Short Communication

Comparative Analysis of the Nuclear Presence of Adhesion/ Growth-Regulatory Galectins and Reactivity in the Nuclei of Interphasic and Mitotic Cells

(lectin / mitose / cell nucleus / Ki67)

O. KODET[1,2], B. DVOŘÁNKOVÁ[1,3], L. LACINA[1,2,3], S. ANDRÉ[4], H. KALTNER[4], H.-J. GABIUS[4], K. SMETANA JR[1,3]

[1]Institute of Anatomy, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic
[2]Department of Dermatovenereology and Venerology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic
[3]Centre of Cell Therapy and Tissue Repair, Second Faculty of Medicine, Charles University in Prague, Prague, Czech Republic
[4]Ludwig-Maximilians-University Munich, Faculty of Veterinary Medicine, Institute of Physiological Chemistry, Munich, Germany

Abstract. Nuclear galectins participate in splicing of pre-mRNA. In this study we detected galectins-1, -2, -3 and -7 and their glycoligands in three types of cells: fibroblasts, cancer epithelial cells and melanoma cells. The results demonstrated that the nuclear expression of distinct types of galectins and their ligands in interphasic nuclei is dependent on the cell type. The extensive binding of labelled galectins-1 and -2 to mitotic cells (around chromosomes, in mitotic spindle and in bridge connecting both daughter cells) suggests their role during the cell division.

Introduction

The intricate spatiotemporal orchestration of diverse proteins underlies the complex array of nuclear activities (Misteli, 2005; Woodcock, 2006; Fedorova and Zink, 2008; Folle, 2008; Austin and Bellini, 2010). Of note, the molecular mechanics and dynamics of intranuclear molecules even involves actin, recently detected inside the nucleus (Castano et al., 2010). At the subcompartment level, this principle of a complex network also holds true for the cell nucleolus (Smetana et al., 2008; Bártová et al., 2010). It is thus an obvious task to profile the presence of nuclear proteins in the long-term quest to tie distinct protein species to functions for nuclear integrity and dynamics. Our interest is focused on a family of endogenous lectins having the β-sandwich fold and reactivity to substituted β-galactosides as well as potently acting as elicitors of biosignalling, i.e. the galectins (Gals) (Gabius, 1987, 2006, 2009a; Villalobo et al., 2006). Members of this family have already been defined as clinically relevant, e.g. galectin-3 (Gal-3) plays a role in cardiac inflammation and dysfunction, involving TGF-β/Smad3 signal routing, or in prognostic histopathological assessments, with relevance to tumour suppressor activities (Plzák et al., 2004; Moisa et al., 2007; Liu et al., 2009; Sanchez-Ruderisch et al., 2010). In addition to glycans as ligands, the lectins thus acting as translators of the sugar code (Gabius, 2009b), they are also capable to bind peptide motifs making them suitable to react with proteins, e.g. galectin-1 (Gal-1) forming a complex with oncogenic H-ras (Rotblat et al., 2004; for recent survey of protein ligands, see Gabius, 2009a). Converging biochemical and immunocyto/histochemical evidence led to the detection of Gals in the nuclei (Gabius et al., 1986, 1988; Laing and Wang, 1988; Dagher et al., 1995; Wang et al., 1995; for reviews see Wang et al., 2004; Smetana et al., 2006; Haudek et al., 2010). At this site, galectins can interact with small nuclear ribonucleoprotein particles (snRNP), with Gemin4 and also with β-catenin, as seen for Gal-3 (Vyakarnam et al., 1998; Patterson et al., 2004; Shimura et al., 2004). Moreover, the transcriptional regulation, e.g. involving the thyroid-specific transcription factor

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Corresponding author: Karel Smetana, Jr., Institute of Anatomy, First Faculty of Medicine, Charles University in Prague, 128 00 U Nemocnice 3, Prague 2, Czech Republic. Phone: (+420) 224 965 873; e-mail: karel.smetana@lf1.cuni.cz

Abbreviations: CRD – carbohydrate recognition domain, Gal – galectin, Gal-1 – galectin-1, Gal-2 – galectin-2, Gal-3 – galectin-3, Gal-BS – galectin-binding site(s), snRNP – small nuclear ribonucleoprotein particles.

