. Only multiply charged (2+, 3+ …) precursor ions were selected for MS/MS. The dynamic exclusion list was restricted to 500 entries, with a maximum retention period of 30 s and a relative mass window of 10 ppm.

Database searches

The mgf-files generated by Xcalibur software (Thermo Fisher Scientific) were used for database searches with the MASCOT search engine (Matrix Science, London, UK; version 2.4) against the SwissProt database (SwissProt 2018_02 (556,568 sequences; 199,530,821 residues)) with taxonomy set to "all". Peptide mass tolerance for database searches was 7 ppm and fragment mass tolerance 0.4 Da. Carbamidomethylation of Cys was set as fixed modification. Variable modifications included oxidation of Met and deamidation of Asn/Gln. Missing one cleavage site due to incomplete trypsin hydrolysis was allowed. Furthermore, proteins were considered as identified, if at least two unique peptides had an individual ion score exceeding the MASCOT identity threshold.

Blast search

In those cases, when database searches resulted in a top-ranking hit with taxonomy other than chicken (e.g., human or bovine), proteins were searched against the UniProtKB database (release 2018_03 of 28-Mar-2018) using the program BLASTP (version 2.2.31+, https://web.expasy.org/blast/) with default values. Corresponding proteins from *Gallus gallus* (chicken) were selected and peptides were aligned with those detected by mass spectrometry.

RT-PCR profiling

Embryos of Brown Leghorn chicken eggs were collected after incubation at 38 °C (starting at 52 h of incubation until hatching) following staging according to the Hamburger and Hamilton classification (Hamburger and Hamilton 1951, 1992; HH staging). In detail, the following embryonic stages were examined following this classification: HH stage 17 (ca. 52–64 h), HH stage 19 (ca. 68–72 h), HH stage 21 (ca. 3.5 days), HH stage 23 (ca. 3.5–4 days), HH stage 25 (ca. 4.5 days), HH stage 27 (ca. 5 days), HH stage 31 (ca. 7 days), HH stage 39 (ca. 13 days), HH stage 41 (ca. 15 days) and HH stage 46 (20–21 days).

Total RNA was isolated (RNeasy® Mini Kit; Qiagen, Hilden, Germany) from chicken embryos/post-hatch chicken (whole head extract for HH stage 21, HH stage 23, HH stage 25 and HH stage 27; whole eye extract for HH stage 31 and lens extract for HH stage 39 and 3-day-old post-hatch chicken), cDNA prepared and then analyzed in RT-PCR assays, as described (García Caballero et al. 2016a). The calculated lengths of the amplified cDNAs are 420 bp for C-GRIFIN and 185 bp for chicken β-actin.

Western blotting

Protein extracts of heads (from chicken embryos at HH stages 25 and 27, respectively), eyes (HH stage 31), or lens (HH stage 39, 3-day-old and 19-week-old post-hatch chickens) were obtained and processed for SDS gel electrophoretic separation and Western blotting, as described previously (García Caballero et al. 2016a). Following gel electrophoretic separation and tank blotting, as described above, non-specific sites on the matrix were blocked by incubation with a solution of TBS-T and 5% (*w*/*v*) powdered milk. Membranes were then incubated overnight at 4 °C with the specific anti-C-GRIFIN IgG fraction, washed thoroughly with TBS-T buffer and exposed to goat anti-rabbit IgG-horseradish peroxidase conjugate (0.5 μg/ml) in blocking solution for 1 h at room temperature and gentle agitation. Signal generation and its documentation were done as described for ligand blotting.

Immunohistochemistry

Embryonic (HH stage 17 to HH stage 46) tissue and eyes from up to 7-day-old post-hatch chickens were fixed in Bouin's solution (71% (*v*/*v*) saturated picric acid, 24% (*v*/*v*) formaldehyde solution (37% (*w*/*v*)), 5% (*v*/*v*) glacial acetic acid) for 1 h per millimeter of tissue thickness. Next, tissues were dehydrated in increasing concentrations of ethanol (70%, 80% and 99%), then isopropanol and finally xylene. These processed specimens were embedded in paraffin wax at 61 °C and serial sections of about 5 μm thickness were obtained, which were mounted on Superfrost® Plus glass slides (Menzel, Braunschweig, Germany). Following an optimized protocol for C-GRIFIN detection (Manning et al. 2017a), sections were deparaffinized and incubated in PBS (pH 7.2) containing 1% (*w*/*v*) carbohydrate-free bovine serum albumin (BSA), supplemented with 5% (*v*/*v*) goat serum (Biozol, Eching, Germany) for 1 h at room temperature in a humidified chamber to saturate antigen/glycan-independent binding of reagents. Sections were then incubated with PBS containing 1% BSA and IgG fraction overnight at 4 °C. Titrations of

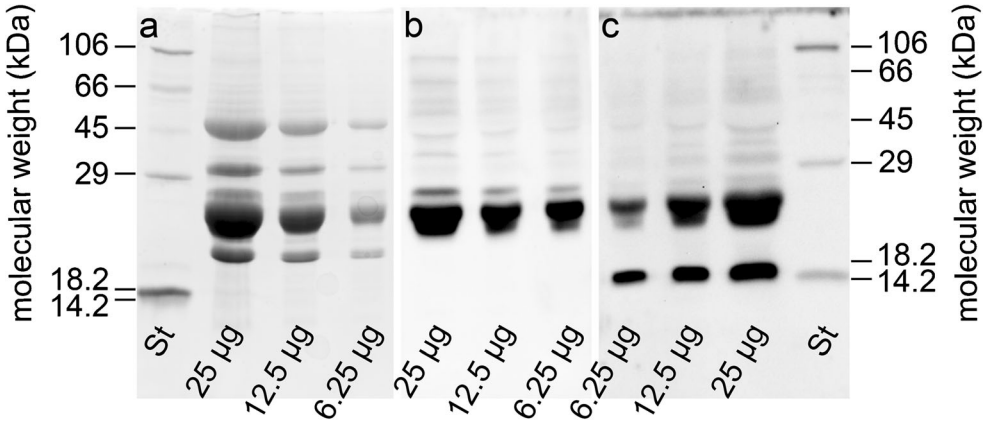


Fig. 1 Ligand blot analysis of lens extract with C-GRIFIN. Extracts of lens obtained from a 19-week-old chicken were separated by gel electrophoresis under denaturing and reductive conditions and proteins were transferred to a nitrocellulose membrane by tank blotting. **a** Protein staining after gel electrophoresis (25 μg, 12.5 μg, 6.25 μg total protein). **b** Blots with samples (25 μg, 12.5 μg and 6.25 μg total protein) were incubated with a solution containing biotinylated C-GRIFIN and, subsequently, exposed to avidin-horseradish peroxidase (please see section "Materials and methods"). **c** Blots with samples (6.25 μg, 12.5 μg and 25 μg total protein) were incubated with a solution containing recombinant C-GRIFIN and, afterwards, with the anti-C-GRIFIN antibody, followed by an incubation step with the corresponding second-step reagent (please see section "Materials and methods"). Molecular weights of markers are indicated both for **a** (left) and **c** (right)

antibodies (0.5–8 μg/ml) to determine the concentration for optimal signal-to-background ratio led to application at 4 μg/ml. After multiple washing steps with PBS, sections were incubated with goat anti-rabbit IgG (whole molecule)-alkaline phosphatase antibody conjugate (0.66 μg/ml; Sigma-Aldrich) for 1 h at room temperature. After removal of unbound antibody by further washing steps, signal was generated by addition of the Vector®Red alkaline phosphatase substrate kit SK-5100 (Biozol) for 20 min at room temperature in a dark humidified chamber. Counterstaining was performed with Mayer's hemalaun, then sections were dehydrated and mounted in Eukitt® (Kindler, Freiburg, Germany). Images were recorded using an AxioImager.M1 microscope (Carl Zeiss MicroImaging, Göttingen, Germany) equipped with an AxioCam MRc3 and MRc digital camera and the software AxioVision version 4.9.

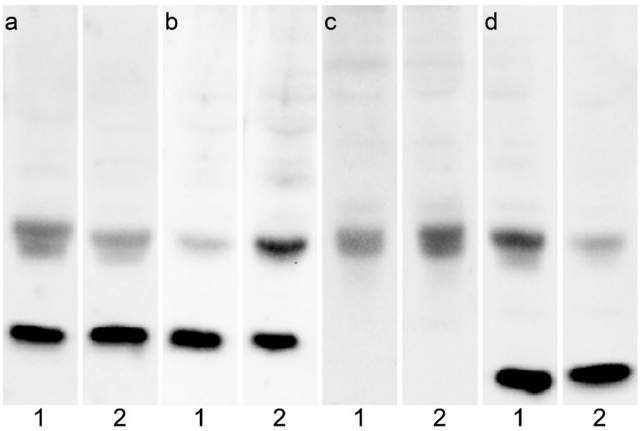


Fig. 2 Ligand blot analysis of lens extract with C-GRIFIN after incubation in the presence of lactose (**a**), mannose (**b**, **c**) and NaCl (**d**). Extract proteins of lens obtained from 19-week-old chickens were separated by gel electrophoresis under denaturing and reductive conditions and proteins were then transferred to a nitrocellulose membrane by tank blotting. **a** Blot was incubated with a constant C-GRIFIN concentration (1 μg/ml) in the absence of lactose (1) or in the presence of 75 mM lactose (2). Immunoprobing led to development of the C-GRIFIN signal as in Fig. 1(b). **b** Blot was incubated with C-GRIFIN as in panel **a**, either in the presence of 75 mM lactose (1) or 75 mM mannose (2). **c** Blot was incubated with biotinylated C-GRIFIN at a constant concentration (1 μg/ml), either in the presence of 75 mM lactose (1) or 75 mM mannose (2) and further processed as described (please see section "Materials and methods"). **d** Blot was incubated with a constant C-GRIFIN concentration (1 μg/ml) with buffer without NaCl (1) or with buffer containing 0.5 M NaCl (2). Each blot was calibrated with the common set of markers for molecular weight assignment (please see Fig. 1)

Post-embedding immunogold labeling (for transmission electron microscopy)

Immediately after obtaining tissue, small pieces (2 mm side length) of 3-/ 7-day-old chicken lenses were fixed overnight at 4 °C in 0.1 M cacodylate buffer (pH 7.4; Sigma-Aldrich) containing 4% paraformaldehyde and 0.1% glutaraldehyde (Sigma-Aldrich). Subsequently, specimens were dehydrated using an ethanol series (30%, 50%, 70%) at −20 °C and embedded in LR White resin (Electron Microscopy Sciences, Hatfield, PA, USA). Ultrathin sections were successively incubated in TBS (pH 7.4), in TBS containing 0.05 M glycine, in TBS containing 0.5% ovalbumin (TBS-ov) and in TBS containing 0.5% fish gelatin. Thereafter, the sections were incubated overnight at 4 °C in TBS-ov containing the anti-galectin IgG preparation (anti-C-GRIFIN/anti-CG-2 10 μg/ml). After carefully rinsing with TBS, sections were finally incubated 1 h at room temperature with goat anti-rabbit IgG conjugated to 10 nm-sized gold particles (BioCell, Cardiff, Wales, UK) diluted in TBS-ov (33.3 μg/ml). Following a

Table 1 Mass spectrometric analysis of proteins obtained by affinity chromatography (further details are given in Supplementary Tables S3 and S4)

			Elution with lactose		Elution with NaCl	
Protein name	Gene name	Species[a]	Mascot score	Sequence coverage (%)	Mascot score	Sequence coverage (%)
δ1-Crystallin	ARLY1	*Gallus gallus*	588	30.5	943	50.9
β-Crystallin B2	CRBB2	*Gallus gallus*	519	59.8	653	63.5
β-Crystallin A2	CRBA2	*Gallus gallus*	410	56.6	443	51.5
β-Crystallin B1	CRBB1	*Gallus gallus*	387	34.5	633	56.3
β-Crystallin B3	CRBB3	*Gallus gallus*	369	37.9	609	57.3
β-Crystallin A3	CRBA1	*Gallus gallus*	279	25.1	341	32.6
α-Crystallin A chain	CRYAA	*Gallus gallus*	193	17.3	263	33.5
Galectin-related protein (C-GRP)	LEGL	*Gallus gallus*	134	11.7	172	17
α-Crystallin B chain	CRYAB	*Gallus gallus*	131	10.3	147	16.7
CG-1A	LEG6	*Gallus gallus*	122	17.2	–	–

[a] Species filtering was set to "all species" (entries for *Gallus gallus* in SwissProt are fragmentary)

further washing step, sections were treated with uranyl acetate and examined with a transmission electron microscope (Leo 906E; Carl Zeiss Microscopy, Oberkochen, Germany). Controls including omission of the first-step reagent (anti-C-GRIFIN/anti-CG-2) and replacement of these antibodies by non-immune rabbit IgG or an irrelevant primary antibody in equimolar concentrations were carried out to exclude antigen-independent labeling.

Combined immuno- and galectin histochemistry

Processing for double staining to detect C-GRIFIN/CG-2 presence and galectin binding by fluorescence microscopy on 5-μm tissue sections started with blocking and antibody incubation, as described above for immunohistochemistry, here also with 0.1% (*v*/*v*) Tween 20. Sections were then processed with biotinylated C-GRIFIN/CG-2 overnight in a humidified chamber at 4 °C. After washing away unbound probe, sections were incubated with a solution of Alexa-Fluor®-568-labeled goat anti-rabbit IgG (whole molecule, 1 μg/ml; Sigma-Aldrich), fluorescein Avidin DCS (20 μg/ml; Biozol) and 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml). After a final series of washing steps, sections were mounted in antifade medium (Vectashield; Enzo Life Sciences, Lörrach, Germany). Systematic titrations of antibodies (0.5–8 μg/ml) and lectins (4–10 μg/ml) were performed to determine concentrations that obtain an optimal signal-to-background ratio (i.e., anti-C-GRIFIN/-CG-2: 4 μg/ml and C-GRIFIN/CG-2: 8 μg/ml). Controls included omission of antibody/lectin from the solution to spot probe-independent staining and co-incubation of biotinylated C-GRIFIN/CG-2 with the haptenic sugar lactose (200 mM) to block glycan-inhibitable binding, hereby excluding dye-mediated signal generation (Kaltner et al. 2018b).

Detection of transcription start point of the C-GRIFIN gene

Total RNA isolated from chicken lens of a 15-day-old (HH stage 41) embryo was processed following the protocol of the GeneRacer™ Kit (Thermo Fisher Scientific). 5′GeneRacer RNA-Oligo was fused to the 5′ end of the mRNA for detection of the 5′ end of the transcript. The sample was reverse transcribed into cDNA by using Super Script II Reverse Transcriptase and oligo-dT primer. This cDNA was used for PCR using the forward GeneRacer 5′Primer and the reverse C-GRIFIN-specific primer GSP1_C-GRIFIN_rev (5′-AGAC AATTCTGGAGCTGGCAAAGCGG-3′), followed by nested PCR using the GeneRacer 5′Nested Primer and GSP2_C-GRIFIN-rev (5′-GACCACGACGCTCCAGCCAGGAC-3′). The amplicon was cloned into the pCR4-TOPO vector

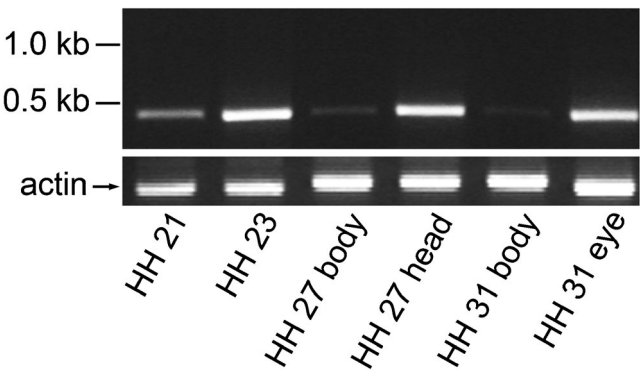


Fig. 3 Expression profiling of C-GRIFIN gene during selected stages of embryonic development by RT-PCR. Detection of C-GRIFIN-specific mRNA (420 bp) in samples of tissue from whole embryo (HH stages 21/23), embryonic head (HH stage 27) and embryonic eye (HH 31). Absence of signal in samples of tissue from bodies of embryos (without heads) at stages HH27/31 served as internal negative control. Loading control was performed by probing for mRNA for actin. Positions of markers for length of RT-PCR product are given (left)

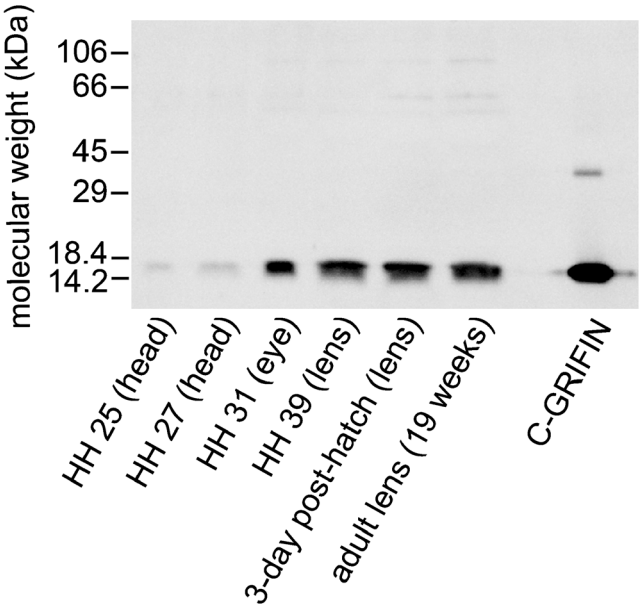


Fig. 4 Expression profiling of C-GRIFIN during selected stages of embryonic development by Western blotting. Detection of C-GRIFIN in samples from embryonic head (100 μg total protein; HH stages 25 and 27, respectively), embryonic eye (50 μg total protein; HH stage 31), embryonic lens (25 μg total protein; HH stage 39), 3-day-post-hatch lens (15 μg total protein) and adult chicken lens (6 μg total protein). A control with purified recombinant protein (C-GRIFIN, 12 ng) is included. Positions of markers for molecular weight determination are included

followed by transformation of the *E. coli* TOP10 strain. Plasmids were purified using a commercial plasmid preparation kit (Stratec Biomedical, Birkenfeld, Germany) and then finally sequenced (GATC, Konstanz, Germany).