TTF-1 or acting on cylin D1 gene expression, is known (Lin et al., 2002; Paron et al., 2003). Shuttling of galectin between the cytoplasm and the nuclei is frequent and operates with galectin-type-dependent characteristics (Davidson et al., 2002; Saussez et al., 2008).

This collective evidence argues in favour of a non-random occurrence of galectins in the nuclei and warrants systematic study. Corroborating the merits of this research line, Gal-1 is even one of the most abundantly expressed proteins in the nuclei of the stem cells (Nasrabadi et al., 2010). Fittingly, this lectin had been detected to be a probe strongly recognizing nuclei of poorly differentiated cells such as adult stem cells and a lectin marking regulation of signal intensity upon senescence in keratinocytes (Purkrábková et al., 2003; Chovanec et al., 2004). The closely related homodimeric galectin-2 (Gal-2) participates in the formation of interchromatin PML bodies in the nuclei of cells under conditions of stress (Dvořánková et al., 2008). In this study, we systematically performed immunocytochemical localization of three homodimeric proto-type galectins (Gal-1, -2 and -7), which have distinct expression profiles in murine tissues (Lohr et al., 2007, 2008), and the chimeric Gal-3 and compared the obtained staining profiles with the presence of corresponding binding sites in three types of cells, i.e. human fibroblasts from the stroma of spinocellular carcinoma, a cell line originating from hypopharyngeal carcinoma (FaDu cells), and melanoma A-2058 cells, during the interphase and mitosis. Blocking experiments with lactose to examine involvement of the lectin site and pre-treatment of tested cells with RNase were also performed.

Material and Methods

Culture of cells

Three types of cells were used, i.e. the FaDu cell line of epithelial cells originating from a tumour of the hypopharynx, the A-2058 (ATCC) cell line of malignant melanocytes from a melanoma lymph node metastasis and cultured human fibroblasts from the stroma of squamous cell carcinoma. The tumour specimen was obtained from a spinocellular carcinoma of the oral cavity treated at the Department of Stomatology of the First Faculty of Medicine (Charles University in Prague, Prague, Czech Republic) after approval by the local ethical committee according to the Declaration of Helsinki and the patient's consent. The fibroblasts were prepared as described and cultured in Dulbecco's modified Eagle's medium (D-MEM, Invitrogen, Carlsbad, CA) containing 10% foetal bovine serum and antibiotics (Biochrom, Berlin, Germany) at a cell density of $3 \times 10^3 cm^{-2}$ at 37 °C and 5% CO_2 from the seventh passage for 24 days (Lacina et al., 2007). The last subculture was seeded on cover slips and cultured to the stage of subconfluency with frequent occurrence of mitosis for three days.

FaDu cells (generous gift of the commercial line by J. Bouček, Academy of Science of the Czech Republic, Prague, Czech Republic) were seeded at a density of 3×10^3 cells/cm^2 and cultured in Minimum Essential Medium with Earle's salt (EMEM, Biological Industries Ltd., Kibbutz Beit Haemmek, Israeli) supplemented with 10% foetal bovine serum and antibiotics (Biochrom) at 37 °C and 5% CO_2 for 18 days (6^{th} passage) and subcultured on cover slips as described above.

A-2058 melanoma cells from the 8^{th} subculture (19 days) seeded at a density of 1×10^3 cells/cm^2 were propagated in DMEM with 10% foetal bovine serum and antibiotics (Biochrom) at 37 °C and 5% CO_2. For microscopic monitoring, the cells were cultured on cover slips for three days to reach subconfluency. Then the cover slips were washed with phosphate buffer, dried in a flow box and frozen for immunocytochemical processing.

Detection of lectins and their binding sites

Cells were fixed by paraformaldehyde in PBS (pH 7.3). All studied types of galectins were detected by non-crossreactive polyclonal antibodies prepared in our laboratory (Kaltner et al., 2002; Saal et al., 2005; Langbein et al., 2007; Čada et al., 2009a, b; Saussez et al. 2009, 2010). The antibodies were used at the standard concentration of 20 µg/ml. Keratin-19 and the Ki67 antigen were visualized by specific monoclonal antibodies (DAKO, Glostrup, Denmark) diluted as recommended by the supplier. Swine anti-rabbit serum labelled by FITC (SwAR-FITC, AlSeVa, Prague, Czech Republic) and FITC-labelled swine anti-mouse serum (SwAM-FITC, AlSeVa) diluted as recommended by the supplier were used as the second-step antibody. Control experiments were performed by testing antibodies for antigens not expressed in these cells and by omission of incubation with first-step antibodies. To detect binding sites for the studied Gals we used biotinylated Gals-1, -2, -3, and -7 including the E71Q mutant of Gal-1 (mutation impairs carbohydrate-binding activity) and the proteolytically truncated version of Gal-3 lacking the collagenase-sensitive section, all probes routinely tested for activity in solid-phase and cell assays (Gabius et al., 1991; André et al., 2006, 2007, 2008). In addition, we included the phosphorylated version of Gal-3 (Kübler et al., 2008; Szabo et al., 2009; Díez-Revuelta et al., 2010), all probes for comparison tested at the concentration of 20 µg/ml. To monitor inhibition of binding, we pre-incubated the galectin-containing solutions in 5 mM lactose (Sigma-Aldrich, Prague, Czech Republic). RNase (Sigma-Aldrich) -treated cells were also used in experiments for detection of galectin-binding sites (Gal-BS). Nuclei were counterstained by the DNA-reactive dye 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). The specimens were finally mounted using Vectashield (Vector Laboratories, Burlingame, CA) and inspected by an Eclipse 90i fluorescence microscope (Nikon, Prague, Czech Republic) equipped with filterblocks for the used dyes FITC, TRITC and DAPI, and a Cool-1300Q CCD camera (Vosskühler, Osnabrück, Germany); data were analysed by computer-assisted image analysis system LUCIA 5.1 (Laboratory Imaging, Prague, Czech Republic).

Results and Discussion

Interphasic nuclei

The presence of galectins and the intensity of staining in interphasic nuclei were dependent on the cell type (Table 1, Fig. 1). On average, only Gals-1 and -3 could be detected in cell nuclei including nucleoli. While Gal-1 was detected in the three studied cell types (Fig. 1A-C), the Gal-3 presence was observed in FaDu cells only (Fig. 1H). No presence of Gals-2 and -7 was observed in the nuclei/nucleoli of the studied cells under the described conditions of culture (Fig. 1D-F, J-L), serving as inherent controls.

Table 1. Localization of galectins and their binding sites in interphasic nuclei and in mitotic cells

	Cell type					
	Fibroblasts		FaDu		Melanoma	
Marker	**Interphase**	**Mitose**	**Interphase**	**Mitose**	**Interphase**	**Mitose**
Gal-1	+/++	+	++	+	±	±
Gal-1-BS (also for E71Q mutant)	++	+	++	+	-	-
Gal-2	-	-	-	-	-	-
Gal-2-BS	++	+	++	+	±	±
Gal-3	-	-	++	+	-	-
Gal-3-BS[a]	-	-	-	-	-	-
Gal-3-BS + RNase	-	-	-	-	-	-
Gal-7	-	-	-	-	-	-
Gal-7-BS	++	-/+	-	-	-	-

[a]no staining was measured either after identical processing with phosphorylated galectin-3 and the proteolytically truncated form