Cloning of C-GRIFIN promoter region and reporter/transcription factor-encoding vectors

Genomic DNA was isolated from F6CC-PR9692 embryonic chicken fibroblasts (F6 cells; kindly provided by Jiri Plachy, Institute of Molecular Genetics, ASCR, Prague, Czech Republic) using the Wizard® Genomic DNA Kit (Promega). A 2509-bp-long region upstream of the ATG of the gene for C-GRIFIN was amplified by PCR with Phusion Polymerase (Thermo Fisher Scientific) with forward (5′-CTAGCTCGAGGCACTGCAGGGAAAGTATGCCTC-3′) and reverse primer (5′-GATCCTCGAGCCTGCTCTCC TGCTCTGTGCTG-3′). This amplicon was inserted into a pCR4-TOPO vector (Thermo Fisher Scientific) by TA cloning, amplified by PCR (forward primer: 5′-CTAG GCTAGCGCACTGCAGGGAAAGTATGCCTC-3′; reverse primer: 5′-GATCAGATCTCCTGCTCTCCTGCTCT GTGCTG-3′) and cloned into vector pGL4 (Promega) containing a luciferase reporter gene, by using restriction sites for *Nhe*I and *Bgl*II (Promega), resulting in the test vector pGL4-C-GRIFINp. Deletion constructs of the promoter region were made by PCR using the pGL4-C-GRIFINp as a template and respective variations of the sequence concerning sections of the forward primer (for respective sequences, please see Supplementary Material, Table S1). Deletions and mutations of potential Maf-binding sites were generated by the Quick-Change method (for respective primer sequences, please see Supplementary Material).

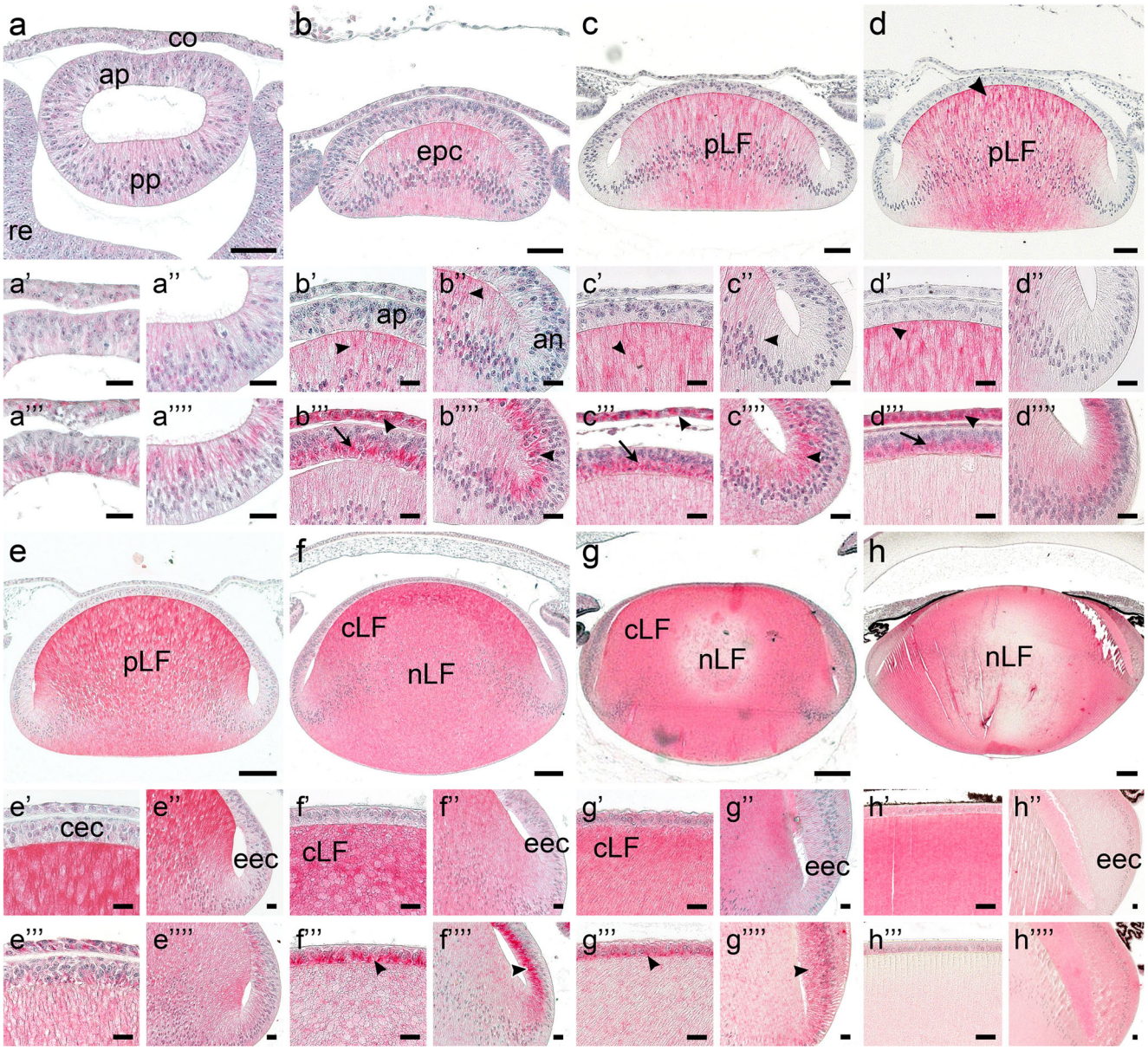
Sequences for the transcription factors L-Maf, MafB and the three Pax6 isoforms were isolated from the cDNA library of a lens from a HH stage 41 embryo by PCR by using the respective primer pairs (for respective sequences, please see Supplementary Material, Table S2). The amplicons were cloned into pcDNA3.1(+) (Thermo Fisher Scientific) using the restriction sites for *Eco*RI and *Xho*I (Promega). Sequences of all constructs were ascertained by sequencing (GATC). In order to normalize transfection efficiency, the *Renilla* luciferase (Rluc)-expressing vector pGL4.74 was used in titrations to reach the optimal quantity of DNA in the transfection sample together with the vector pGEM®-T Easy (Promega).

Cell culture

Chicken fibroblast cells (see above) were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Sigma-Aldrich) supplemented with 8% fetal calf serum, 2% chicken serum and 1% mixture of penicillin/streptomycin/glutamine at 37 °C and 5% CO_2. To run transfection and luciferase assays, 1×10^5 cells per well were seeded in 24-well plates 24 h prior to transfection.

Transfection and luciferase reporter assay

F6 cells were transfected in the presence of the jetPEI transfection reagent (VWR, Darmstadt, Germany) at a concentration of 4 μl/μg plasmid DNA (for plasmid details, please see above) according to the protocol of the manufacturer. Analyses of luciferase (firefly and *Renilla*) expression were performed applying the Dual-Luciferase® Reporter Assay System (Promega). Twenty microliters of cell lysate was used for the measurement in Nunc® MicroWell™ 96-Well Plates (VWR) 24 h post-transfection with sequential addition of 50 μl of solutions of first firefly and then *Renilla* luciferase substrate. Luminescence detection was performed using the plate reader Infinite F200 (Tecan, Männedorf, Switzerland) with a period of monitoring of 5 s for both signals. Transfection with the pGL4-C-GRIFINp vector was first performed in a range of 0.3–300 ng, keeping pGL4.74 quantity constant at 10 ng and total applied DNA at 500 ng to optimize transfection conditions in relation to DNA/luminescence light unit ratio. Routinely, 20 ng of pGL4-C-GRIFINp was used. pcDNA 3.1 vectors containing transcription factor-encoding cDNAs as inserts were applied in the respective experiments



in a range of 0.1–30 ng. In each series, measurements were done at least in triplicate in three independent series. Data are presented as mean ± SEM; statistical assessment of significance was performed by the unpaired *t*-test.

Results

C-GRIFIN interactions monitored by ligand blotting

A hallmark of the functionality of tissue lectins is their capacity to interact with distinct counterreceptors via lectin-glycan/protein recognition (Gabius et al. 2016; Manning et al. 2017b). As a first step to reveal such specificity for C-GRIFIN, proteins of adult chicken lens were subjected to ligand blot analysis after gel electrophoretic separation (the profile of extract proteins shown in Fig. 1a). Monitoring was performed in parallel with biotinylated probe/avidin-peroxidase conjugate and the natural protein detected immunochemically. Independent of a binding of the used probe to extract proteins, each detection system gave rise to a distinct signal: a protein in the lane with standards, i.e., the $(GlcNAc)_n$-binding lysozyme (Fig. 1b) and (endogenous) C-GRIFIN present in the extracts (Fig. 1c). Their presence established internal controls to exclude false-negative results.

In addition to these bands that ascertain the functioning of the detection systems, strong signals appeared due to a pairing between probe and extract proteins at certain sites of the blot membrane. Examining the lanes with extract proteins, biotinylated or unlabeled C-GRIFIN both associated with ligands

◀ **Fig. 5** Immunohistochemical localization of C-GRIFIN and CG-2 in fixed cross-sections through developing and post-hatch chicken lens. Anti-C-GRIFIN-stained sections of the complete (developing) lens are shown in each overview (**a**–**h**). Enlarged views (**'**–**""**) of distinct regions stained with anti-C-GRIFIN (**'**, **"**) and anti-CG-2 (**"'**,**""**) antibodies are assigned to each microphotograph. **a**, **b** Documentation of the lens vesicle stage. **a** Weak and rather homogeneous staining for C-GRIFIN in the cells of the anterior part (ap, **a'**) and comparatively slightly stronger intensity in cells of the posterior part (pp, **a"**) of the lens vesicle at HH stage 19. Presumptive retina (re) and cornea (co) were negative. Supranuclear CG-2 positivity in cells of the presumptive cornea, the anterior (**a"'**) and posterior (**a""**) parts of the lens vesicle. **b** Moderate intensity of signals for C-GRIFIN presence in elongated posterior cells (epc) that fill the cavity of the lens vesicle at HH stage 21; these cells were predominantly positive in regions close to the anterior part of the lens vesicle (arrowheads in **b'**, **b"**). Cells of the anterior part (**b'**) and epithelial cells in the region of the presumptive annular pad (an, **b"**) were negative. Strong staining for CG-2 in the developing corneal epithelium (arrowhead, **b"'**), supranuclearly in cells of the anterior part, which mature to central epithelium (arrow, **b"'**) and to equatorial epithelial cells at the developing annular pad (arrowhead, **b""**), whereas elongated posterior cells presented weaker staining intensity (**b"'**). **c**–**e** Formation of primary lens fiber cells (pLF). **c** Ribbon-like and diffuse staining pattern in the primary lens fiber cells at HH stage 23 with stronger intensity in lens fiber cells located in central regions of the lens (arrowhead, **c'**) than in those closer to the annular pad (arrowhead, **c"**). Supranuclear signals for CG-2 presence were more intense in cells of the presumptive cornea (arrowhead, **c"'**), anterior part (arrow, **c"'**) and at the annular pad (arrowhead, **c""**) than in primary lens fiber cells (**c"'**). **d** Similar staining pattern for C-GRIFIN at HH stage 25 as in (**c**) but further elongated primary lens fiber cells showed increased staining intensity at the contact site to the central epithelial cells (arrowheads in **d** and **d'**). Epithelial cells at the annular pad were free of signal for C-GRIFIN (**d"**). Processing sections with anti-CG-2 led to strong staining intensity in the developing cornea (arrowhead, **d"'**), in the anterior part (arrow, **d"'**) and in the cells of the annular pad (**d""**), while less intense staining was seen in the primary lens fiber cells (**d"'**). **e** Staining intensity for C-GRIFIN in primary lens fiber cells is strongest at HH stage 27. Central (cec, **e'**) and equatorial (eec, **e"**) epithelial cells are negative but supranuclearly positive for CG-2 (**e"'**, **e""**) with lower intensity than in less-advanced developmental stages. Staining in the primary lens fiber cells remained at a medium level of intensity. **f**–**h** Formation of secondary fiber cells in the lens cortex. **f** Stronger immunoreactivity in secondary cortical lens fiber cells (cLF, **f** and **f'**) than in nuclear primary lens fiber cells (nLF) and equatorial epithelial cells (**f"**) at HH stage 31, an early period of secondary lens fiber cell formation. **g** C-GRIFIN-positive cortical lens fiber cells (**g** and **g'**) together with partially negative nuclear lens fiber cells and equatorial epithelial cells (**g"**) at HH stage 39. Further reduction of the level of C-GRIFIN presence in the nucleus was followed by a complete loss of staining in this region in a 3-day-old post-hatch chicken lens (**h**). At this stage, a strong signal was detected in cortical lens fiber cells (**h'**). Equatorial epithelial cells were weakly stained (**h"**). Signal intensity for CG-2 presence in central and equatorial epithelial cells (at the annular pad) decreases from strong (arrowheads in **f"'**/**""**) to moderate (arrowheads in g"'/""), while extent of staining in cortical lens fiber cells decreases from moderate (**f"'**) to weak (**g"'**). Post-hatch chicken lens fiber cells are negative (**h"'**) and only equatorial epithelial cells are weakly stained (**h""**). Concentration of anti-C-GRIFIN and -CG-2 was 4 μg/ml. Scale bars are 20 μm (**'**–**""**), 50 μm (**a**–**d**), 100 μm (**e**, **f**) and 200 μm (**g**, **h**)

in the mass range characteristic for β-crystallins (from 23 kDa for the A_2-subtype to 27.3 kDa for B_1) in a concentration-dependent manner (Fig. 1b, c). Further assays with the unmodified protein as a sensor revealed a reduction of signal intensity by presence of cognate sugar lactose (Fig. 2a) but not by mannose tested as non-cognate (osmolarity) control (Fig. 2b). Increase of the sugar (lactose) concentration from 75 to 200 mM and addition of a glycoprotein to the sugar-containing solution did not further decrease signal intensity. The same susceptibility pattern was seen, too, when applying biotinylated C-GRIFIN as a probe (Fig. 2c). A negative effect on signal intensity was also seen by raising the ionic strength of the buffer (0.5 M NaCl), a means to interfere with polar interactions between proteins (Fig. 2d). As the broadness of the band attests, more than a single protein will most likely be involved in C-GRIFIN binding and inert proteins will also be present on the matrix within this range. These experiments thus inform us about actual binding of C-GRIFIN to denatured and matrix-presented lens proteins. In order to identify the biochemical nature of proteins in this mass range, to be able to trace (much) less abundant proteins with affinity to C-GRIFIN and to avoid application of a denaturation step, we proceeded by carrying out affinity chromatography of lens extract on resin harboring immobilized C-GRIFIN.

C-GRIFIN interactions monitored by combining affinity chromatography and MS analysis

Lens extracts of native proteins were incubated with resin that presents covalently conjugated C-GRIFIN. Proteins bound to the receptor on the affinity resin (or complexes formed with a binding partner) were eluted using lactose or NaCl after thorough washing to remove any non-interacting material. The protein populations in the two eluted fractions, obtained in parallel by processing aliquots of extract solution and resin suspension, were then subjected to tryptic fragmentation, peptide profiling and protein identification by data bank-based protein identification. The experimental data led to matches that satisfied the stringent criteria for identification. The complete list of binding proteins of C-GRIFIN is presented in Table 1 (for additional information on protein matches and peptide sequences, please see Supplementary Material, Table S3 and Table S4). Since the Swissprot database has presently not yet reached the level of complete information on the chicken proteome, the taxonomy was also set to "ALL" for database searches with the MASCOT search engine, inevitably resulting in prominent hits of proteins of mammalian, especially human and bovine origin. In these cases, a BLAST search against the UniProtKB database was conducted. Corresponding proteins from chicken were selected and peptides were aligned with those detected by mass spectrometry so that any species-specific (diagnostic) peptide(s) would show up. Several non-chicken proteins known as environmental contaminants like keratins from hair and skin or salivary/sweat-derived proteins such as zinc α_2-glycoprotein were also detected but, following common practice, not included in Table 1.

The two modes of elution yielded similar patterns, with differences in ranking. Proteins of two groups are present in both fractions: (i) α-, β-, and δ-crystallins and (ii) two other members of the galectin family that are present in the annular pad and also in lens fibers of the main lens body. Based on peptide assignment, an interaction with crystallins, alone or in complexes, appears likely to occur in the lens. C-GRIFIN can thus be referred to as a lens constituent with a capacity to interact with distinct proteins in situ, making involvement in structural organization and cellular functions possible. These findings prompted us, building on previous documentation of C-GRIFIN presence and localization in adult lens (García Caballero et al. 2016a; Manning et al. 2018a), to explore these features during tissue development starting with biochemical means on material from HH stage 21 up to adult tissue.

C-GRIFIN expression monitored by RT-PCR and Western blot analysis

When performing expression profiling by RT-PCR analysis, material of HH stage 21 yielded weak positivity (Fig. 3). The panel of specimen then covered HH stage 23 (whole body; positivity exclusively in head), embryonic day 5 (HH stage 27; separated in head and body) and day 7 (HH stage 31; separated in eyes and body) (Fig. 3). As previously shown for samples of adult chicken (García Caballero et al. 2016a), extracts prepared from eyes (at HH stage 31) or head (at HH stage 27) were positive. The data revealed presence of C-GRIFIN-specific mRNA in substantial quantity from HH stages 21 and 23 onwards. Presence of protein could be detected in extracts as a faint band with material from head at HH stage 25 onwards, the signal from extract of eye (at HH stage 31) being conspicuously strong (Fig. 4).

The presented results set the stage for immunohistochemical monitoring in the course of development, which will answer the question on the distribution of C-GRIFIN in the ocular lens at different stages.

C-GRIFIN localization monitored by immunohistochemistry

Since the sensitivity of protein detection differs between methods, we processed tissue specimen starting at the stage of the lens vesicle (HH stage 19) by immunohistochemistry. Weak to medium staining was seen in sections at this stage. Graded signal intensity with a tendency for an increase was detected in sections when moving from the anterior to the posterior part (Fig. 5a, a', a"). Anti-CG-2 antibodies used as control for the specificity of the immunodetection labeled anterior and posterior cells supranuclearly (Fig. 5a"', a""). Serving a second purpose, we applied parallel processing with this antibody to figure out whether another member of the galectin family (for biochemical details, please see Kaltner et al. 2008), which is only weakly immunopositive exclusively in the annular pad of adult chicken lens, (Manning et al. 2018a) is expressed in development and whether with a similar or a different developmental course of distribution relative to C-GRIFIN.

Weak RT-PCR signals at HH stage 21 (Fig. 3) can be interpreted as indicators of C-GRIFIN presence in elongated posterior cells, especially observed in regions in direct vicinity to the anterior part, whereas anterior cells are no longer positive (Fig. 5b, b'). Lack of staining in the developing annular pad is also documented in panel b". In contrast, CG-2 was detectable in the anterior region and the developing annular pad (Fig. 5b"', b""), underscoring the specificity of the immunohistochemical reaction. In the adult lens different from C-GRIFIN, CG-2 had been reported to be present in the annular pad, the epithelium and the lens body (Manning et al. 2018a). Obviously, the distribution profiles of these two homologous proteins diverged early in development.