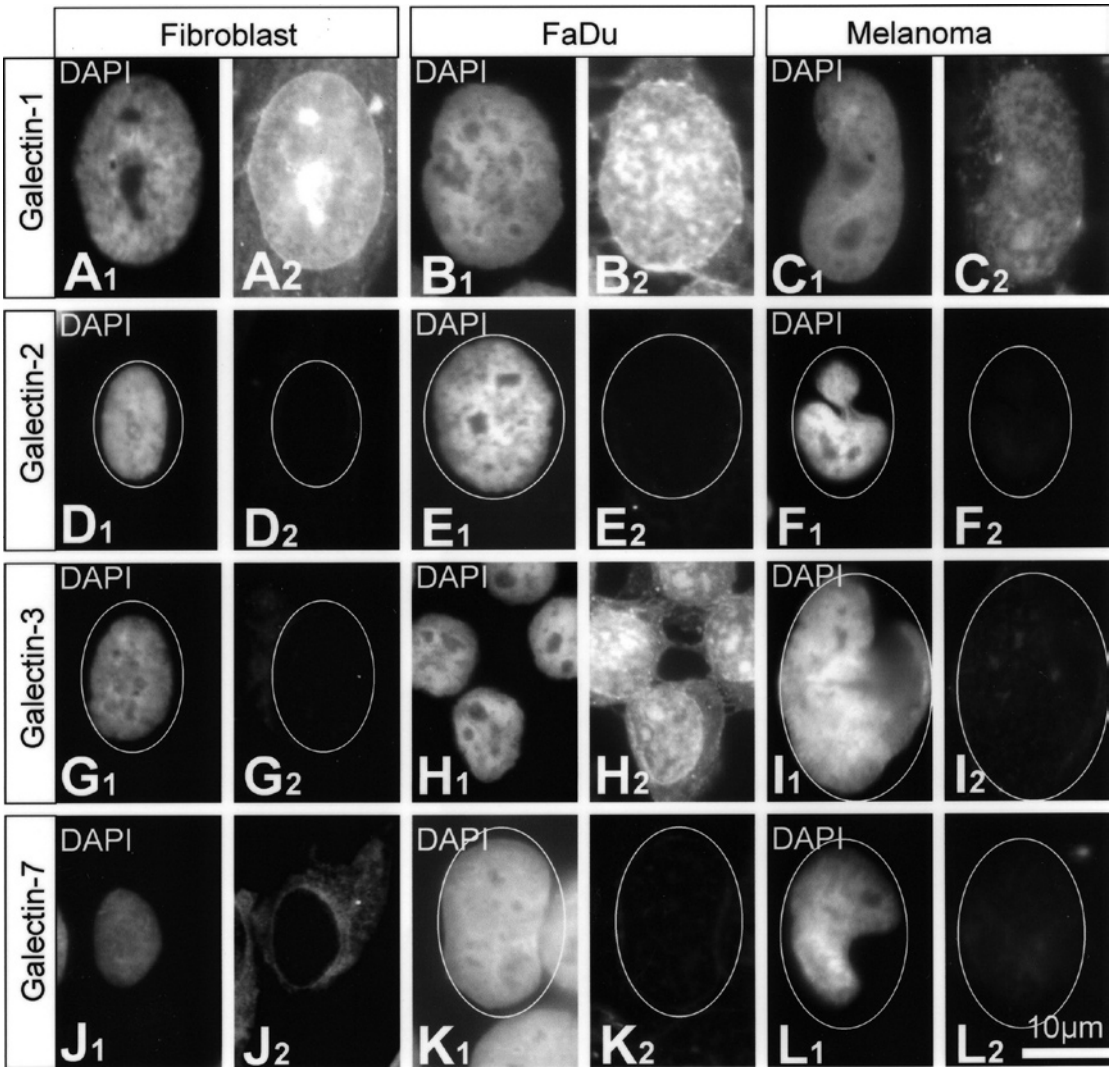

Fig. 1. Detection of Gal-1 (A-C), Gal-2 (D-F), Gal-3 (G-I) and Gal-7 (J-L) in the nuclei of fibroblasts, FaDu epithelial tumour cells and melanoma cells. Results of DAPI staining are also presented.