Proceeding in embryogenesis to HH stage 23 (positivity in primary lens fiber cells; Fig. 5c, c'), HH stage 25 (with increase at contact site to central epithelial cells; Fig. 5d, d') and HH stage 27, signal intensity in the lens primary fiber cells is strongest at stage 27 (Fig. 5e). In contrast, cells of the anterior part and of the annular pad (at HH stages 23 and 25; Fig. 5c, d; parts'/") as well as of central and equatorial epithelial cells (for HH stage 27) are negative (Fig. 5e',"); these cell populations, however, present positivity for CG-2, with decreasing intensity (Fig. 5c–e; parts"'/""). Again, the different courses of signal intensity are evidence for lack of influence of any antigen-independent mechanisms on staining.

At HH stage 31, a period of formation of secondary lens fiber cells, the resulting cortical cells are more strongly positive than nuclear primary lens fiber cells (Fig. 5f, f'), a tendency further accentuated during the days of incubation, at HH stage 39 (Fig. 5g, g') and the day 3 post-hatch (Fig. 5h, h'). Both central and equatorial epithelial cells remained mostly negative (Fig. 5f–h; parts'/"). During this period, CG-2 presence also becomes lowered, as illustrated in Fig. 5f, g, (parts "',"") so that the status of the post-hatch lens is weakly positive (Fig. 5h, parts "',"").

Viewed at the level of electron microscopic resolution at this stage, C-GRIFIN is localized diffusely in the cytoplasm, close to cell membranes, in cisternae of endoplasmic reticulum and in intercellular spaces (Fig. 6a, b; enlarged area in panels a', b'). Comparatively less frequent, gold granules as an indicator for CG-2 presence were seen associated to the endoplasmic reticulum and lens fiber cell membrane (Fig. 6c). These patterns were maintained in specimens at day 7 post-hatch (Supplementary Material, Fig. S1). In order to visualize galectin presence together with sites of affinity for the galectin, fluorescence microscopy was performed.

Localization of C-GRIFIN and binding sites by fluorescence microscopy

Combination of visualization of C-GRIFIN presence immunohistochemically and of accessible sites for C-GRIFIN binding by biotinylated lectin, as used in ligand blotting (Fig. 1a), facilitated monitoring the two profiles and the detection of regions of overlap. Controls for absence of antigen-independent staining and for inhibition of galectin binding by cognate sugar are documented in Supplementary Material, Fig. S2a, b. As performed in light and electron microscopy, CG-2 was processed accordingly as further internal control.

As shown in panels a–f of Fig. 7, weak intensity of the diffuse signal was characteristic at HH stage 19 and more intense at HH stage 21, predominantly in the apical part of the elongated posterior cells (please see also corresponding Fig. 5b, b'). In contrast, the green signal for C-GRIFIN binding covered the entire area. Overlap of signals occurred at HH stage 21 in a small region at the apical surface of elongated posterior cells (Fig. 7f). The insets to Fig. 7c, f document differences in this respect to CG-2, especially an overlap in the presumptive cornea at HH stage 19 and then also in the developing cornea at HH stage 21. In both cases, though, presence of accessible binding sites covered the complete region (Fig. 7b, e).

The intensity of signals of galectin to primary lens fiber cells decreases during successive stages, probably due to their masking by the cognate lectin (Fig. 7g–l). Therefore, epithelial cells of the developing cornea and cells of the central lens epithelium remained positive in galectin histochemistry. Of note, an overlap was apparent at apical contact sites of the lens fiber and central epithelial cells (Fig. 7g, h; please see also

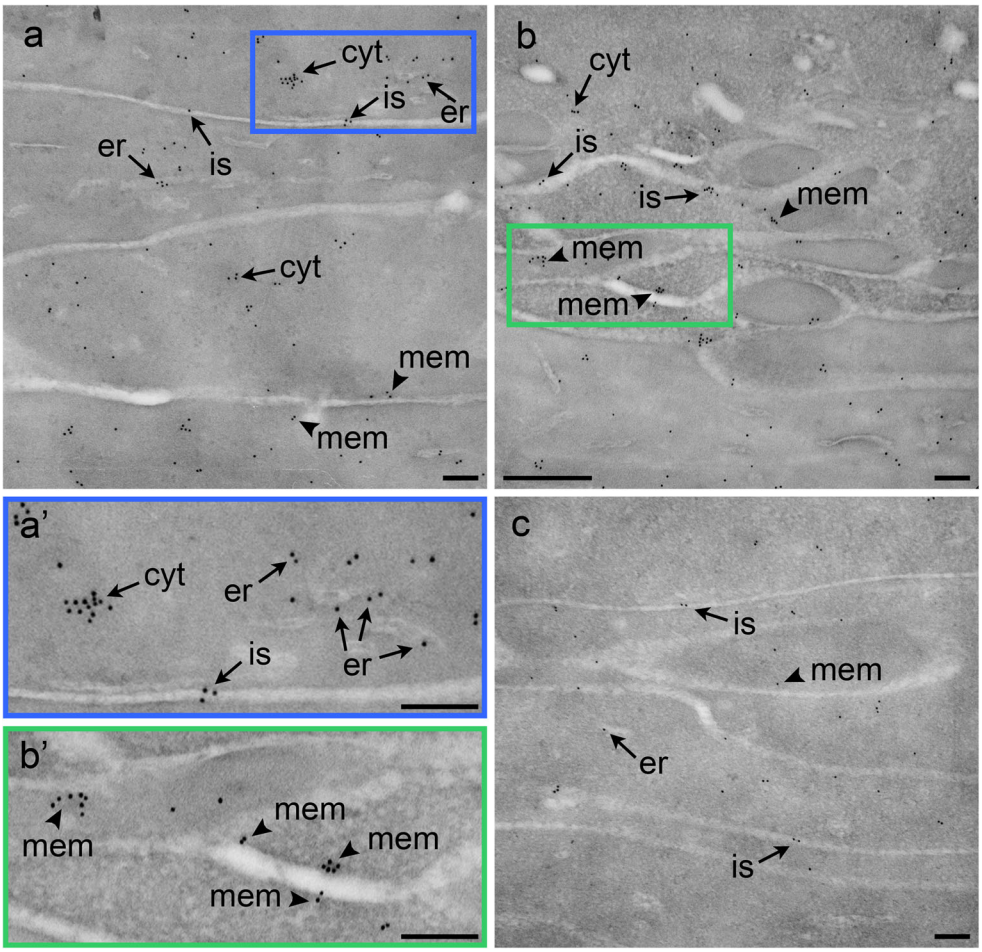
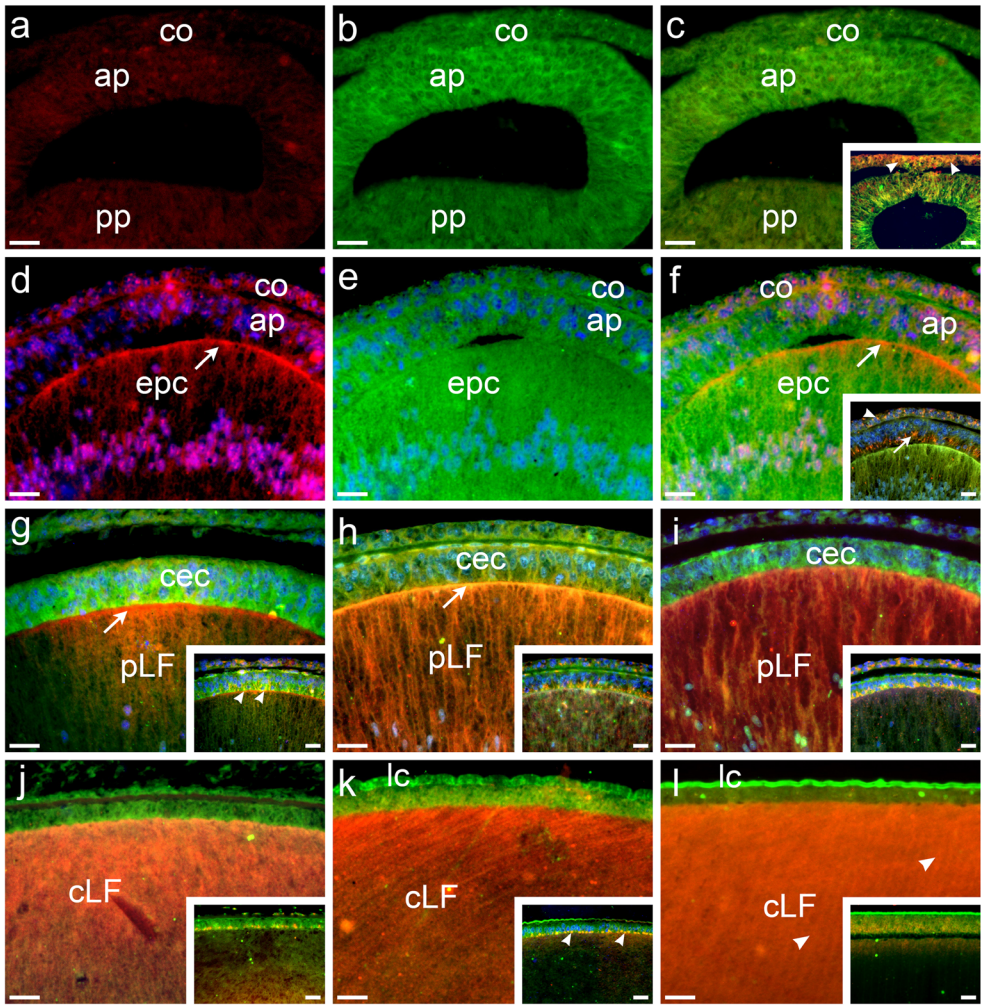