Interphasic-nuclei fibroblasts and FaDu cells, the latter results extending previous results with labelled Gal-1 (Smetana et al., 2006), were reactive with this type of lectin; melanoma cells presented nearly no Gal-1-BS (Table 1, Fig. 2A-C). While Gal-1-BS in the nucleoli were detected in fibroblasts (Fig. 2A), the signal for binding to the nucleolar region in FaDu cells was very weak, if present at all (Fig. 2B). Lactose, the pan-galectin hapten inhibitor, inhibited intensity of the signal to a certain extent, revealing an involvement of the lectin site (Fig. 2D). A very strong inhibitory effect for Gal-1 binding was seen after pre-treatment of the studied cells by RNase (Fig. 2E). The same inhibitory effect of RNase on the binding was similarly recorded for Gals-2 and -7. When the Gal-1 mutant (E71Q) with impaired lectin activity was used instead of the wild-type protein, no decrease of binding was registered (Table 1, Fig. 2F). Similar to Gal-1, binding of Gal-2 (Table 1, Fig. 2 G-I) to cell nuclei of melanoma cells was observed. All forms of Gal-3 (i.e. full-length, truncated and phosphorylated) were unable to react with nuclei of the tested cell types, serving as inherent control for specificity (Table 1, Fig. 2 J-L). Gal-7-reactive epitopes were visualized in the nuclei of about a third of all fibroblasts, in that case of cells with rather small size (Table 1, Fig. 2M).

Mitotic cells

Throughout the mitotic period when the nuclear material had been condensed to chromosomes, these were surrounded Gal-1-positive material (all studied cell types) and Gal-3 (FaDu cells only), respectively. Interestingly, these two Gals were also strongly present in the region connecting the cells in the process of telophase (Table 1, Fig, 3A). When the cells were stained by biotinylated Gals as probes, Gals-1/-2-BS were seen to be present in all studied cell types, with the lowest intensity down to negativity in melanoma cells (Table 1). Of note, binding sites for Gals-1/-2 were mainly present around the chromosomes (Fig. 3B). However, we also observed a signal for both types of reactivity to the mitotic spindle, but in only 10 % of the cells (Fig. 3C). The addition of lactose nearly completely inhibited the binding of both Gals to cells including the chromosomal region (Fig. 3D). The same effect was observed after RNase pre-treatment, except for the stalk connecting both daughter cells (Fig. 3E). When we compared the localization patterns of Gals-1/-2-BS with the distribution of the Ki67 antigen known as marker of proliferating cells, a very similar topography was determined (Fig. 3F). Again, the tested Gal-3 forms were not reactive in FaDu cells (Table 1), underscoring Gal-type-dependent staining. Evidently, despite structural homology each galectin exhibits its own reactivity profile. Mitotic fibroblasts bound Gal-7 with very weak affinity (Table 1). In summary, this study extends our previous reports on Gal parameters in the nuclei, with special emphasis given here to a cell-type comparison and monitoring of mitosis. A salient result is the inherent specificity among the closely related proteins.

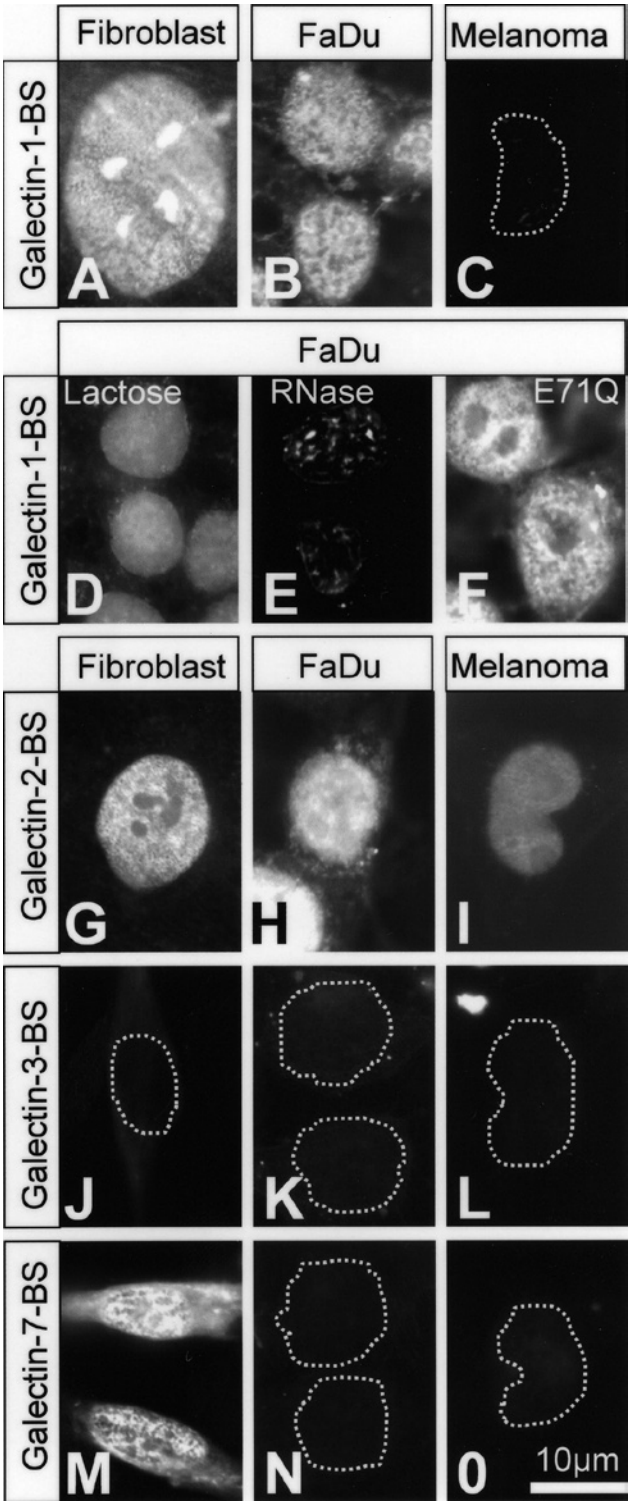

Fig. 2. Detection of binding sites for Gal-1 (A-E), for the Gal-1 mutant E71Q (F), for Gal-2 (G-I), for Gal-3 (J-L) and for Gal-7 (M-O) in fibroblasts, FaDu epithelial tumour cells and melanoma cells. Pre-treatment of cells with 5 mM lactose slightly and with RNase strongly reduced binding of Gal-1 to the cell nuclei. Impairment of lectin activity by mutation in the carbohydrate recognition domain of Gal-1 (E71Q) has no inhibitory effect on Gal-1 binding to the nucleus.