Fig. 6 Illustration of electron microscopic immunogold labeling for C-GRIFIN (**a**, **a'**, **b**, **b'**) and CG-2 (**c**) in ultrathin sections of lens fiber cells from 3-day-old post-hatch chicken lens. Positivity for C-GRIFIN was detected in lens fiber cells in longitudinal (**a**) and in transversal (**b**) directions diffusely in the cytoplasm (cyt, arrows), close to cell membranes (mem, arrowheads) over intercellular spaces (is, arrows) and within cisternae of the endoplasmic reticulum (er, arrows). (**a'**, **b'**) Enlarged views of selected regions from **a** (blue box) and from **b** (green box). **c** Reflecting the comparatively less-intense light microscopical staining for CG-2 than for C-GRIFIN (shown in Fig. 5h'–""), gold granules were sparsely distributed when detecting CG-2 presence, here in association with endoplasmic reticulum (er, arrow), lens fiber cell membranes (mem, arrowhead), and over intercellular spaces (is, arrows). Scale bars are 0.2 μm



enhanced immunopositivity in the apical parts of primary lens fiber cells in Fig. 5c, c', d, d'). While immunopositivity typically resided in secondary lens fiber cells in the characteristic lamellar pattern from HH stage 31 onwards (also observable in Fig. 5g', h'), remaining signals for binding of labeled C-GRIFIN were registered in central epithelial cells and the developing lens capsule (Fig. 7j–l). Regarding CG-2, only rather small areas of overlap were seen, restricted in extension and signal intensity by the typical decrease in level of expression (insets to Fig. 7j–l; please also see extent of reduction in staining intensity in corresponding Fig. 5f"', g"' and h"').

The immunohistochemical analysis by light and fluorescence microscopy delineated a spatiotemporally distinct course of C-GRIFIN expression and distribution. The presented results for the lens-specific galectin, together with the previously documented sharing of sequence motifs in the promoter region of 10 genes for eye proteins such as four crystallins (García Caballero et al. 2016a), shape the hypothesis for an impact of MARE/Maf (total sharing) and Pax6 (8 of 10 promoters positive) binding on transcriptional activity of the C-GRIFIN gene.

C-GRIFIN gene expression monitored by reporter assays

In order to provide first insights into the molecular basis of transcriptional regulation on the level of control by transcription factors, we performed reporter assays using a promoter sequence region that spans 2509 bp from this gene's ATG. The transcription start point was found 29 bp upstream of the ATG, as shown in Fig. 8(a), a result experimentally obtained in all six clones analyzed.

The next feature of the cloned section of C-GRIFIN's promoter is presence of a TATA box 32 bp upstream from the start point of transcription. Its functionality is demonstrated by activity assays using mutation at/deletion of this

◀ **Fig. 7** Combined immunohistochemical and galectin histochemical staining by anti-C-GRIFIN/-CG-2 and biotinylated C-GRIFIN/CG-2 visualized using fluorescently labeled second-step reagents. Anti-C-GRIFIN/-CG-2 was detected with Alexa-Fluor®-568-labeled goat anti-rabbit IgG (red) and biotinylated galectin with a fluorescein-avidin conjugate (green). Immunohistochemical detection of C-GRIFIN (**a**, **d**). Very low and rather homogeneous signal intensity in the anterior (ap) and posterior (pp) parts of the lens vesicle was seen at HH stage 19 (**a**; please see corresponding illustrations in Fig. 5a, a', a"). At HH stage 21 (**d**), the anti-C-GRIFIN-dependent reaction became comparatively stronger. At this stage, C-GRIFIN was detected in the cytoplasm of elongated posterior cells (epc), here most prominently in the apical region adjacent to the central epithelial cells (arrow; please see corresponding illustrations in Fig. 5b, b', b"). C-GRIFIN binding led to moderate signal generation distributed homogeneously in the cytoplasm of presumptive cornea, anterior and posterior parts of the lens vesicle at HH stage 19 (**b**) and in presumptive cornea, the anterior part of the lens vesicle and elongated posterior cells at HH stage 21 (**e**). **c**, **f** Overlay of staining patterns for C-GRIFIN presence and binding at the HH stages 19 and 21, respectively. Due to the weak antibody-dependent signal, no significant overlap was seen at HH stage 19 (**c**). Positivity for CG-2 (please see corresponding illustrations in Fig. 5a"') and accessible binding sites for CG-2 at HH stage 19 revealed an overlap predominantly in the presumptive cornea (arrowheads, inset to **c**). At HH stage 21, localization and reactivity of C-GRIFIN in elongated posterior cells resulted in a limited overlap in their apical parts (arrow, **f**). Very weak overlap of CG-2 presence and accessible binding sites was detected supranuclearly in cells of the anterior part of the lens (arrow) and cytoplasmically in developing cornea (arrowhead) at HH stage 21 (inset to **f**; please see also corresponding illustration in Fig. 5b"'). **g**–**i** Signals for C-GRIFIN presence were detected in primary lens fiber cells (pLF) with increasing intensity in lenses from HH stage 23 (**g**), 25 (**h**), to 27 (**i**), which were well-ordered at HH stage 23 (please see also corresponding illustrations in Fig. 5c, c')/HH stage 25 (please see also corresponding illustrations in Fig. 5d, d') and appeared in a loosened ribbon-like staining pattern at HH stage 27 (please see also corresponding illustrations in Fig. 5e, e'). Reactivity to C-GRIFIN was observed in approximately the same intensity in the corneal and central epithelial cells of these three developmental stages, with a low degree of overlap in lens fiber cells in apical parts at HH stages 23/25 (arrows, **g**, **h**). At HH stage 23, CG-2 presence (inset to **g**) was predominantly detected in the cornea and supranuclearly in the central epithelium (please see also corresponding illustration in Fig. 5c"'), here leading to regions of distinct overlap with CG-2 binding sites (arrowheads), which was weaker in HH stages 25 (inset to **h**, please see also corresponding illustration in Fig. 5d"') and 27 (inset to **i**; please see also corresponding illustration in Fig. 5e"'). **j** C-GRIFIN positivity in early secondary cortical lens fiber cells (cLF) seen in transversal direction at HH stage 31 (please see also corresponding illustrations in Fig. 5f, f'). No overlap was seen with staining by biotinylated C-GRIFIN, which was limited to the central epithelium and lens capsule (lc). In contrast a weak overlap of anti-CG-2 (please see also corresponding illustration in Fig. 5f"')/biotinylated CG-2-dependent signals (inset to **j**) was found supranuclearly in the central epithelium. **k**, **l** Staining pattern of C-GRIFIN in secondary cortical lens fiber cells (cLF) at HH stage 39 (**k**, please see also corresponding illustrations in Fig. 5g, g') is indicative of the typical lamellar arrangement seen in the 3-day-old post-hatch lens (arrowheads, **l**, please see also corresponding illustrations in Fig. 5h, h'). As in (**j**), no overlap with staining due to accessible C-GRIFIN-binding sites, which was confined to cells of the central epithelium and lc, was observed. Probing the sections with anti-CG-2 and biotinylated CG-2 (insets to **k**, **l**) led to weak overlap (arrowheads) supranuclearly in central epithelial cells at HH stage 39 (**k**, please see also corresponding illustration in Fig. 5g"'), which was further decreased in the 3-day-old (post-hatch) lens (**l**; please see also corresponding illustration in Fig. 5h"'), in parallel with the reduction of CG-2 presence in these epithelial cells. Concentration of biotinylated C-GRIFIN and CG-2 was 8 μg/ml and of anti-C-GRIFIN and anti-CG-2 IgG was 4 μg/ml. Scale bars are 20 μm

site (Fig. 8b). Looking at the sequence of the cloned region, in view of the reported presence of matches with putative expression-enhancing activity that had been inferred by analogy considerations (García Caballero et al. 2016a), motifs with likely affinity for Maf proteins, especially L-Maf and for Pax6 appear to be relevant as potential regulators for C-GRIFIN gene expression. After determining the optimal plasmid amount encoding C-GRIFIN promoter for activity assays by titrations (Supplementary Material, Fig. S3), we ran reporter assays to detect an influence of these sites on transcriptional activity.

The functional relevance of the occurrence of six MAREs, their presence reported previously (García Caballero et al. 2016a) and their positions illustrated in Fig. 8(a), was first probed by stepwise deletions of sections of the promoters. Upon loss of three sites (in the 1257del variant), the extent of stimulation by L-Maf decreased (Fig. 9a). This indication for functional relevance was put to the test by deletions confined to the actual MAF-binding site and by targeted mutation. The mutational impairment at sites 2, 3, or 4 and their deletion led to marked reductions of signal generation in the presence of L-Maf in each case, pointing to the possibility of functional cooperation and redundancy (Fig. 9b). In contrast to L-Maf, the external addition of MafB or the three Pax6 isoforms had a comparatively small stimulatory effect (Supplementary Material, Fig. S4). However, when tested in binary mixtures at a 1:1 ratio (at 30 ng), presence of the two Pax6 isoforms X2 and X3 but not of Pax6(5a) accounted for an increase in relative activity (Supplementary Material, Fig. S5). This result points to the possibility for functional cooperation between transcription factors in the regulation of expression of this lens-specific protein.

Discussion

Considering that GRIFIN is a lens-specific galectin present in a substantial amount, "it is rather remarkable that so little is known about a possible physiological role for this protein" (Barton et al. 2009). Having previously reported (i) its representation as single-copy gene throughout vertebrates, (ii) strong immunoreactivity confined to semicircular fibers of the main lens body and, comparatively weaker, also found in the transition zone between this region and annular pads in adult chicken (García Caballero et al. 2016a) and (iii) structural stability and rigidity for C-GRIFIN in crystals and in solution (Ruiz et al. 2018), we continued to explore its biochemical characteristics as well as start to determine spatio-temporal expression properties during development. Knowing the nature of potential binding partners and the course of expression, especially at early stages in development, can provide clues to shape a concept for GRIFIN's role(s) in situ. After all, cDNA for GRIFIN had first been detected in

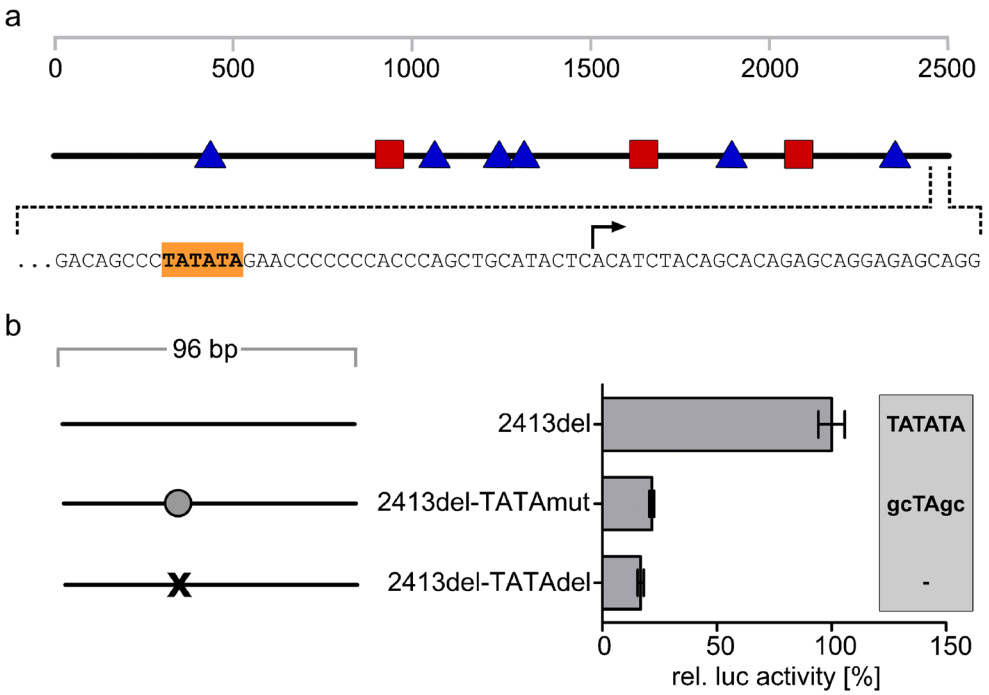


Fig. 8 Overview of relevant motifs in the cloned section of C-GRIFIN's promoter (**a**) and functional activity of its TATA box (**b**). **a** Schematic representation of the relative distribution of putative sites with affinity to Maf proteins, i.e., MARE/Maf (blue triangles) or Pax6 (red squares) on the 2509-bp-long sequence. Shown enlarged is the section preceding the ATG with the transcription start point (arrow) and the TATA box (colored in orange). **b** Engineered versions of the cloned promoter sequence and impact of deletions/mutations on activity. Truncation of cloned promoter section to 96 bp, mutation of TATA site and its deletion established the test panel (left). Activity values for each plasmid are shown in the graph on the right (luc: luciferase). Values are given as mean ± SEM

differential display by quantitative changes in post-conception (PC) stages of murine lens development (PC day 50 vs days 33.5 and 29.5; Ogden et al. 1998). To add a note of caution, the immunohistochemical study of another galectin present in lens development on specimen from man, mouse and rat, i.e., chimera-type galectin-3 (Gal-3), has yet taught the lesson that the course of expression varied, precluding extrapolations between species (Dahm et al. 2003).

Ligand blotting works by presenting electrophoretically separated extract proteins on the surface of a matrix to the probe at relative abundance levels that reflect protein presence in the tissue. In order to exclude an effect of protein modification by labeling, application of C-GRIFIN was performed via two protocols. The experimental series run in parallel with biotinylated and label-free C-GRIFIN obtained very similar results. A strong signal in the broad mass range, into which β-crystallins fall, was detected. Its intensity could be lowered by co-incubation of C-GRIFIN with cognate sugar or 0.5 M NaCl. This inhibition by lactose should not be simply interpreted as a sugar-dependent process, because the canonical contact site for the glycan with its central Trp residue can also interact with a complementary peptide motif, as suggested for Gal-3-bcl-2 recognition (Yang et al. 1996; Akahani et al. 1997). Likewise, structurally related β-sandwich plant lectins found in protein bodies (organelles containing highly ordered storage forms of legumin and vicilin) are known to connect to their binding partners in situ via glycan/ionic strength-interfereable mechanisms (Rüdiger and Gabius 2001). Of note, accomplishing the dense packing and also contact between storage proteins and protein body membranes, assumed to be mediated by leguminous lectins (Einhoff et al. 1986; Freier and Rüdiger 1987; Kummer and Rüdiger 1988; Wenzel et al. 1993; Schecher and Rüdiger 1994), is phenomenologically comparable to a putative galectin assignment in locally organizing crystallin or lens fiber cell arrangements. In this respect, physical interaction of the lectin to relevant proteins of lens fiber cells needs to be documented, at best without involving a denaturation step.

Toward this end, affinity chromatography of lens extract over C-GRIFIN-presenting resin was performed, using both modes of elution in parallel when processing extract. The dominant presence of β-crystallins in the eluted fraction is in line with the data obtained by ligand blotting. In addition, α- and δ-crystallins are identified in the pool of eluted proteins, shown in Table 1. Working with tagged human αA-crystallin as bait in a transgenic mouse model, GRIFIN-αA-crystallin recognition had first been described using chemical cross-linking, size exclusion chromatography and filtration assays (Barton et al. 2009). The affinity of binding between bovine

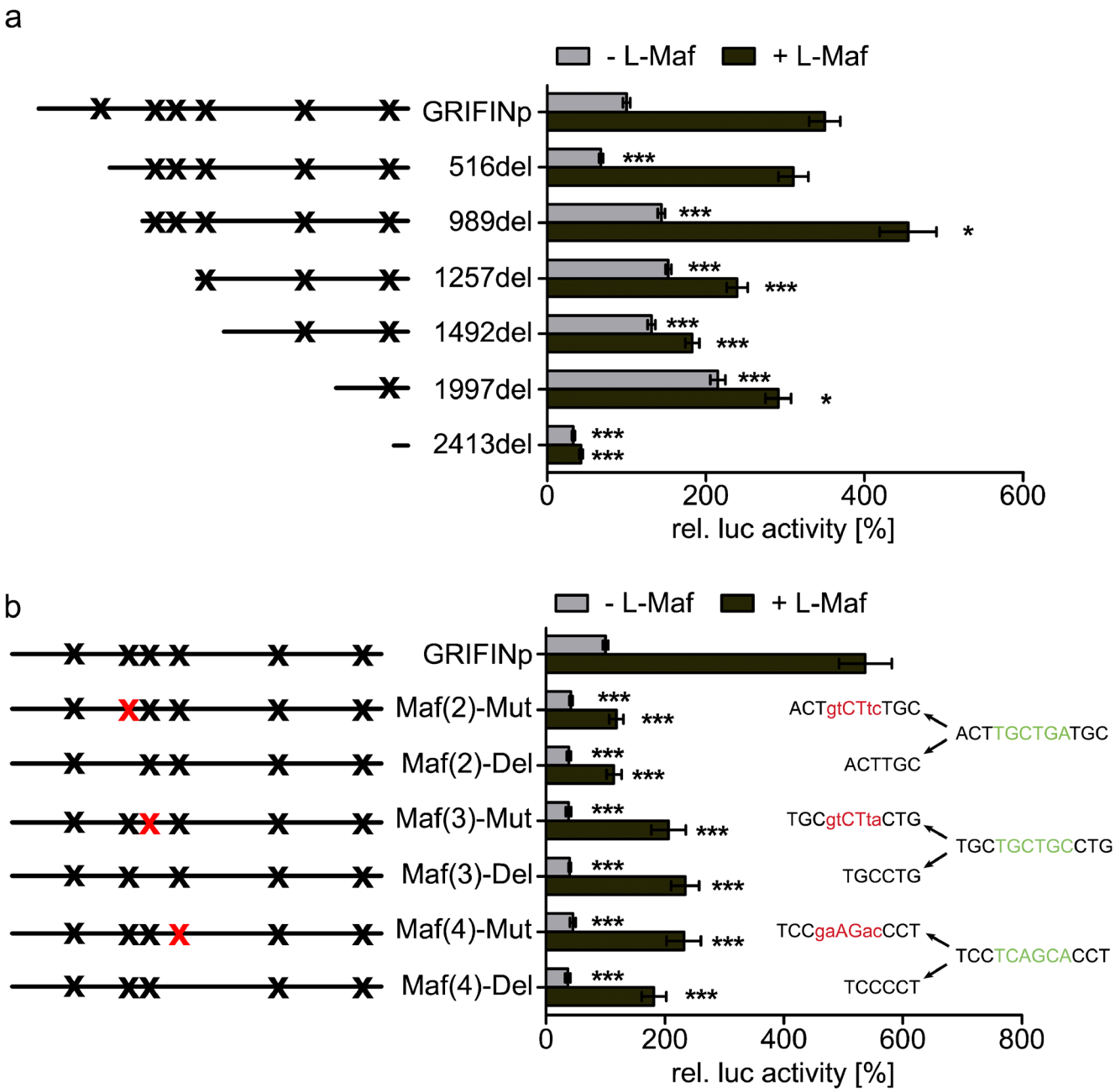


Fig. 9 Effect of engineering the presence of sites with putative affinity to L-Maf (black cross) by general stepwise deletion (**a**) or deletion/mutation (**b**) on promoter activity. **a** Subsequent nucleotide deletions removed sites with putative affinities to L-Maf in a stepwise manner (left: schematic illustration; right: activity values). **b** Targeted deletion/mutation of three distinct sites with putative affinity to L-Maf (left; position of mutation marked by red cross) and activity of the processed promoter sequence (right; nature of deletion/mutation given in detail). Data are presented as mean ± SEM. Statistical significance for comparison of control (wild-type promoter) vs sequence variant is given by asterisks (*$0.05 > p > 0.001$; **$0.01 > p > 0.001$; ***$p < 0.001$)