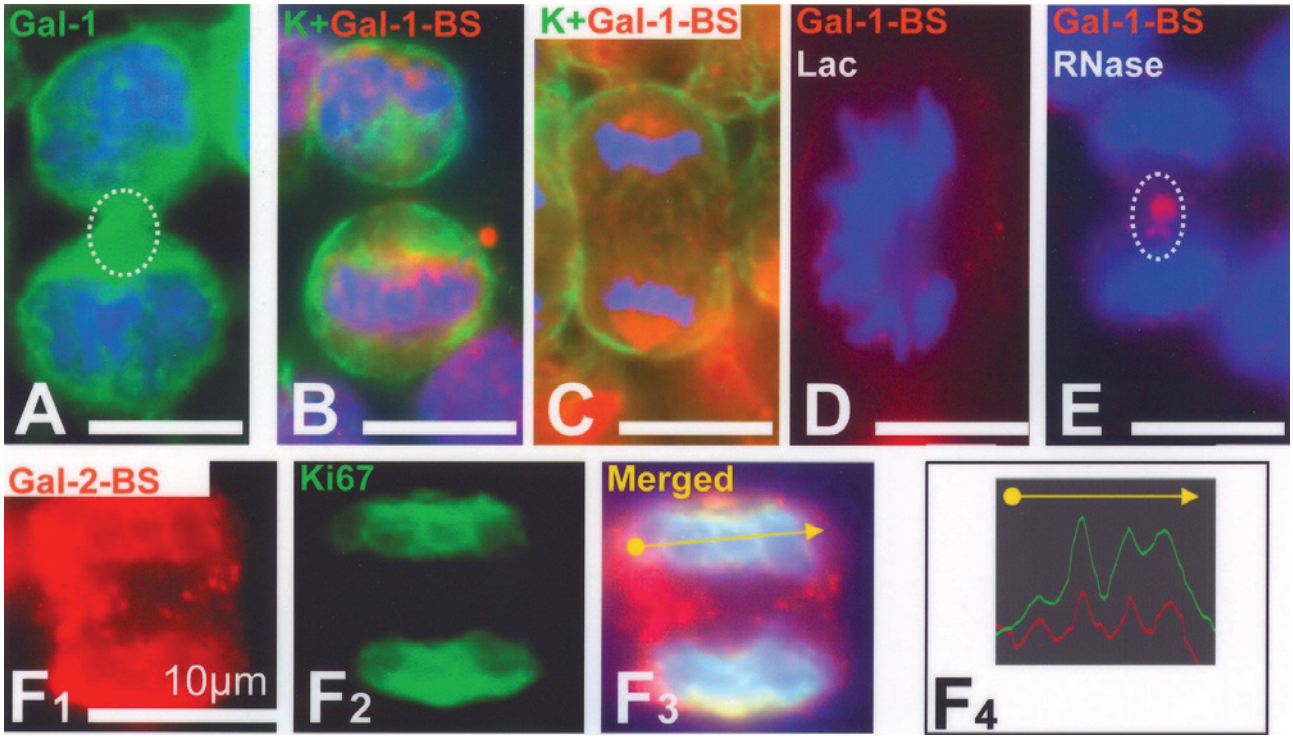

Fig. 3. Detection of Gal-1 (green signal, A), of binding sites (BS) for Gal-1 (red signal; B-E) and for Gal-2 (red signal, F), of keratin-19 for cell staining (green signal; B, C) and of Ki67 (green signal; F) to identify mitotic cells. DNA is visualized by DAPI stain. Chromosomes appear surrounded by Gal-1 staining that is also present in the stalk between daughter cells (dashed ellipse) (A). Galectin-1-BS clearly form the boundary of the area containing chromosomes (B) and are also present in the mitotic spindle (C). Pre-incubation with lactose strongly inhibits Gal-1 binding (D). The same effect was also observed after pre-treatment with RNase, where positivity is present only in the region between both cells (E). Gal-2-BS (F1) and Ki67 (F2) positivity resides in very similar parts of cells surrounding the region in which the chromosomes are located. Fluorescence intensity measured upon detection of Ki67 and Gal-1-BS at the site of the yellow line (F3) exhibits rather similar peak positions (F4) that indicate their very close signal topology.

Regarding cell mitosis, we describe positivity around the chromosomes, in the mitotic spindle and in the stalk connecting daughter cells prior to complete cytokinesis. This "perichromosomal envelope" also contains proteins important for the control of cell proliferation such as the Ki67 antigen, with similar signal distribution. Whether these parameters may be involved in the condensation of chromatin to chromosomes and the organization of kinetochore, which require cooperation of diverse proteins (Oegema and Hyman, 2006; Hudson et al., 2009; Kitagawa, 2010), is presently unclear. Of note, the potential of Gals to cross-link ligands, with ensuing physiological consequences and exquisite specificity when e. g. presented in microdomains (Wang et al., 2009; Kopitz et al., 2010), may be instrumental in this respect. Noted disparities between the proto-type and chimeric Gals, which differ in this capacity leading to functional competition (Kopitz et al., 2001; Sanchez-Ruderisch et al., 2010), serve as an argument along this line and give direction to further experimental examination. Also, our results encourage us to pursue the investigations by monitoring the tandem-repeat-type Gals-4, -8, and -9 (Delacour et al., 2005; Cludts et al., 2009; Solís et al., 2010).

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