α_L-crystallin and murine GRIFIN was found to be 6.5 ± 0.8 μM in Scatchard analysis, with a stoichiometry of complex formation of one GRIFIN subunit to a crystallin tetramer. Since various crystallins had been reported among the 38 proteins constituting α-crystallin complexes with the tagged human protein (Barton et al. 2009), it is a reasonable possibility that not all of them directly interact with the bait. The same reasoning, considering the stickiness among crystallins, holds true when identifying binding partners for C-GRIFIN by affinity chromatography.

Present on the list of eluted proteins, too, are two members of the galectin family, i.e., *g*alectin-*r*elated *p*rotein (LEGL, C-GRP) and CG-1A (LEG6, formerly called C(G)-16). Homodimeric (proto-type) CG-1A was first detected by its

potent hemagglutination activity in extracts of pectoral muscle cultures of 12-day-old chick embryo and then purified (Teichberg et al. 1975; Den and Malinzak 1977; Nowak et al. 1977) monomeric C-GRP was traced by partial expression sequence tag information (AJ453496) leading to cloning, its sequence being conserved among vertebrates at an exceptionally high level (Cooper 2002; García Caballero et al. 2016b; Manning et al. 2018b).

When considering (i) the ability of rat GRIFIN subunits to switch partners between tagged and untagged homodimers (Ogden et al. 1998) and (ii) the recent discovery of formation of heterodimers consisting of the carbohydrate recognition domains of human galectin-1 and galectin-3 (Miller et al. 2018), the detection of the mentioned chicken galectins in the eluted fraction obtained by affinity chromatography points to an actual potential of inter-galectin recognition involving C-GRIFIN in the lens body. Such an interaction may be operative intracellularly. Since CG-3 was not detected as a binding partner in affinity chromatography, inter-galectin binding appears to harbor selectivity. As an alternative to a direct contact, an association of a galectin, which is present in lens fibers, i.e., CG-1A (Manning et al. 2018a), to crystallins in complexes bound to C-GRIFIN is a possibility. The same can hold true for C-GRP, when considering the previously reported presence of this member of the galectin family in the annular pad of adult lens (Manning et al. 2018a). Of note, the recent report on activity of human GRP as a stimulator of migration and proliferation of human breast cancer cells indicates a physiological potential for C-GRP (Zheng et al. 2018). Galectin activity is also being unveiled in the cytoplasm. Mammalian galectin-3 and galectin-8, to add a special cytoplasmic function, act as sensors inside cells, tracking down damage to endomembranes and activating autophagy (Hong et al. 2018). This activity involves association with an adaptor, i.e., tripartite motif (TRIM) protein 16 for Gal-3 (Chauhan et al. 2016) and nuclear dot protein 52 (NDP52) for Gal-8 (Wittrup et al. 2015; Falcon et al. 2018).

In immunohistochemistry, the posterior part of the lens vesicle gives rise to the C-GRIFIN-positive primary lens fiber cells. In contrast to negative central and equatorial epithelium, which originate from cells of the anterior part of the lens vesicle, these elongating cells present a ribbon-like and diffuse staining (up to HH stage 27). When the cavity of the lens vesicle was entirely filled with elongated, neatly aligned primary lens fiber cells, formation of C-GRIFIN-positive lens fiber cells in the cortex started. The herein documented cytoplasmic presence is corroborated for mammalian lens by Barton et al. (2009) and GRIFIN occurrence in the insoluble fraction reported from analysis of rat nuclear lens fiber cells by Ueda et al. (2002) can be due to its association to crystallins or membrane compounds. As noted above, the occurrence of two other CGs in our list of binding partners raises interest to explore whether such complexes will form in situ so that a corresponding monitoring is of interest. In fact, the comparatively small number of the set of galectins in chicken with seven well-characterized proteins makes this organism well-suited for comprehensive immunohistochemical fingerprinting. It will guide a detailed comparison of regulatory mechanisms for transcription among galectins. As documented herein, C-GRIFIN joins the list of lens-specific genes actually controlled by L-Maf and also affected by Pax-6, as α- and δ-crystallin genes are (Cvekl et al. 1994, 1995). Since L-Maf, in embryogenesis first detectable in the lens placode at HH stage 11 and preceding δ1-crystallin gene expression, is a master regulator of essential genes in lens initiation and development (Ogino and Yasuda 1998; Reza et al. 2002, 2007; Yoshida and Yasuda 2002; Reza and Yasuda 2004), its activity on C-GRIFIN gene expression in our test system alone or in combination with Pax6 can be interpreted to signal such a role for C-GRIFIN.

In fact, ideas for essential roles have been put forward for mammalian galectins beyond GRIFIN. Human Gal-3 is an integral constituent of mammalian ocular lenses, suggested to be involved "in adhesion processes and in the regulation of programmed organelle elimination during lens cell differentiation" (Dahm et al. 2003). Since it should also be considered that the differentiation into secondary lens fiber cells is linked to cell elongation, here desmosomes are found between elongating but not mature fibers (Piatigorsky 1981; Lo 1988), cell connections based on them may be essential in a distinct phase of the dramatic changes occurring along this route (Kuszak 1995) and galectins may be involved. Among them, the concept for a participation of these bi- or oligovalent proteins in junctions has precedents: mammalian galectin-4 has first been referred to as adherens junction protein (Chiu et al. 1994; Wasano and Hirakawa 1995), human Gal-3 that forms oligomers in complexes with counterreceptors (for structural information and review of the literature, please see Flores-Ibarra et al. 2018) co-localizes with desmoplakin/desmoglein (Hrdlicková-Cela et al. 2001; Plzák et al. 2002; Jiang et al. 2014) and associates with MP20, a component of junctions between lens fiber cells (Gonen et al. 2000, 2001). Probing into the possibility for an association of especially galectin-3 to cadherins during the differentiation process leading to formation of crystalline lens (Ferreira-Cornwell et al. 2000) or to the cortex adherens components (Straub et al. 2003) is thus an attractive aim when extending investigation of CGs beyond C-GRIFIN with this study design. Homodimeric galectin-1, too, is present in lens epithelium, assumed to function "as a regulator of cell growth, differentiation and apoptosis as well as playing a role in cell adhesion" (Wang-Su et al. 2003). Since avian orthologues for galectin-1 and galectins-3 and two further members of the galectin family, i.e., C-GRP and CG-8, have been detected in adult chicken lens (Manning et al. 2018a), the presented work on C-GRIFIN gives clear direction to characterizing the developmental

course of expression of each of the seven galectin proteins in the lens.

What is more, the internal control with CG-2 convincingly attests the importance of these measurements by showing strong expression during embryogenesis, for example at HH stage 21 (Fig. 5b"'), whereas CG-2 detection is confined to the annular pad in the adult chicken and this at a low level (Manning et al. 2018a). Of relevance, hemagglutination assays on organ extracts of several chicken tissues such as brain and retina revealed pronounced developmental changes with organ-specific characteristics (Kobiler and Barondes 1977; Eisenbarth et al. 1978); another reason to study expression profiles in detail comprehensively at various HH stages. Regional differences seen at the adult stage, e.g., epithelial positivity only for CG-3 and CG-8 (Manning et al. 2018a) and the known anti-apoptotic activity of mammalian Gal-3 let it become likely that new functional implications will emerge by making these distribution profiles available.

Equally promising, the comparison of regulatory factors/promoter sequence motifs that control gene expression between lens-specific C-GRIFIN and the other six CGs that are widely present in various tissues as well as respective reporter assays is postulated to trace functional similarities and differences, especially for CG-3. This chicken galectin is in closest vicinity to C-GRIFIN in a phylogenetic-tree diagram (Houzelstein et al. 2004; García Caballero et al. 2016a) but abundant in the lens epithelium and annular pad (Manning et al. 2018a). Interestingly, its gene transcription starts at three sites (Kaltner et al. 2011), whereas a single site is seen for C-GRIFIN. Faced with the challenge to crack the sugar code (Gabius 2017), studying distribution profiles of galectins during embryogenesis and flanking these results with biochemical data, as reported herein, eventually reaching the level of the complete network, will help in connecting these proteins and their counterreceptors to mechanisms of cellular development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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